UNIVERSITY OF CRETE

"A Role of RGS9 in determining synaptic plasticity in addiction"

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ΕΥΧΑΡΙΣΤΙΕΣ

Αρχικά θα ήθελα να ευχαριστήσω την επιβλεπούσα καθηγήτρια μου κ. Ζαχαρίου Βενετία για την εξαίρετη ικανοτήτα της να διεγείρει την πλαστικότητα των νευρώνων μου όσον αφόρα τις συμβουλές και την στήριξη της σε αυτό το ταξίδι γνώσεων. Επιπλέον, αυτή η εργασία δεν θα μπορούσε να ολοκληρωθεί χωρίς την συμβόλη των επίτιμων καθηγητών μου κατά την διάρκεια των μαθημάτων του μεταπτυχιακού προγράμματος.

Επίσης, θα ήθελα να ευχαριστήσω την Δήμητρα, την Μάρω, την Βίκυ, την Σέβη και την Ολυμπία για την ανταλλαγή των γνώσεων τους και την άψογη συνεργασία. Τέλος, ένα μεγάλο ευχαριστώ οφείλω στην οικογένεια μου που με στηρίζει ανέκαθεν.

ABSTRACT

Drug addiction is considered to be a major social-economic issue since it is characterized as a disease that currently affects more than 30 million people in the USA and Europe. Although, scientists have elucidate the involvement of several brain regions and signaling pathways that are involved in drug of abuse actions, still drug dependence pathways have not been fully characterized. In particular, members of the regulators of G protein signaling (RGS) have been implicated in addiction, with a particular interest of RGS9-2 having an essential role in opiate dependence, regarding its expression in nucleus accumbens. In this present study, RGS9-2 role is investigated in determination of proximal and distal dendritic spine density in neuronal population of dorsal striatum, (a region functionally related to elements of addiction) in locomotor morphine sensitization paradigm. Specifically, morphine leads to decreased proximal mushroom spine density in RGS9 knockout mice and in proximal/distal stubby density. Also, morphine affects spine morphology, regarding head and neck diameter of dendritic spines. More specifically, morphine administration results in decreased thin and stubby head diameter in proximal dendrites and in a decrease in total spine head diameter in distal dendrites. Moreover, proteins that are involved in synaptic and structural plasticity are also regulated by morphine administration in the dorsal striatum of RGS9 wild type mice, whereas aberrant regulation of synaptic proteins in response to morphine is observed in the dorsal striata knockout mice. Taken together, the present study elucidates opiate actions in synaptic plasticity and provides further evidence of a potent role of RGS9-2 in the molecular mechanisms underlying addiction.

1. INTRODUCTION

1.1 PATHOPHYSIOLOGY OF ADDICTION

Opiate addiction is widely characterized as a chronically pathological state, with several relapsing events that result mainly due to either illicit opioid abuse or by drug prescription for the treatment of acute and chronic pain. According to the Manual of Mental Disorders {DSM-IV} of the American Psychiatric Association, substance dependence, such as opioid addiction is characterized by tolerance dependence (escalating doses intake for the desired effect), withdrawal symptoms, craving (intense occupation for opioid abuse in order not to experience withdrawal symptoms) and drug relapse (failure to quit drug abuse). Nowadays, drug dependence and addiction are thought to be two separate processes regulated by different molecular mechanisms in various brain circuits {1}.

Initially, drug addiction develops due to the pleasant effects of substance usage, which then are followed by rewarding events that finally convert to the aversive symptoms of withdrawal. These addictive behaviors impact molecular changes in neuro-circuits sustained drug use leads to biological neuroadaptations that may explain the drug seeking behaviours even after a prolonged drug absence. It is believed that acute or chronically administration of a drug displays its own properties of promoting activation or inactivation signaling events and thus contributing to the development of different neuroadaptative changes in brain regions involved in addiction {2}. Current research focuses on the discovery of these molecular mechanisms in brain regions involved in the pathophysiology of addiction (Fig. 1) after either acute drug usage, thus resembling the positive drug reinforcement or chronically drug abuse depicting the negative drug reinforcement {3}.

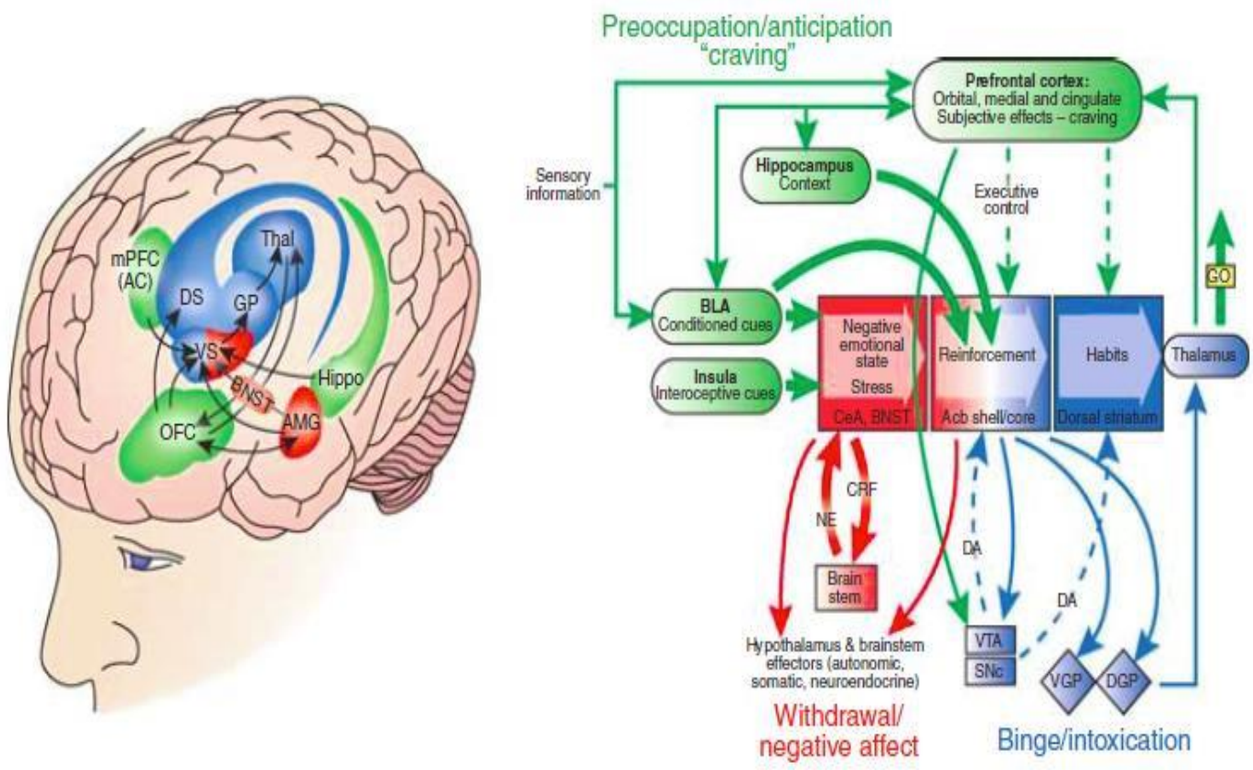


Figure 1: Brain neurocircuits involved in the acute and continuous drug abuse. Acute drug usage (binge/intoxication stage) requires the activation of the mesolimbic dopamine pathway that involves VTA projections to nucleus accumbens (NAc shell and core), prefrontal cortex (PFC) and amygdala (CeA). Positive reinforcing effects by drug of abuse are mediated by activation of dopamine and opioid peptides. Dorsal striatum plays a pivotal role in the association between drug reinforcing effects and drug-stimulus events resulting in anticipation processes. On the other hand, the amygdale, which project to the hippocampus and brainstem, mediate the negative reinforcing drug effects through activation of several neurotransmitters such as norepinephrine (NE), corticotropin-releasingfactor (CRF) and dynorphin {4}

1.2 BRAIN NEURO-CIRCUITS INVOLVED IN ADDICTION

Electrical brain stimulation have shown that all drug of abuse mediate their actions through the mesocorticolimbic dopamine pathway {5}, where dopamine cell bodies in the ventral tegmental area (VTA) project to various limbic brain regions, including nucleus accumbens (Nac) , hippocampus, amygdala and cortical structures like prefrontal cortex, dorsal striatum (DS) and anterior cingulate. Involvement of drug abuse in regulating dopamine pathways was confirmed by intracranial place conditioning experiments, where opioids have been shown to be directly self administered into the VTA {6}. Moreover, self-administration of both opiates and psychostimulants, stimulate the extracellular release of dopamine particularly in nucleus accumbens but also in dorsal striatum, indicating that mesolimbic dopamine pathway is involved in the acute reinforcing effects of drug abuse {7}. On the contrary, depletion of D1 receptor in mice have the opposite effects where mice failed to self administered cocaine, whereas intravenous opioid agonist administration lead to persistent positive reinforcement effects {8}, supporting the idea that opioids may exert their actions through an additional molecular mechanism other than mesolimbic dopamine pathway. Indeed, morphine conditioned place preference with rats, stereotaxically infused with hydroxydopamine (6-OHDA-neurotoxic compound targeting dopaminergic and noradrenegic neurons) into NAc, showed that rats acquire morphine CPP, inspite dopamine depletion {9}. Thus, it is believed that opiate drug mediate their actions for elevating dopamine release in the NAc by indirect activation of dopaminergic neurons in the VTA through hyperpolarization of inhibitory GABAergic neurons (90% percent of MSN are GABAergic neurons) {10}. Optogenetic studies show that MSN have an inhibitory, opioid sensitive role regulated by $GABA_A$ receptors and target non-dopaminergic neurons in the VTA {11}. To extend this notion, morphine directly inhibits VTA GABAergic neurons expressing mu opioid receptors located in rostromedial tegmental nucleus leading to dopaminergic release from VTA neurons {12} that project to other regions other than NAc like prefrontal cortex and amygdala. This could explain evidence of persisting opioid reward even after lesion in NAc {13}. All these data provide an insight in molecular mechanisms that are triggered after acute opioid exposure and can be persistent in case of chronic drug abuse.

Neuro-adaptations in the mesolimbic dopamine signaling appear to be common in all chronic actions of drug abuse. Absence of drug abuse is responsible for decreased dopamine signaling in the NAc {14} but in the case of continuous presence of dopamine signaling, other molecular mechanisms and brain regions are activated leading to drug- seeking behaviors. Specifically, it seems that drug induced maladaptations after chronic exposure implicate dorsal striatum {15}, another important brain region for motivational processes. On the other hand, dorsal striatum it is believed not to be involved in the acute reinforcement drug state. Indeed, human neuro-imaging studies show an elevation in DA release in this specific region, and not in the ventral striatum, in cocaine-addicted subjects after cocaine-stimulus {16}. Taken in account that chronic drug abuse that basically reflects craving and that dorsal striatum is implicated in habit induced behavioral processes (cue-related learning), molecular neuro-adaptations in dorsal striatum promote the habit of addicted subject for drug seeking {17}.

Continuous drug of abuse represent the negative reinforcement drug effects that are present in the absence of drug use. Common withdrawal symptoms of addicted subjects are dysphoria, stress, irritability, tremor, sweating and temperature changes, all depict the level of addiction and emerge the need for drug seeking. Drugs of abuse, mainly display their actions through biochemical circuits that are activated in the amygdala. Particularly, excitotoxic lesion of the amygdala in rats, reveal that moprhine failed to induce withdrawal in a stimuli dependent environment {18}. Amygdala plays a pivotal role in memory processes and emotional reactions and it is subdivided in key structures {(bed nucleus of the stria terminalis (BSTN)}, central nucleus of the amygdala (CeA) and medial subregion of the nucleus} with unique functions in addiction through projection to hippocampal and brainstem areas. Emotional features like stress during drug absence lead to the activation of Hypothalamic-Pituitary-Axis (HPA) and to activation of Corticosterone Releasing Factor (CRF). Particularly, blockage of CRF in the CeA eliminates opiate withdrawal symptoms {19}. On the other hand, knock out mice for the CRF receptor elicit pronounce opiate withdrawal compared to their wild type littermates {20}. Other molecules like norepinephrine in the BSTN and dynorphin in the NAc {21} contribute to molecular neuroadaptations involved in experiencing negative symptoms of drug absence.

As mentioned above, addiction is a pathological state, where processes of learning and memory play an important role, since one key feature of addiction is relapse and involves strong association between drug-related cues with drug taking {22}. Indeed, over the last decade there has been much effort to connect neuronal activity that represents synaptic plasticity with actions mediated by drug of abuse. Thus, there are several studies supporting that addiction is directly linked with synaptic plasticity, since drug of abuse alter synaptic plasticity in mesocorticolimbic brain circuit {23}.

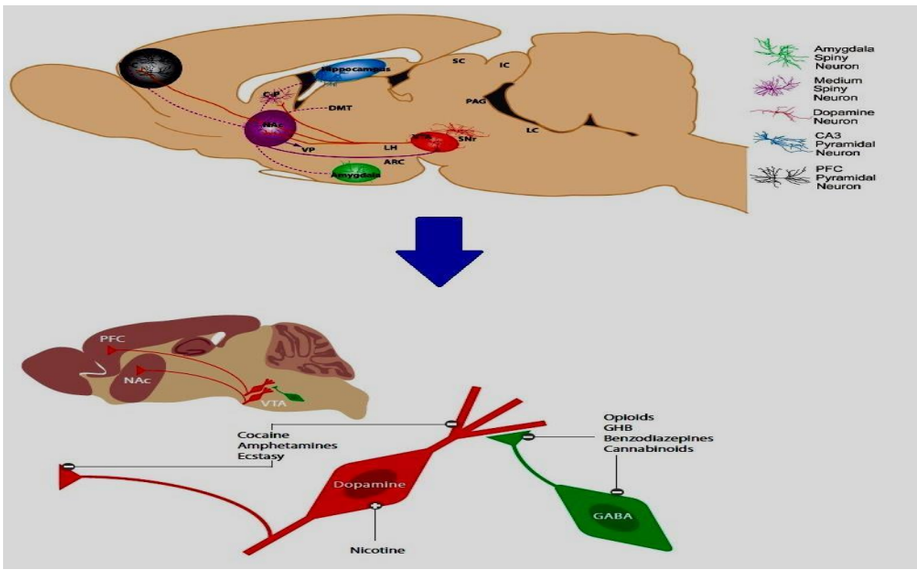


Figure 2: Opioids are known to act directly through dopaminergic VTA projections to NAc, PFC and hippocampus, pathways that are depicted with orange lines. Moreover, GABAergic medium spiny neurons project from NAc to the VTA, where they activate opioids receptors in the VTA. Indirect GABAergic afferents are also involved in the opioid actions that project to amygdala spiny, pyramidal PFC and CA3 hippocampal neurons (purple lines) {adapted from 23, 24}.

1.3 SYNAPTIC PLASTICITY

Ramon y Cajal was the first to describe in Purkinje cells the presence of small protrusions from the dendritic shaft of neurons, termed as dendritic spines {25}. Later on, the presence of these distinct areas was reported in almost every type of neuron in the mammalian brain, including medium spine neurons of the striatum. Dendritic spines are representative structures mediating molecular processes involved in synaptic plasticity. Synaptic plasticity is a term first introduced by Hebb in 1949 that described it as a functional process, where neurons communicate with each other, forming a complex brain network to control learning and memory of neuronal cells. Further studies indicated the role of synaptic plasticity in either synaptic formation or synaptic elimination, orchestrating neuronal activity {26}. In excitatory synapses two basic structures are involved in synaptic transmission. The first part includes the exocytotic compartment of presynaptic cells that releases neurotransmitters into the synaptic cleft and the other one is comprised of the plasma membrane of postsynaptic cells that contains several receptors that initiate signal transduction upon neurotransmitter binding. Excitatory synapses are formed in the head of dendritic spines, which are fine structures divided into different specialized compartments. The most important niche of dendritic spine is the post synaptic density (PSD) located in the plasma membrane of dendritic spine head, containing several neurotransmitter receptors, cell signaling molecules such as phosphatases and kinases, adaptor proteins and cell adhesion molecules, all participate in synaptic transmission regulation. Also, the spine cytoplasm contains recycling endosomes in order to participate in the synaptic strength by controlling the recruitment and the turnover rate of signaling molecules located in the PSD.

Dendritic spines are typically classified as mushroom, thin and stubby, depending on their morphology and size. Mushroom spines have small necks and are characterized by large, wide heads, while thin are described as spines with wide necks and small heads. Lastly, stubby spines lack the presence of head and are located near the dendritic shaft. Another dendritic spine form is filopodia, described as long thin structure without the presence of head and they are proposed to be the initial form of every type of dendritic spine.

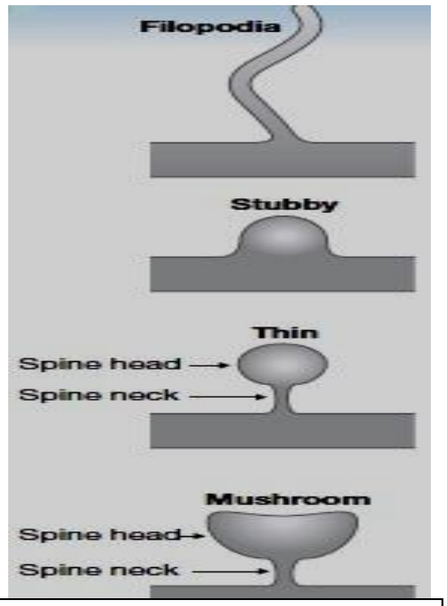


Figure 4 : Classification of dendritic spine morphology {27}.

Given the assumption that dendritic spines are dynamic and not stable structures, the categorization of spines into three subtypes can be diverse due to their variability in their morphology. Their shape and size can change, depending on the signaling molecular events that are triggered within the dendritic spines and there is a strong correlation between shape, structure and function of dendritic spines. It has been proposed that the spine morphology depends on cytoskeleton machinery. Particularly, microtubules (heterodimers of a and b tubulin subunits) and microfilament (polymerized F-actin) structures are essential for the structural plasticity of

dendritic spines {28}. Two-photon photoactivation of GFP (PAGFP) fused to actin revealed that actin polymerization in the spine head is responsible for enlargement of the spine and thus for controlling its volume {29}. Given the wide range of protein families involved in organization of actin in dendritic spines, neck and head compartments of dendritic spines are differentially organized based on actin reconstitution. Specifically, proteins participating in orchestrating spine morphology are involved in actin polymerization {30}, membrane trafficking and receptor endocytosis {31).

Of particular interest seem to be Rho GTPase proteins that are involved in the remodeling of actin cytoskeleton by catalyzing GTP hydrolysis to GDP. Particularly, Rac1 is involved in regulating spine volume, through stabilization of F-actin filaments. Activation of Rac1 leads to phosphorylation and subsequent activation of LIMK1 (LIM kinase 1) which inhibits the activity of cofillin, a protein responsible for the induction of actin de-polymerization {32}. This inhibition results in increased spine morphology and synaptic function {34}. On the other hand, RhoA GTPase protein activation mainly inhibits dendritic branching and induces decreases spine volume. It seems like Rac1 and RhoA are acting in parallel, since spine enlargement require both increased activity of Rac1 and decreased activity of RhoA {35}. On the other hand constitutive active Rac1 is responsible for spine shrinkage {36}. Interestingly, Rac1 activity has been implicated in the pathology of stress disorders and depression, as there is downregulation of Rac1 in the NAc in stress-resilient mice that results in increased stubby density {37}.

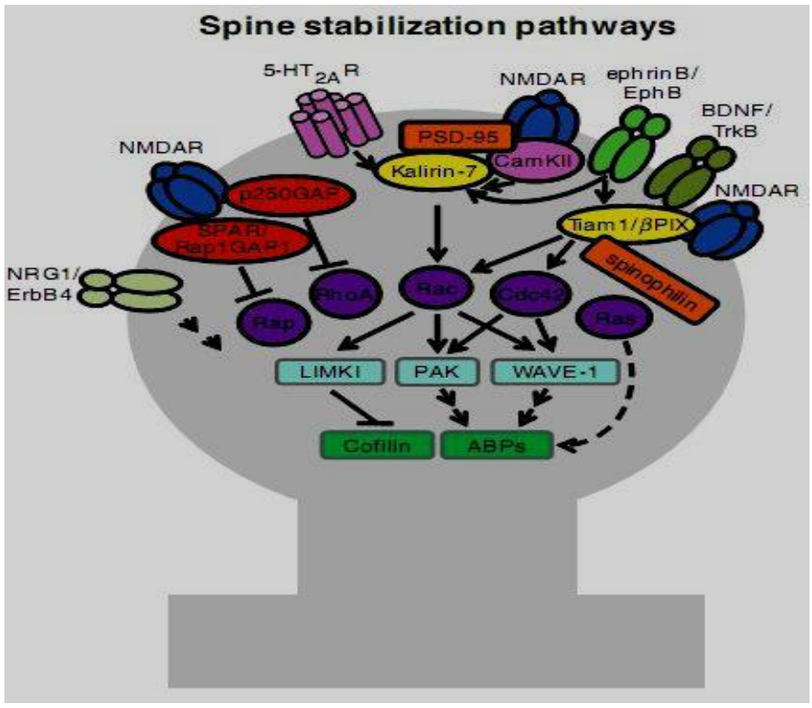


Figure 5 : Molecular mechanisms involved in the regulation of dendrtitic spine morphology {33}**.**

Also, spinophilin, a scaffold protein, has been implicated in actin organization within dendritic spines {38}. As a binding partner of Phosphatase Protein 1 (PP1) that modulates glutamate receptors {39}, spinophilin is responsible for the recruitment of PP1 in plasma membrane. Regarding to dendritic spines morphology determination, it has been shown that spinophilin knockout mice exhibit elevated spine density in caudate-putamen compared to their wild type littermates, while in hippocampal cultured neurons there is a much earlier developmental presence of filopodia in the absence of spinophilin expression {40}. Thus, spinophilin protein acts as a positive regulator of spine morphogenesis by forming complexes with Tiam1 (Rac Guanine Nucleotide Exchange Factor: Rac-GEF) that regulates Rac1 protein actions {41}. As shown in Figure 5, multiple regulating pathways are triggered in the different

compartments of dendritic spines that positively or negatively affect via regulation of cellular cytoskeleton, the morphology and size of dendritic spine, determining synaptic plasticity.

In general, synaptic plasticity is characterized by two separate functions but yet necessarily connected processes, the long-term potentiation (LTP) and long—term depression (LDP). LTP is the ability of neurons to potentiate synaptic transmission by high frequency stimuli, whereas LDP results in weakening of synaptic strength by low frequency stimulation. Both LTP and LDP are based on stimulation of certain synaptic receptors and the availability of neurotransmitters in excitatory and inhibitory synapses. The molecular organization of actin cytoskeleton in dendritic spines is regulated by LTP and LDP synaptic strength, by the presence and subsequent activation mainly of AMPA type glutamate receptors in the tip of spine heads. Specifically, LTP induced by activation of AMPA receptors is responsible for spine head enlargement and spine neck shortening and widening {42}. AMPA receptors are multimeric ionotropic receptors composed of heterotetrameric complexes from combination of GluR1-4 subunits. Fast excitatory transmission results in translocation of AMPA receptors from recycling endosomes to the plasma membrane of post synaptic density {43}. Moreover, the combination of each hetero-complex can determine the cellular localization and the turnover rate thus affecting the magnitude of synaptic strength. High resolution studies have shown that GluR1 and GluR2 subunits are synthesized in dendritic spines and LTP enhanced their synthesis rate {44}. Moreover, the AMPA GluR1 and GluR2 hetero-complexes are translocated in synaptosomal membranes after LTP {45}. GluR1 subunit seems to be sufficient to produce LTP since GluR1 homomeric AMPA receptors are Ca2+ permeable and GluR2 containing AMPA receptors are impermeable to Ca2+ {46}. More specifically, GluR1 intracellular protein levels do not incorporate into PSD. Instead, ready-state exocytic pools located near dendritic membrane are fused to PSD membrane or GluR1 move laterally from dendritic shaft membrane to synaptic sites in order to properly function after LTP induction {47}.

In addittion, NMDA (N-methyl-D-aspartate) receptors are involved in LTP. NMDA ionotropic glutamate receptors are hetero-tetramers composed by two NR1, or NR2 and NR3 subunits, (NR3 subunit has an inhibitory role). Binding of glutamate leads to activation of NMDA receptors, which are now permeable due to ion concentration change of Mg2+ that causes intracellular increases in the concentration of Ca2+ {48}.This elevation triggers the activation of several molecular pathways. Most notable is the activation of signaling kinases such as CamKII (calsium-caldmodulin-dependent protein

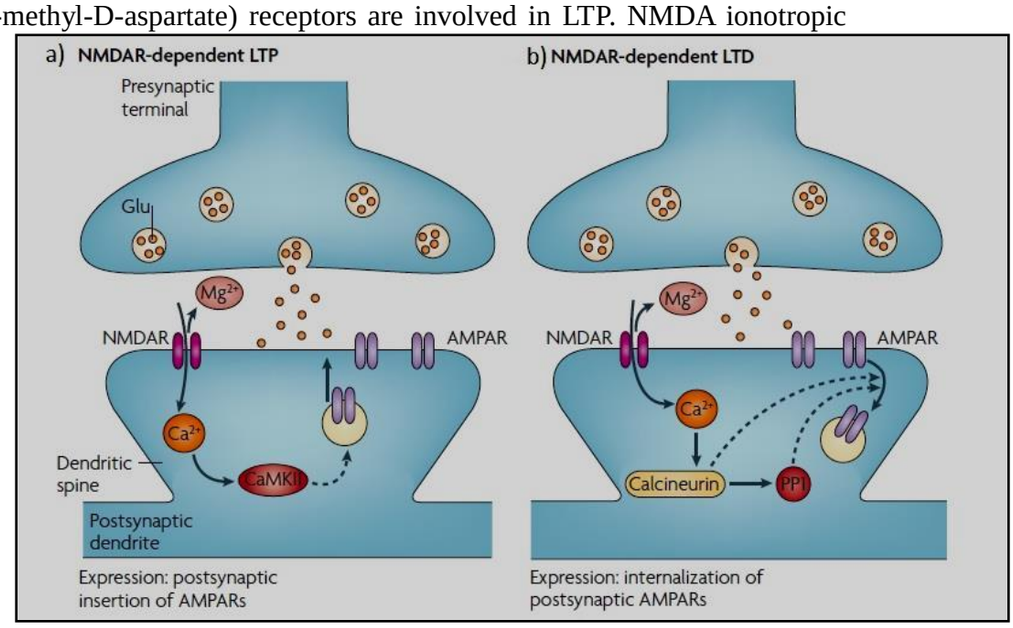


Figure 6 : Molecular mechanisms mediating synaptic plasticity {49}

kinase II) and PKA (Protein Kinase A). Indeed, there is a positive correlation between LTP magnitude with CamKII protein levels in post synaptic densities {50}, whereas mice lacking expression of CamKII alpha subunit, fail to develop proper LTP induced structural plasticity {51}. Similarly, CamKII expression is increased with regard to spine enlargement {52}. CamKII is believed to mediate its action through interaction with NR2B subunit of NMDA receptors {53}. It is suggested that anchorage of CamKII in the NR2B subunit {54} serves as a docking site for Kalirin-7, a brain specific guanine-nucleotide exchange factor (GEF). In this way, phosphorylated Kalirin-7 activates Rac1, thus promoting spine actin polymerization {55}. On the other hand, CamKII is inhibited mainly by PP1 (Phospatase Protein 1) that dephosphorylates the catalytic residue Thr286, only when CamKII is present in the PSD, otherwise PP2A is responsible for the dephophorylation of soluble CamKII in the cytoplasm (56). Also, spinophilin has been shown to interact directly or indirectly with CamKII protein in striatal tissue {57}. Interestingly, dopamine depletion in dorsolateral striatum leads to increased association of spinophilin with PP1 {58}.

More specifically, autophosphorylated form of CamKIIa subunit at Thr286 is required for NMDA receptor dependent LTP induction {59}. Other scientific groups support that kinase activity of CamKIIa is not necessary for pre-synaptic plasticity, showing its actions as a scaffold protein for the assembly of docking proteins in NMDA receptor {60}. On the contrary, CamKII kinase activity seems to be required for AMPA mediated LTP since experiments have shown that activated phospo-CamKIIa phosphorylates AMPA receptors at Ser831 of GluR1 subunit leading to increased ion conductance {61}. In addition, CamKII is important for AMPA receptor trafficking {62}, since CamKII inhibition prevents GluR1 plasma membrane insertion and promotes GluR1 presence in synaptic pools {63}. It seems that CamKII prevents diffusion of AMPA receptors and is responsible for the accumulation of AMPA receptors at specific synaptic sites. This process is independent of GluR1 phosphorylation at Ser831, since mutant form of GluR1 is found in PSD after LTP induction Also, Cloquet and his collegues supported that CamKII seems to be important for the phosphorylation of other assembling proteins, like PSD-95 (a post-synaptic marker) and stargazin {63} that belongs to AMPA receptor regulatory family (TARP), necessary for the regulation of downstream signaling pathways {64}. This scientific group showed also that GluR1 Ser831 phosphorylation was not efficient to mediate LTP induction {63}. To extend this notion, replacement of the C terminal cytoplasmic tail of GluR1 that contains Ser831 residue with the cytoplasmic tail of GluR2 subunit did not affect single cell LTP induction, whereas impairment in AMPA trafficking had the opposite results, supporting that existence of synaptic AMPA pools and that receptor subunit substitution could mediate LTP function {65} Other scientific groups support that AMPA insertion in PSD requires CamKII-AMPA interaction and association of GluR1 with a protein containing PDZ domain {66}. PDZ domains are secondary structures, important for formation of scaffold complexes and are present in almost every protein present in the PSD that is involved in organizing glutamate receptors, therefore controlling LTP {67}.

Scientists have focused more in the involvement of LTP and its correlation with dendritic spine morphology, since little is known about the molecular mechanisms underlying spine shrinkage during LTD {68}. Compared to LTP, long-term depression requires AMPA

internalization. This process depends on two signaling events {69}. The first one is the de-phosphorylation of GluR1 AMPA subunit at Ser845 (Protein Kinase A phosphorylation site) that resulted in increased internalization rate {70}. Knock in mice bearing mutations for both Ser845 and Ser831 do not elicit NMDA dependent LTD and generate decreased synaptic strengths {69}. Secondly, activation of protein phospatase 1 (PP1) and protein phosphatase 2 (PP2) are key players mediating LTD. More specifically, mutations in PP1 domains necessary for the protein interaction abolish NMDA-dependent LDP {71}, while disrupted interaction of PP1 with spinophilin, among other proteins, leads to increased NMDA and AMPA receptor responses, suggesting that PP1 is active only when it is bound with its partners {72}. In particular, spinophilin seems to be important in LTD, since knock out spinophilin mice do not show LTD responses {40}. Moreover, GSK3β (Glycogen Synthase Kinase 3β), a molecular target of PP1 seems also very important in the induction of LPD, since GSK3β co-immunoprecipates with AMPA receptors after LTP induction and during LDP GSK3β is activated by dephosphorylation of Ser9 by PP1 {73}.

Taken all together, several molecular mechanisms are crucial for determining dendritic spine number and morphology and these characteristics of neuronal cells depict synapse formation, maintenance and elimination, allowing neuronal connectivity to maintain behavioral processes. Thus, it is assumed that aberrant molecular signaling involved in these processes often leads to the development of several maladaptations that occur in brain disorders such as drug addiction or neuro-degenerative diseases such as Alzheimer or Parkinson disease. So, further research must be done, in order to elucidate the exact mechanisms involved in the formation and maintenance of dendritic spines.

1.4 REGULATION OF SYNAPTIC PLASTICITY IN DRUG ABUSE NEUROBIOLOGY

It is widely accepted that drug addiction affects behavior by inducing neuro-adaptations due to persistent synaptic changes in related neuronal systems involved in the chronic reinforcement drug effects. More specifically, in most cases, drug of abuse mediate their actions by altering LTP and LTD processes in excitatory synapses thus altering molecular pathways involved in learning and memory, since persistent drug usage alters homeostatic intracellular pathways. For example, drugs of abuse that generally decrease neuronal activity enhance molecular mechanisms participating in the elevation of synaptic strength, whereas chronic drug intake that increase neuronal firing initiate homeostatic regulation programs conferring decreased synaptic strength {74}. The activation of such signaling events trigger molecular adaptations, that are depicted either in the neuronal morphology or in the pattern expression of specific neuronal cell types located in brain regions affected by drug of abuse actions {75}.

In general, all drug of abuse, including psychostimulants and opioids enhance excitatory synaptic transmission in dopamine cell neurons in an NMDA dependent manner as a consequence of AMPA subunit upregulation {75,76}, whereas non addictive drugs do not have the same results in increasing AMPA/NMDA ratio of excitatory synaptic currents {76}. These effects are mediated predominantly by GluR1 subunit since overexpression of this specific subunit in the ventral tegmental area (VTA) increased locomotor sensitization activity after

morphine treatment compared to LacZ overexpressing animals that were also treated with morphine. On the contrary, overexpression of GluR2 subunit did not produce morphine sensitivity {78}. Additionally, subcutaneous administration of chronic morphine resulted in elevated levels of GluR1 subunit in the VTA {79}. Moreover, phosphorylation of GluR1 at Ser845 was increased in dorsal striatum in a locomotor behavioral paradigm after pretreatment with increasing morphine doses {80}, further supporting a role of GluR1 AMPA subunit in synaptic plasticity induced by opioids. On the other hand, acute morphine has been shown to participate in the inhibition of GABAergic inhibitory neurons that project to dopaminergic neurons in the VTA. This type of plasticity requires activation of NMDA receptors in dopamine neurons and the release of NO (Nitric Oxide) by NOS (Nitric Oxide Synthase). The uptake of NO by GABAergic neurons leads to GABA release and LTP_{GABA} by activation of guanylyl cyclase activity (GC). Morphine inhibits LTP mediated by the neurotransmitter GABA by inhibiting the actions of NO {81}. It seems like VTA GABA neurons are required for the initiation of aversion behaviors, since inhibiting GABA neurotransmitter release leads to reward related behaviors that are connected with learning processes {82}.

Altered synaptic strengths by drugs of abuse regulated by AMPA subunits have been also correlated with dendritic spine morphology of medium spiny neurons and also with changes in cell soma size of VTA neurons. As shown in Figure 7, chronic morphine treatment results in decreased spine density of NAc MSN {83), hippocampal pyramidal neurons {84}, medial neurons in prefrontal cortex {85}. More specifically, repeated morphine treatment in cultured hippocampal cells decreased expression of synaptic AMPA receptors and also in hippocampal neurons cultured from mice lacking expression of mu opioid receptor there was an increased spine density compared to their wild type littermates, even with morphine treatment {84}. Regarding cell body alterations induced by opiates, decreased VTA cell size soma was associated with sustained downregulation of IRS-2 (Insulin Receptor Substrate 2) Akt pathway. This particular morphological change persisted for two weeks after morphine withdrawal {86}. On the other hand, no change in spine density was found in neurons located in primary somatosensory cortex (S1) {87} Compared to opiates, psychostimulants have the opposite effects in the dendritic spines density in the same regions compared to morphine, where chronic cocaine leads to increased spine density {83}. However, little is known about the molecular mechanisms involved in the differential regulation of dendritic spine morphology induced by these drugs of abuse, taken in account that both opiates and psychostimulants share common regulatory programs for altering synaptic strengths and they induce behavioral responses such as tolerance, withdrawal symptoms and relapse events.

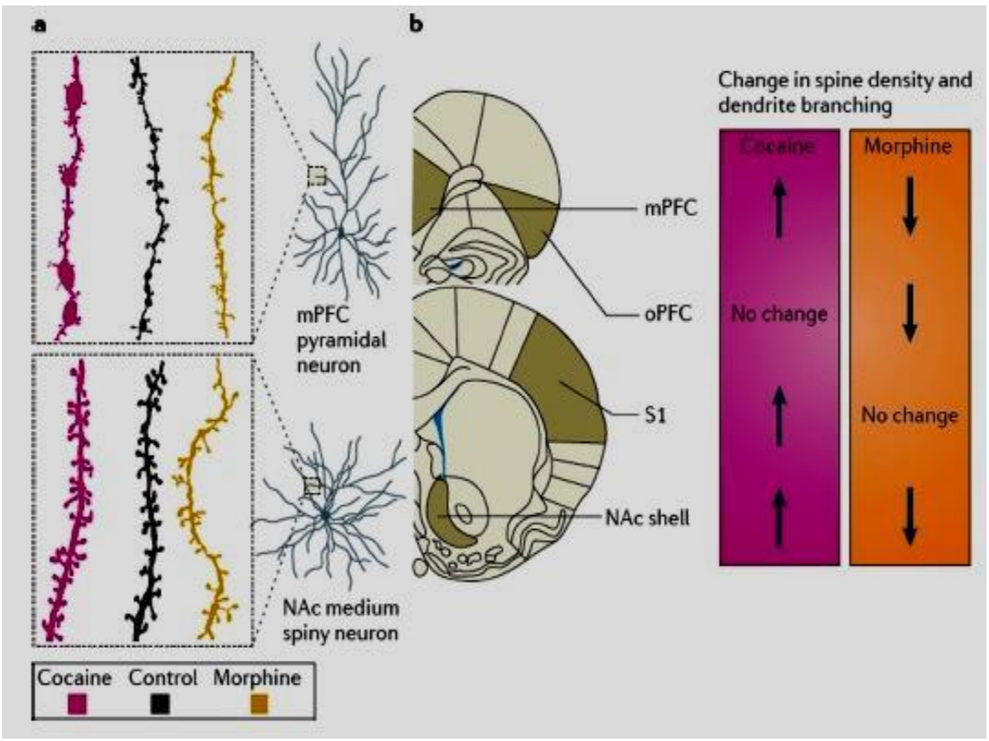
In particular, chronic cocaine induced upregulation of CamKIIa in NAc shell where protein levels of phoshorylated GluR1 at Ser831 were also elevated, a substrate of autophosphorylated form of CamKIIa, but no difference of the autophophorylated form of CamKIIa in protein levels were found {88}. On the other hand, acute cocaine results in elevated phospho Thr876 CamKIIa in dorsal striatum {89}. Regading CamKII activity by opiates, both inhibition of CamKII and calcineurin in hippocampal neurons reverse loss of dendritic spines induced by morphine treatment. Moreover, morphine is responsible for the translocation of CamKII in dendritic spines, further confirming the implication of CamKII in morphine's action

{90}. Interestingly, CamKIIa is essential for ΔfosB induction through stabilization of ΔfosB by phosphorylation. Subsequently, activated form of ΔfosB protein leads to CamKIIa upregulation after chronic cocaine treatment {87}. ΔfosB is a transcriptional factor upregulated by morphine in nucleus accumbens but also in the dorsal striatum and its overexpression leads to elevated morphine sensitivity {91} and increased dendritic spine density {92}.

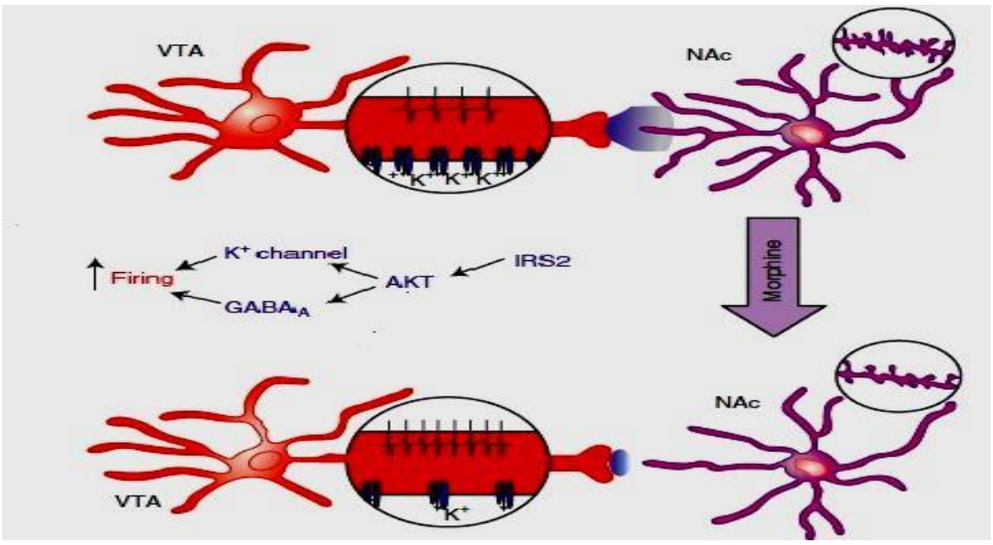
Unlike psychostimulants, in opiate-induced plasticity, the transcription factor that seems to be important for morphine actions is NeuroD, since overexpression of this molecule attenuated the effect of morphine in decreased spine density {93}. NeuroD is substrate of CamKII, which phosphorylates NeuroD at Ser336. This specific phosphorylation is not required for the regulation of gene expression but is important for dendritic growth {94}. Another key molecule involved in molecular pathways stimulated by drugs of abuse is BDNF (Brain Derived Neurotrophic Factor). As a neurotrophic factor, BDNF synthesis in dendrtitic spines is essential for spine maturation and spine head enlargement {95}. In contrast, in cultured cells BDNF overexpression leads to instability of dendritic spine density {96}.As mentioned above chronic morphine leads to decreased dopamine neuron cell size soma in the VTA and this effect is reversed by BDNF infusion in the VTA {86}. Although until now there is not known direct relationship between BDNF-dendritic spine regulation and opiates, BDNF is negatively regulated by chronic morphine and induces VTA dopamine neurons excitability through decreased expression of BDNF since DA neurons from AAV-Cre-VTA mice for *bdnf* gene display enhanced burst firing rates compared to AAV-GFP mice {97}. On the other hand, acute morphine or chronic cocaine administration have been shown to upregulate BDNF expression in the nucleus accumbens {98}. In addition, BDNF downstream molecular pathways have been shown to participate in chronic morphine actions, such as PLCγ (Phosholipase Cγ), PI3K (Phosphatidylinositol 3 Kinase) and MAPK (Mitogen Activated Protein Kinase). Chronic morphine administration results in elevated levels of PLCγ and decreased protein expression of PI3K, with the latter change to be responsible for the decreased VTA cell soma size, via downregulation of IRS-2-Akt signaling {86}. As shown in Figure 8, this molecular adaptation is believed to increase fire rating of VTA neurons through upregulation of potassium channels.

Apart from molecular changes in key neurotrophic pathways induced by abuse drug, there is evidence that they also participate in the regulation of dendritic spine morphology regarding remodeling of actin cytoskeleton, since molecules participating in regulation of actin branching and spine growth could be key players in drug abuse actions. More specifically, active Rac1 has been found to be down-regulated after chronic cocaine treatment and knocking down Rac1 specifically in the NAc increased density of thin spines via enhanced activity of cofilin. Moreover, Rac1 overexpression in nucleus acccumbens reduced locomotor activity of cocaine treated animals, showing that Rac1 repression is important for chronically actions induced by cocaine {99}. Similarly, in hippocampal neurons chronic morphine leads to decreased levels of active Rac1 {84}.

All together, these molecular signaling alterations by drug abuse are depicted in dendritic spine density and morphology in brain regions implicated in drug addiction. Unraveling molecular changes in determination of dendritic spine morphology and density is very important since drug of abuse are responsible for altering homeostatic regulation expression patterns that are important for the induction of LTP or inhibition of LDP and thus for altering synaptic transmission.



<u>Figure 7:</u> Altered dendritic density after exposure to psychostimulant and opiates. Increased spine density is induced by cocaine in medial PFC, primary somatosensory cortex (S1) and in NAc shell, without altering it in orbital PFC. On the other hand, morphine decreases dendritic density in the same regions, except the S1 region where no change is observed {100}.



<u>Figure 8</u>: Molecular adaptations induced by chronic morphine in the VTA neurons, leading to decreased VTA cell size soma and increased firing rates from VTA neuronal population {101}.

1.5 G -PROTEI N COUPLED RECEPTOR SIGNALING

G-protein coupled receptors represent the largest family protein members of cell surface receptors. These seven trans-membrane receptors are expressed almost in every cell type and are responsible for the regulation of several molecular pathways involved in crucial physiological processes, such as neurotransmission, differentiation, proliferation and inflammation. However disruption GPCR signaling leads to disease development. Approximately, 40% percent of prescribed drugs target these receptors based on their unique expression pattern and ligand selectivity, which act either as agonists or antagonists. {102}.

Upon agonist binding, GPCRs undergo a conformational change that allows interaction with heterotrimeric G proteins. Crystalographic studies have shown that each ligand promotes specific conformational changes in order GPCR to mediate the assembly of different combinations of effector molecules {103}. The formation of transient ligand-GPCR-G protein complex activates G proteins, thus leading to activation of downstream signaling pathways. G proteins comprise of three different subunits (α, β and γ). Ga subunit act as GTPase that further triggers signaling molecules after dissociation with Gβ and Gγ subunits that together form a dimer. Ga subunits can be classified into four major categories, depending on the signaling transduction. Gas is responsible for the stimulation of adenylyl cyclase resulting in increased cyclic AMP, while Gai/o inhibits adenylyl cyclase and voltage-gated calcium channels but activates potassium channels and MAPK pathway (Mitogen Activated Protein Kinase). Moreover, Gaq is responsible for the activation of PLCβ (Phospholipase Cβ) and inhibition of GIRK (Inward rectifying potassium channels). Moreover, Gβγ can also trigger the activation of several protein molecules. On the other hand, deactivation of G protein results from hydrolysis of bounded GTP to GDP from the Ga subunit. Subsequent G protein activation is blocked due to the occupation of GPCR with phosphorylation kinases. Thus, GPCRs are being subjected to phosphorylation events in the C terminal cytoplasmic domain, leading to GPCR desensitization followed by endocytosis, which is the main characteristic of GPCR {104}.

1.6 INSIGHTS IN OPIOID RECEPTORS : MU OPIOID RECEPTORS

Opioid receptors are well characterized receptors, thought to be involved in several molecular pathways in the CNS contributing to the development of addiction. They have been classified into three categories, referred to as μ, δ and κ opioid receptors with distinct pharmacological properties. Chen and his colleagues were the first to clone mu opioid receptors that belong to the GPCR family and display sensitivity towards adenylyl cyclase regulation {105}. Endogenous ligands, such as beta endorphin and encephalin, are the primary agonists of MOR, whereas morphine, heroin, fentanyl and methadone are considered to be the exogenous administered opioids that bind with high affinity to MOR {106}.

MOR is expressed widely in the central nervous system (CNS) as long as in the peripheral nervous system (PNS). Specifically, MOR mRNA is expressed mainly in the thalamus, striatum, locus coereleus the solitary nucleus {107} and the spinal dorsal horn {108}. Interestingly, MOR

mRNA expression in the rat caudate putamen nucleus begins at embryonic day 13 {109}. Similarly, MOR is localized, within the first postnatal week, in dendrite plasma membrane and in dendro-dentrite junctions, which are membrane regions containing several channels responsible for the diffusion of metabolites and second messenger molecules. MOR localization in dendritic spines also correlates with synaptogenesis in this specific brain region {110}.

Morphine administration in mice lacking expression of mu opioid-receptor revealed that only this opioid receptor subtype is responsible for morphine's action, since deletion of MOR blocked analgesia in several antinociceptive behavioral experiments {111}.Activation of MOR by morphine binding, leads to association of MOR with Gi/o subunits, following activation of several downstream signaling molecules including the G protein- coupled inwardly rectifying pottasium channels (GIRK). Cultured studies with hippocampal neurons have shown that morphine up-regulates expression and induces localization of $GIRK_2$ in dendritic spines {112}, indicating that GIRK2 activation could be a mediator for the analgesic effects of morphine {113}. Another research group, associated the localization of $GIRK_2$ in dendritic spines with the Gβγ-RGS7 complexes in the CA1 hippocampal region {114}, where Gβγ interacts with the C terminal cytoplasmic tail of GIRK, together with the GGL domain of R7 RGS (Regulators of G Protein) family protein members {115}. Taken into account the R7 RGS expression pattern in the striatum, RGS9-2 could be a player for regulation of GIRK activation in the striatal dendritic spines.

Upon activation of MOR by agonist binding, residues in MOR cytoplasmic domain can be phosphorylated by different kinases, such as G protein-coupled receptor kinases (GRKs), second messenger-regulated kinases (PKA, PKC), CamKII and ERK1/2. Depending on the ligand, these phosphorylation events can trigger activation of several intracellular mechanisms and affect the internalization rate of MOR, thus contributing in opioid tolerance. It is well documented, that MOR phosphorylation after morphine administration triggers assembly of β-arrestin2, an adaptor proteins necessary for receptor trafficking to proteasome {116}. Notably, β-arrestin2 is located mainly in the post-synaptic densities and it is widely expressed in the striatum in order to regulate G protein-coupled neurotransmitter receptors {117}. Interestingly, morphine treatment in knock-out mice for *β-arrestin2* gene, with a subanalgesic dose for wild type animals, resulted in a sustained MOR signaling {118}.

All the above signaling molecules contribute to MOR densensitization, triggering its endocytosis in order to be recycled. Failure to promote endocytosis, decreased MOR cell surface expression or inhibition of MOR functional signaling are the main reasons for conferring opioid-receptor tolerance in CNS.

1.7 REGULATORS OF G PROTEINS (RGS)

Regulators of G proteins are consider to act as GAPs (GTPase Accelerating Protein) and comprise a large protein family that can be further categorized into eight subfamilies with distinct domains and certain functions for each subfamily as shown in Figure 10. Their classification depends on the RGS domain homology and their common domains apart from RGS domain that are responsible for their subcellular localization, protein stability and their additional functions except from their GAP activity, such as protein-protein interactions.

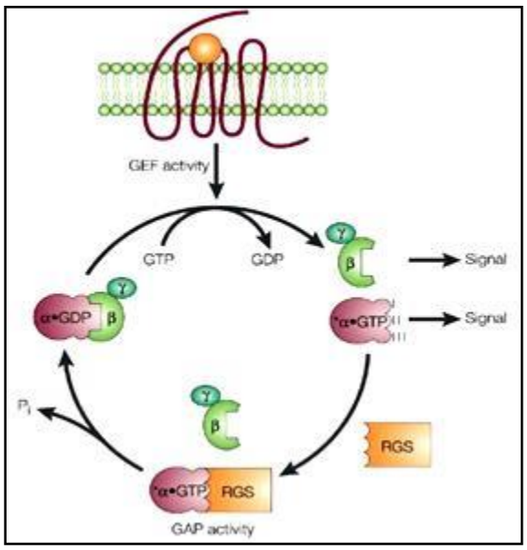


Figure 9 : Molecular mechanism of RGS protein signaling {125}.

The catalytic activity of RGS proteins is depended on the conserved RGS domain which is responsible for the direct interaction of RGS proteins with the Gα subunit of G proteins. RGS proteins are proposed to act by two different mechanisms. Firstly, they terminate Gα protein signalling by accelerating GTP hydrolysis and secondly they act as effector proteins by inhibiting G protein activity.

Moreover, most of RGS are expressed in the CNS, with distinct role in the regulation of molecular pathways {119}, but each RGS protein is characterized by distinct expression pattern and regulation specificity towards Gα activity, GPCR proteins and other cellular signaling molecules. Despite their differences in RGS pattern expression, RGS are important for regulating important physiological processes. For example, RGS9-2 and RGS4, are both expressed in NAc but RGS9-2 seems to modulate opioid responses {120} compared to RGS4 which is important for regulating morphine actions in the locus coeruleus {121}. Lastly, disrupted RGS signaling has been implicated in several neuropathological their dysfunction is associated with several neuropathological conditions such as addiction, schizophrenia {122} depression (123} and Parkinson disease{124}.

1.8 RGS9 -2 MOLECULAR SIGNALING IS INVOLVED IN ADDICTION

RGS9-2 is a 72-kDa protein, member of R7 subfamily and apart from its catalytic conserved RGS domain that confers specificity for Gαi/o subunits {126}, also contains several non-catalytic domains. The first non-catalytic domain is the G-protein γ-like (GGL) region that binds to G protein β5 subunit, thus contributing to the stability and proper folding of the RGS9-2, since deletion of Gβ5 gene blocks the expression of RGS9-2 and RGS7 at the protein level {127}. Also, studies in cultured cells have shown the nuclear localization of RGS9-2, due to the proline rich motif (SH3-like), with or without the formation of Gβ5-RGS9-2 complexes {128}. This proline-rich encoding motif is only present in the RGS9 mRNA isoform {129} and it is

crucial for the assembly of signaling proteins, which may be necessary for G protein-mediated signaling occurred solely in brain-specific regions and not in retinal photoreceptor cells. Moreover, RGS9-2 structure includes the Disheveled, Egl-10, Pleckstrin (DEP) domain, which is approximately 90 amino acids and it is an important region for the interaction of RGS9-Gβ5 complexes with the adapter protein R7BP (R7 Binding Protein). This specific interaction contributes to the cytoplasmic relocation of these proteins to the plasma membrane and to post-synaptic density fractions via palmitoylated cysteines in the C terminus of R7BP {130} and protects RGS9-2 from proteasome degradation {131, 132}.

RGS9-2 is a splice variant of *Rgs9* gene that is expressed mostly in the CNS, {120}, with RGS9-2 being involved in antinociceptive signaling by modulating sensitivity of the mu opiod receptor {133}. Specifically, RGS9 mRNA is highly expressed in the striatal brain regions (e.g. caudate putamen (dorsal striatum), nucleus accumbens (ventral striatum), olfactory tubercle) and is present in minimal amounts in the hypothalamus, neocortex, dentate gyrus and medial amygdala {119,134}. Taken in account the expression pattern, RGS9-2 protein seems to mediate the molecular responses of several GPCRs in different brain regions, but RGS9-2 role has been mostly investigated in the striatum, which is a key region for molecular mechanisms involved in addiction. Interestingly, RGS9-2 protein levels were decreased in the nucleus accumbens (NAc) and dorsal striatum with chronic morphine treatment. Deletion of RGS9-2 enhanced sensitivity to morphine reward using the place preference condition paradigm (CCP), while over-expression of RGS9-2 particularly in NAc had the opposite effect in mice. In contrast, over-expression in dorsal striatum failed to restore the phenotype seen in CCP. Also, RGS9 KO mice showed enhanced morphine induced analgesia and more severe morphine withdrawal symptoms compared to their WT littermates {120}. To extend this notion, cell cultures studies have shown that RGS9-2 directly interacts with MOR after acute morphine treatment and suppresses MOR actions by decreasing its endocytosis rate {135}. Pharmacological manipulations with opiates have also indicated that RGS9-2 participates in MOR actions, by regulating Gai3 subunit signaling after acute morphine treatment, whereas acute fentanyl administration leads to association of RGS9-2 with Gαq, an effect seen also in chronic morphine application {136}.

RGS9-2 role has been also investigated in dopamine signaling since RGS9-2 is widely expressed in medium spiny neurons and in cholinergic interneurons in dorsal striatum, and results in a decreased signaling of D2 receptors leading to inhibition of Ca^{2+} currents {137}. Another study supporting the idea that RGS9-2 modulates D2 receptor signaling came from -in situ hybridization studies which confirmed that D2 receptors co-localize with RGS9 in striatal neurons {138}. In fact, RGS9-2 inhibits D_3 receptor subtype through interaction with β-arrestin2, which is required for the specificity of this inhibition by RGS9-2 {139}. Additionally, behavioral experiments that study dopaminergic functions (rotation behavior) showed that administration of D_2 agonist resulted in an enhanced circling behavior towards the side of HSV-LacZ expression and not RGS9-2 in nucleus accumbens. Similarly, cocaine treatment, which acts though D1 or D2 receptors decreased locomotor activity in RGS9-2 over-expressed animals compared to animals that were treated with control, whereas RGS9 knock out animals showed significantly increased locomotor activity compared to their WT litter-mates. Chronic cocaine treatment resulted in reduced RGS9-2 protein levels in the striatum. Also, deletion of *Rgs9* gene

did not alter protein expression of D2 receptor {138}.

These data indicate that RGS9-2 negatively regulates dopamine signaling. Furthermore, human studies have shown that dopamine levels are inversely correlated with RGS9-2 protein levels that also are elevated in putamen after chronic L-dopa treatment, a drug administered in Parkinsonian patients with several side effects including dyskinesia {140}. Lastly, in a primate model, striatal over-expression of RGS9-2 diminished the dyskinesia symptoms, whereas parkinsonian RGS9 KO mice are more sensitive to L-dopa side effects compared to their WT littermates {141}.

2 . MATERI ALS AND METHODS

2.1 ANIMALS

Mice were housed in a facility and kept on a 12-hour light/dark cycle, with food and water available ad libitum. Animal handling and experiments were in accordance to the guidelines of the Institutional Animal Care and Use Committee of the University of Crete. Experiments were carried out with RGS9 wild type and RGS9 knock out male mice that were 2-3 months old. Mice were injected subcutaneously (s.c.) with morphine sulfate. Doses were prepared daily, and morphine was diluted in saline.

2.2 LOCOMOTOR ACTIVITY

Locomotor sensitization assay is used to measure the progressive increase in locomotor activity that is caused by repeated drug exposure. Locomotor activity has been described to be linked with drug induced plasticity, since locomotor sensitization can be persistent even after withdrawal. For testing morphine locomotor sensitization, mice were tested blindly at the same time each day. The locomotor activity chamber was a plastic cage (28 × 17 × 12 cm) and horizontal movements were measured with photocell beams. Mice were habituated for the first three days with saline injections prior to locomotor activity testing. In days four, five and six, mice were injected with morphine 10mg/kg subcutaneously and were placed into the chambers after morphine and saline injections and locomotor activity was recorded for 30 minutes.

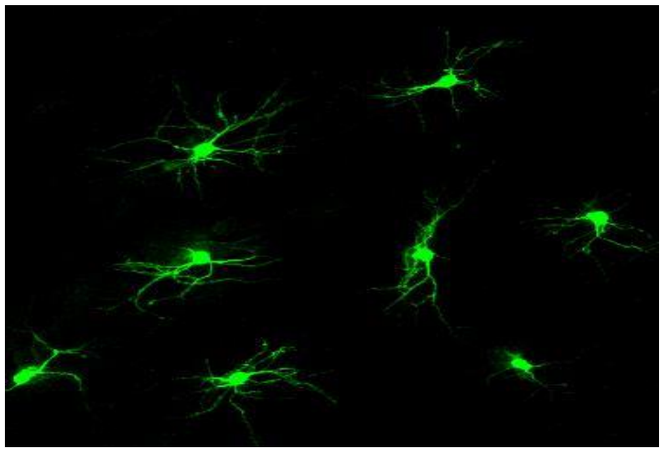
2.3 CELL MICROINJECTION

Thirty minutes after the end of the third morphine locomotor sensitization session, mice were anesthetized with pentobarbital and perfused with 4% paraformaldehyde and 0.125% glutaraldehyde in 0.1 M phosphate saline buffer. Brains were sectioned into 250 μm slices. Cells in dorsal striatum were impaled with a micropipette containing 5% Lucifer Yellow (LY, Molecular Probes), injected with 1–10 nA of current, and mounted (Vectashield) for confocal microscope.

2.4 CONFOCAL IMAGING

Proximal dendrites were defined as the middle portions of terminal dendrites, and images were 25±2 μm away from soma. Distal dendrites were defined as the dendrtitic tip and images were taken 10μm away from proximal dendrites. From each animal, 25 proximal and 10 distal dendrites from average 5 neurons from each hemisphere were imaged. The vast majority of distal dendrites were imaged from the same cells that were included in the proximal dendrite analysis. First, a whole image was captured as a guide for correctly tracking the neurons with a 10X objective on an inverted Zeiss LSM 710 confocal microscope (fig. 11) and then Z-stacks were performed using a using a 100×1.4 NA oil objective. The size of each voxel was 0.033×0.033×0.33 μm3 voxel size in order to acquire a 45μm x 6,3 μm image of each dendrite.

Figure 10: 10X image of neurons located in dorsal striatum.



2.5 STATISTICAL ANALYSIS

Deconvolution of images was performed with AutoDeblur (MediaCybernetics) and spine analysis was carried with the semi-automated software NeuronStudio (http://research.mssm.edu/cnic/tools-ns.html), which analyzes dendritic length, spine number, and spine head and neck diameter. NeuronStudio further classifies spines into three major morphologic types: thin, mushroom, and stubby. Excel, Matlab, and GraphPad Prism were used. Total and subtype spine densities were calculated by dividing the total number of spines by the length of the dendritic segment. The average proximal or distal spine density and (head and neck) diameter for each neuron was then calculated followed by the total average density/diameter for each brain region in each animal. Statistical differences were measured using two-way ANOVA.

2.6 CELL FRACTIONATION PROTOCOL- WESTERN BLOT

Striatal tissue from locomotor sensitized mice was taken thirty minutes after their last session. Samples were sonicated in 0.32M sucrose buffer containing 1% SDS and 0.1% protease, phosphatase and proteasome inhibitor (MG132, Sigma Aldrich. An aliquot of 100µl was obtained for homogenate cell fractionation. Subsequently, samples were centrifuged (1000g) for 5min at 4o C. Supernatant was kept and centrifuged (16000g) for 20min at 4o C to obtain crude synaptosomal cell fractionation. Pellet was resuspended in 0.1mM CaCl2 buffer. Then, incubation of synaptosomal cell fractionation was performed for 20min at 4o C with a hypotonic buffer containing 1M Tris (ph 6), 10% Triton X-100 and 0.1mM CaCl2. Samples were then centrifuged (40.000g) for 30min at 4o C. Synaptic junctions were obtained by resuspending pellet in a buffer containing 20mM Tris and 1% Triton X-100. After, incubation of synaptic junction was followed for 20min at 4oC. Then, samples were centrifuged (40.000g) for 30min at 4o C and post synaptic density pellet was resuspended in 1% SDS. For western blot, samples were quantified using the Lawry method (Bio-Rad). Then the lysates were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Transferring proteins were performed with polyvinylidene fluoride (PVDF) membranes (Bio-Rad). Membranes were incubated with 1X PBS-T (Phosphate Buffered Saline Tween-20) containing 3% skim milk, for 1 hour at RT. Primary antibody incubation was performed at 4oC overnight. The following day, membranes were washed with 1X PBS-T, and then were incubated with horse peroxidase labeled goat anti- rabbit IgG (Jackson ImmunoResearch) or horse peroxidase labeled anti-mouse IgG (1:20.000) Invitrogen for 1h at R.T. Bands were visualized with SuperSignal West Dura substrate (Pierce). Primary antibodies used: rabbit GluR1 (1:5000), rabbit GluR2 (1:3000), rabbit phospho-CamKII (1:1000) mouse CamKIIa (1:2000), rabbit LimK1 (1:1000), rabbit Rac1 (1:1000). Gb5 was used as control, since it have been shown that morphine does not regulate Gb5 protein expression{120}

3 .RESULTS

3.1 LOCOMOTOR ACTI VITY

In order to evaluate the locomotor response to morphine administration of RGS9 wild type and knockout mice, locomotor activity of these mice was measured during habituation with saline injections(days 1-3) and during morphine injections (days 4 and 5). As shown in Figure 11, morphine enhanced locomotor activity to both genotypes compared to saline-treated animals, but in day 5 the locomotor activity of RGS9 knockout mice was increased compared to RGS9 wild type mice that received morphine injections

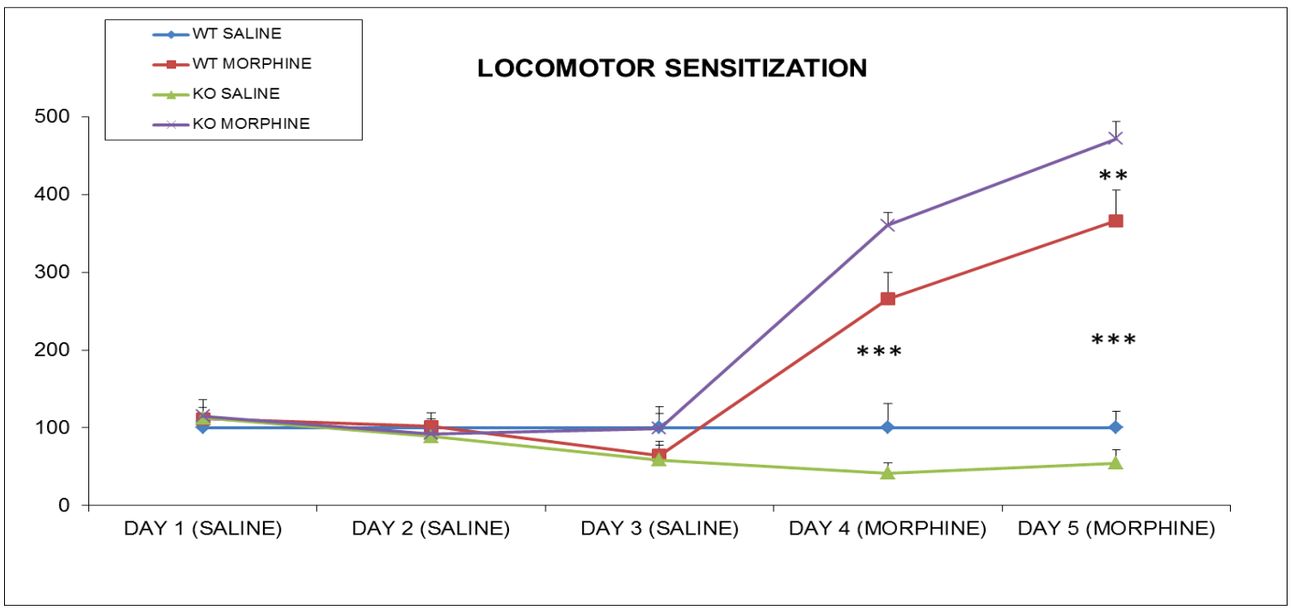
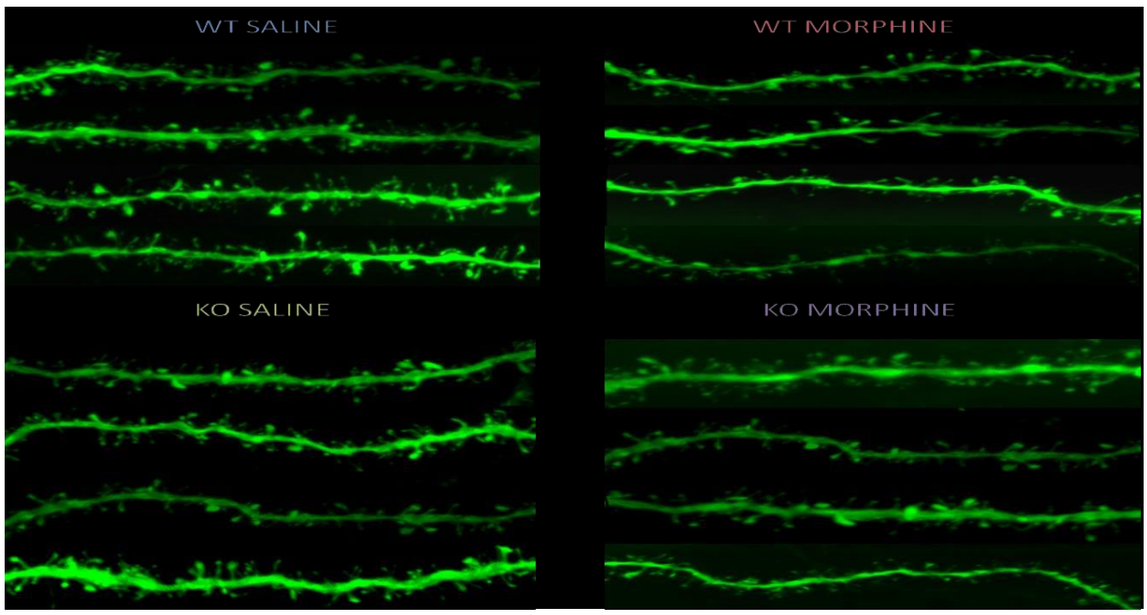


Figure 11: Morphine-injected RGS9 knockout mice exhibit enhanced locomotor activity compared to RGS9 wild type mice that were also treated with morphine. Morphine was administered s.c. at 10mg/kg, (Day 4: ***p<0.0001 for treatment, p=0.2817 for genotype, p=0.8739 for interaction)/ (Day 4: ***p<0.0001 for treatment, **p=0.0066 for genotype, p=0.3074 for interaction). Values expressed as average ± SEM (normalized to WT SAL) and analyzed using two-way ANOVA. (WT: wild type, KO: knockout) (n=5-7).

3.2 CONFOCAL IMAGING

Considering the effect of opiates in altering spine density and moprhology in various brain regions important for drug abuse actions, such as nucleus accumbens, we wanted to observe the effect of morphine sensitization in determining proximal or distal dendritic spine density in the neuronal population of dorsal striatum in the presence or the absence of RGS9-2 protein expression. Representative pictures are shown in fig.13 (a for proximal and b for distal dendrites)

a) ***PROXIMAL DENDRITIC SPINES***



a) DISTAL DENDRITIC SPINES

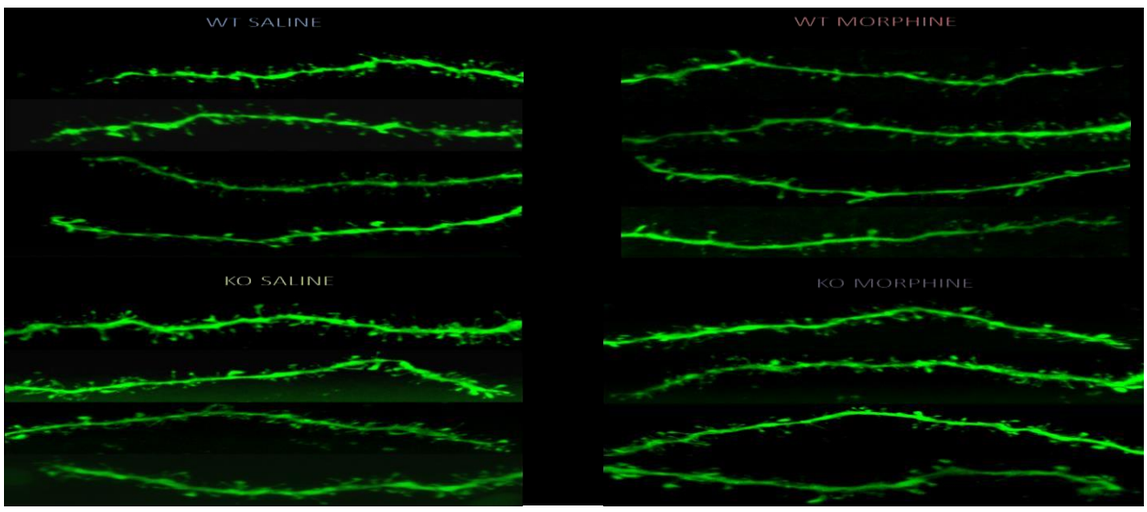


Figure 12: Representative pictures of proximal and distal dendrites in RGS9 wild type and knockout mice treated either with saline or morphine. (WT: wild type, KO: knockout)

3.2.1: EFFECTS OF MORPHINE TREATMENT IN SPINE DENSITY OF PROXIMAL DENDRITES OF RGS9 WT AND KO MICE

As mentioned in the introduction, morphine induces structural plasticity changes, including alterations in dendritic spine density. Proximal dendrites in dorsal striatum neurons, belonging to morphine locomotor sensitized mice, were analyzed and measured for their total dendritic spine density as well as for each spine subtype, i.e. mushroom, thin and stubby. In particular, morphine did not alter total and thin spine density (Fig. 13b and d), whereas it resulted in decreased mushroom density only in the absence of RGS9-2 protein signaling. Notably, RGS9-2 signaling pathway is not essential for decreasing mushroom spine density, since saline treated RGS9 knockout mice display the same basal levels for mushroom density with saline treated RGS9 wild type mice (Fig. 13a). Lastly, stubby density was affected negatively by morphine only in RGS9 knockout mice compared to their morphine treated RGS9 wild type. On the other hand, morphine did not alter stubby density in saline treated mice. Also, stubby spine density is not altered among genotypes that were drug differentially treated (Fig. 13c).

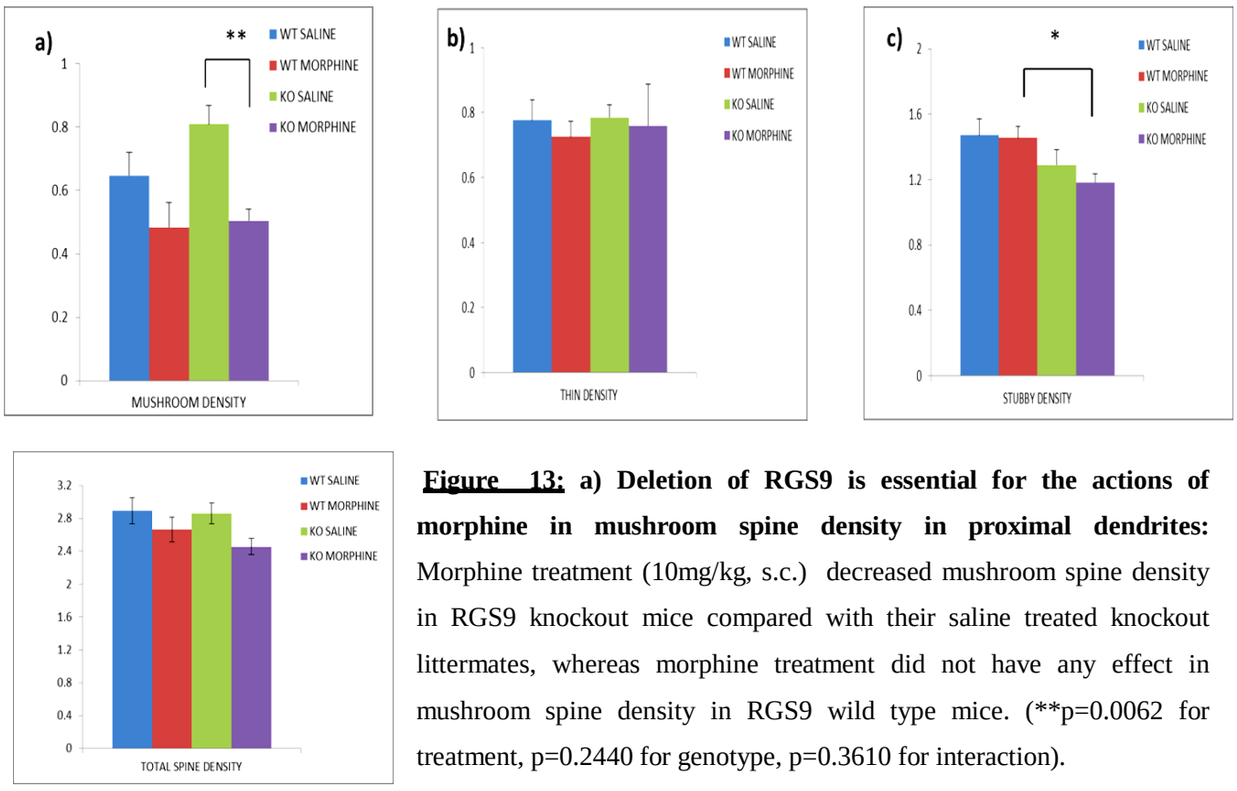


Figure 13: a) Deletion of RGS9 is essential for the actions of morphine in mushroom spine density in proximal dendrites: Morphine treatment (10mg/kg, s.c.) decreased mushroom spine density in RGS9 knockout mice compared with their saline treated knockout littermates, whereas morphine treatment did not have any effect in mushroom spine density in RGS9 wild type mice. (**p=0.0062 for treatment, p=0.2440 for genotype, p=0.3610 for interaction).

b) Morphine treatment did not alter thin spine density in RGS9 wild type or RGS9 knockout mice: Morphine treatment did not affect thin spine density in RGS9 knockout mice as compared to their saline treated knockout mice or in RGS9 wild type mice (p=0.5621 for treatment, p= 0.7423 for genotype, p=0.8380 for interaction).

c) RGS9 mediates morphine action for increasing stubby spine density: Morphine treated RGS9 knockout mice displayed decreased stubby density compared to morphine treated RGS9 wild type mice. (*p=0.0367 for genotype, p=0.5573 for treatment, p=0.6587).

d) Morphine did not alter total spine density in RGS9 wild type and knockout mice: Subcutaneous morphine treatment (10mg/kg) did not affect total spine density between genotypes, although there is a tendency in decreased spine density in RGS9 knockout mice by morphine administration. Moreover, RGS9 is not essential for determining total dendritic spine density (p=0.0610 for treatment, p= 0.4460 for genotype, p=0.5873 for interaction) Values expressed as average ± SEM and analyzed using two-way ANOVA (WT: wild type, KO: knockout)

3.2.2: EFFECT OF MORPHINE TREATMENT IN PROXIMAL DENDRITIC HEAD DIAMETER OF RGS9 WT AND KO MICE.

As described above morphine treatment resulted in decreased density of mushrooms and stubby spines in proximal dendrites. Next, to examine if morphine treatment or deletion of Rgs9 gene results in alterations of dendritic spine morphology, head diameter of each spine subtype was measured. Morphine administration resulted in decreased mushroom diameter only when RGS9-2 protein signaling pathway was not disrupted (Fig. 14a). Moreover, decreased head diameter of thin and stubby spines in proximal dendrites was observed in both genotypes (Fig.14b and c). Finally, RGS9 defective signaling does not affect head diameter in all spine types in proximal dendrites (Fig.14).

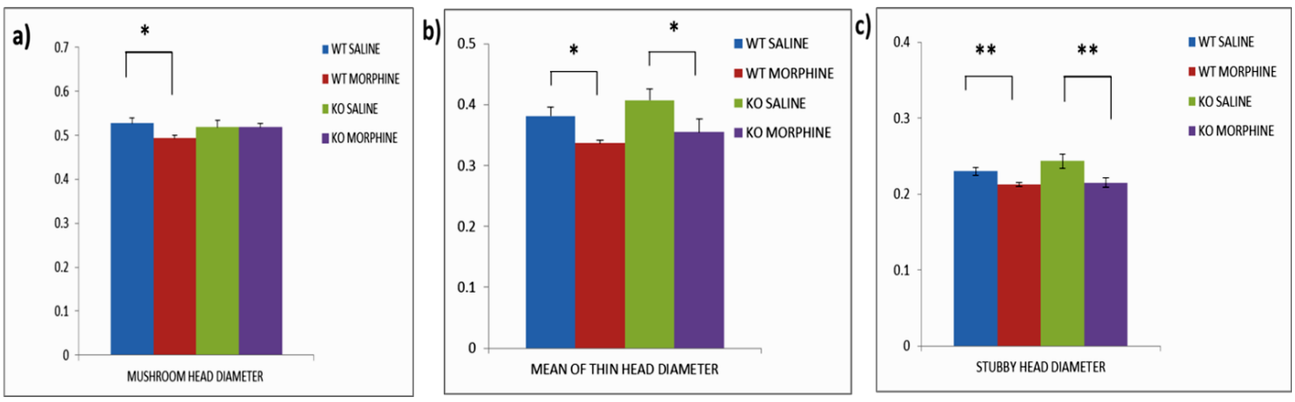


Figure 14: Morphine treatment alters spine morphology: a) Subcutaneously administered morphine (10mg/kg, s.c.) decreases mushroom head diameter in RGS9 wild type mice compared with their saline treated wild type littermates, whereas morphine treatment did not have any effect in this type of spine in RGS9 knockout mice. (*p=0.0364 for treatment, p=0.118 for genotype, p=0.9884 for interaction).

b and c) Morphine treatment leads to significant change in the head diameter of thin and stubby dendrtitic spines. Specifically, mice that received morphine, regardless of their genotype, exhibited decreased thin and stubby head diameter compared to the saline-injected littermates (thin spines: *p=0.0116 for treatment, p= 0.226 for genotype, p=0.8080 for interaction)/(stubby spines: *p=0.0093 for treatment, p= 0.3296 for genotype, p=0.4756 for interaction). Values expressed as average ± SEM and analyzed using two-way ANOVA (WT: wild type, KO: knockout)

3.2.3: EFFECT OF MORPHINE TREATMENT IN DENDRITIC NECK DIAMETER OF SPINES IN PROXIMAL DENDRITES OF RGS9 WT AND KO MICE.

Next, we examined if morphine treatment results in neck diameter alterations in spines located in proximal dendrites of RGS9 wild type and RGS9 knockout mice. While RGS9-2 protein seems to be a positive regulator of neck spine diameter, since knockout animals show decreased diameter under basal conditions, morphine did not confer any alterations in total spine neck morphology, taken in account that saline treated RGS9 mice exhibit differences in basal levels of spine neck diameter (Fig. 15a) On the other hand, morphine did alter mushroom neck diameter in RGS9 knock out animals (Fig. 15b), indicating that absence of RGS9-2 protein mediates morphine actions important for neck diameter in this specific spine subtype of the proximal dendrites.

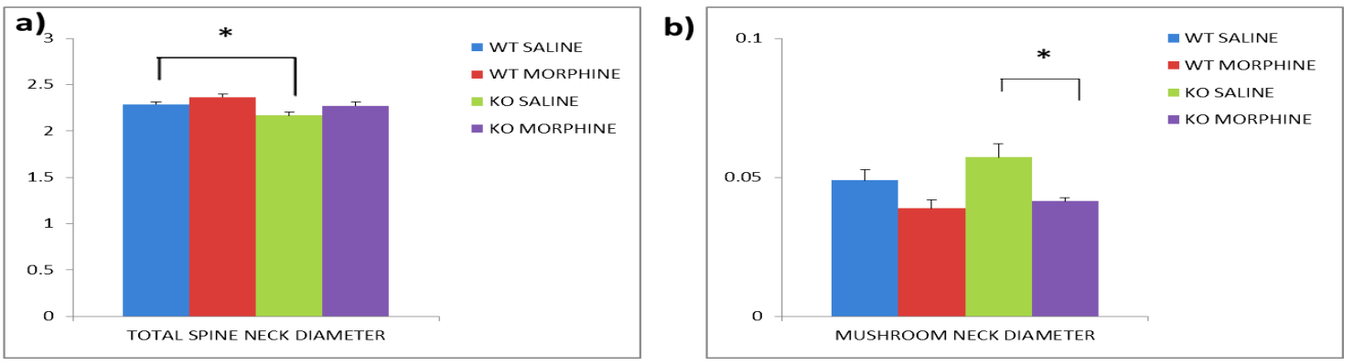


Figure 15: a) Altered spine neck diameter of proximal dendrites in RGS9 wild type and knockout mice: Deletion of Rgs9 gene decreases total spine neck diameter (*p=0.00271 for treatment, *p=0.0120 for genotype, p=0.7259 for interaction). **b) Morphine decreases mushroom neck spine morphology in RGS9 knockout mice:** Subcutaneously administered morphine (10mg/kg, s.c.) treatment significantly decreased mushroom neck diameter in RGS9 knockout mice compared with their saline treated littermates. On the other hand morphine resulted in a slight but not significant decrease in neck diameter in RGS9 wild type mice. Values expressed as average ± SEM and analyzed using two-way ANOVA (*p=0.0171 for treatment, p= 0.2808 for genotype, p=0.5736 for interaction). (WT: wild type,KO:knockout)

3.2.4 EFFECTS OF MORPHINE TREATMENT IN SPINE DENSITY OF DISTAL DENDRITES OF RGS9 WT AND KO MICE

Having seen the effects of morphine administration in synaptic plasticity of dendritic spines located in proximal dendrites of neurons in RGS9 wild type and knockout mice, we wanted to determine the effects of morphine treatment in dendritic spine density of distal dendrites that are defined as the dendritic tip and are located 10μm away from proximal dendrites. Specifically, morphine affects negatively mushroom spine density only in the absence of Rgs9 gene (Fig. 16a), whereas it had no effect on thin distal spine density (Fig. 16b), as seen also in proximal dendrites (Fig. 13a and b respectively). Moreover, stubby spine density was decreased by morphine administration in both genotypes, showing that RGS9 could participate in molecular signaling activated by morphine administration (Fig. 16c). On the other hand, morphine treatment did not result in changes in total spine density between genotypes.

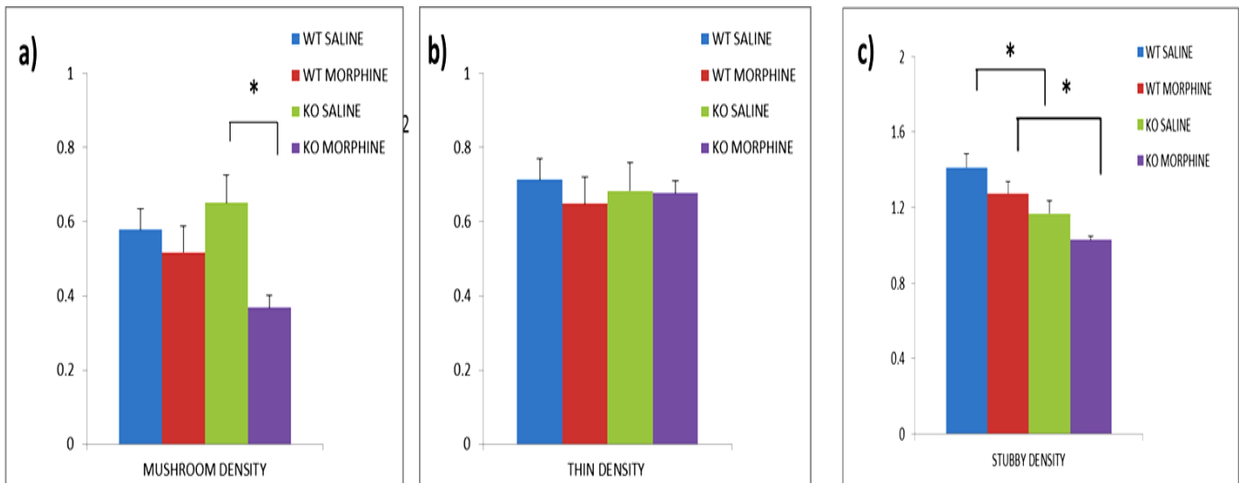


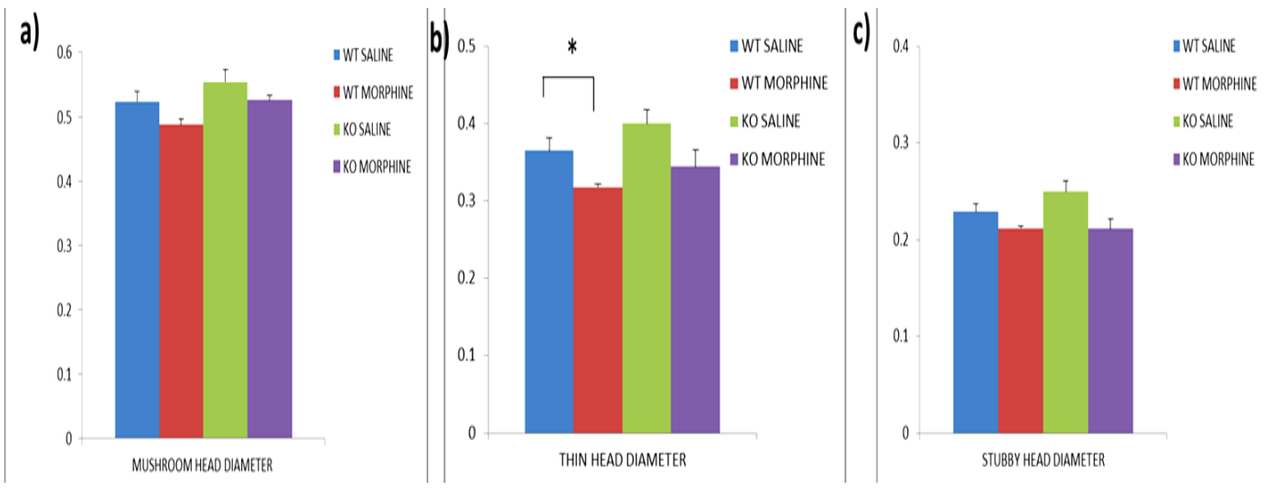
Figure 16:

a) Deletion of RGS9 is essential for the actions of morphine in mushroom spine density in distal dendrites: Morphine treatment (10mg/kg, s.c.) decreased mushroom spine density in RGS9 knockout mice compared with their saline treated knockout littermates, whereas morphine treatment did not have any effect in mushroom spine density in RGS9 wild type mice. Note that saline treated RGS9 knockout mice display the same basal levels for mushroom density with saline treated RGS9 wild type mice. (*p=0.0308 for treatment, p=0.6108 for genotype, p=0.1485 for interaction). **b) Morphine treatment did not alter thin spine density in RGS9 wild type or RGS9 knockout mice:** Morphine treatment did not affect thin spine density in RGS9 wildtype or knockout mice as compared to their saline treated controls. Moreover, thin spine density is not under the control of RGS9-2 protein, since deletion of the RGS9 gene did not affect thin spine density. (p=0.0810 for treatment, *p= 0.0043 for genotype, p=0.9760 for interaction)

c) RGS9 is an important mediator of morphine actions for decreasing stubby spine density in distal dendrites: Morphine administration decreased stubby spine density between genotypes (*p=0.0367 for genotype, p=0.5573 for treatment, p=0.6587) **d) Morphine did not alter total spine density in RGS9 wild type and knockout mice:** although there is a tendency for decreased spine density in both genotypes by morphine administration (**p=0.0067 for treatment, p= 0.14420 for genotype, p=0.7576 for interaction). Values expressed as average ± SEM and analyzed using two-way ANOVA (WT: wild type, KO: knockout) (WT: wild type, KO: knockout)

3.2.5: EFFECTS OF MORPHINE TREATMENT IN HEAD SPINE DIAMETER OF SPINES IN DISTAL DENDRITES OF RGS9 WT AND KO MICE.

Moreover, as observed in proximal dendrites, morphine results in alterations in spine morphology. Specifically, morphine decreased head diameter of thin subtypes in RGS9 wild type mice, without affecting thin head diameter of RGS9 knockout mice (Fig. 17b), suggesting that RGS9-2 protein is important for morphine action in determining head diameter of this specific spine subtype. On the contrary, head diameter of total spines in distal dendrites was reduced in RGS9 knockout mice by morphine administration (Fig. 17d), demonstrating that deletion of Rgs9 gene is essential for morphine actions in head diameter of spines in dendritic tip. Lastly, morphine treatment failed to induce alteration in head diameter of mushroom and stubby spines (Fig. 17a and c respectively).



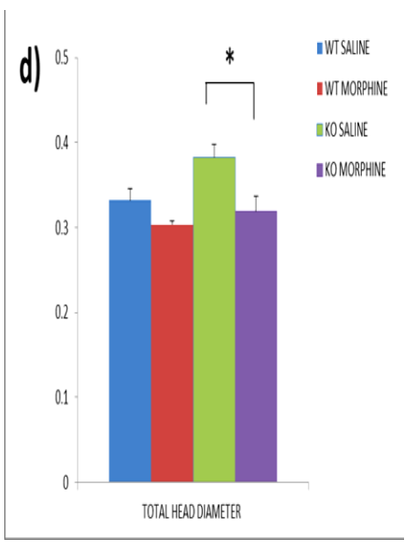


Figure 17: Morphine did not affect the head morphology of mushroom and stubby spine subtypes regardless of the genotype (a and c) (a: p=0.0682 for treatment, p=0.0554 for genotype, p=0.8350 for interaction). (c: p=0.0704 for treatment, p=0.3105 for genotype, p=0.2859 for interaction). **Morphine treatment leads to reduction of thin head spine in distal dendrites of RGS9 wild type mice** compared to their wild type littermates that were treated with saline (b) (**p=0.0075 for treatment, *p=0.0095 for genotype, p=0.8075 for interaction). Generally, morphine caused a significant reduction of head spine diameter of distal spines in total (d) (**p=0.0054 for treatment, *p=0.0366 for genotype, p=0.2489 for interaction). Values expressed as average ± SEM and analyzed using two-way ANOVA (WT: wild type, KO: knockout).

3.2.6: EFFECTS OF MORPHINE TREATMENT IN NECK SPINE DIAMETER OF SPINES IN DISTAL DENDRITES OF RGS9 WT AND KO MICE

Regarding neck diameter of spines located in distal dendrites, RGS9-2 expression is important for total spine neck morphology since deletion of *Rgs9* gene leads to decreased neck head diameter, whereas morphine treatment does not result in any alteration of neck spine diameter (Fig. 18a). Specifically, morphine failed to induce morphology alterations in neck diameter in mushroom spine subtype in distal dendrites (Fig. 18b).

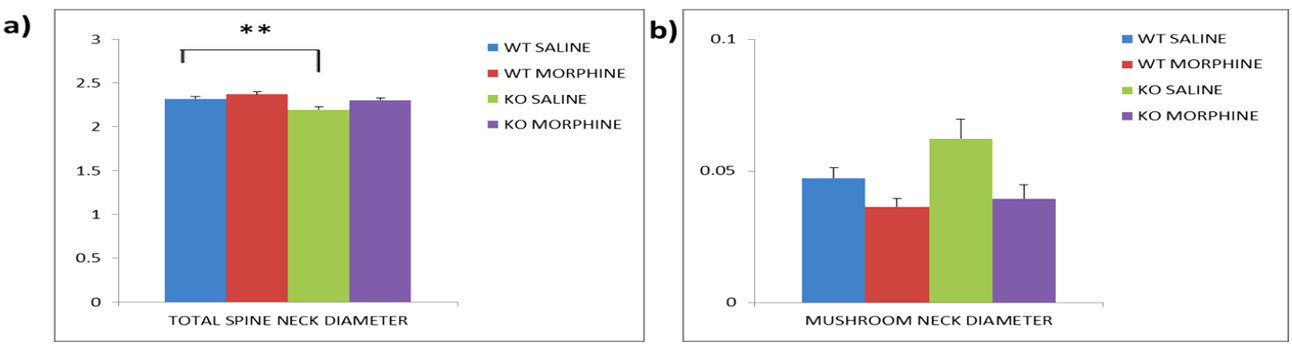


Figure 18: Morphine did not affect the neck morphology of total spine subtypes (a) or mushroom neck diameter (b) regardless of genotype in distal dendrites. (a: p=0.2180 for treatment, **p=0.0067 for genotype, p=0.3599 for interaction) (b: p=0.0718 for treatment, p=0.1545 for genotype, p=0.3429 for interaction). Values expressed as average ± SEM and analyzed using two-way ANOVA (WT: wild type, KO: knockout) (WT: wild type, KO: knockout)

3 .3 BIOCHEMICAL RESULTS

3.3.1 SYNAPTIC PLASTICITY RELATED PROTEIN EXPRESSION IN REGARD TO MORPHINE ADMINISTRATION AND DELETION OF Rgs9 GENE.

In order to further evaluate the effects of morphine actions in density and morphology of dendritic spines of RGS9 wild type and knockout mice, cell fractionation protocol and western blots were performed to observe whether these neuronal adaptations are depicted in altered expression at the protein level of molecules that participate in synaptic plasticity, such as AMPA GluR1 and GluR2 subunits and CamKIIa, thus correlating spine density with synaptic plasticity magnitude. Specifically, GluR1 containing AMPA receptors are important mediators of synaptic plasticity because elevated GluR1 protein expression correlates positively with induction of Long term Plasticity (LTP), due to higher conductance properties compared to GluR2 containing AMPA receptors. As shown in Fig 19a, morphine leads to increased GluR1 protein levels in homogenates from RGS9 wild type animals compared to their knockout littermates, whereas GluR1 protein expression remained unaffected by morphine in synaptosomes (Fig 19b) and in post synaptic densities (Fig 19c). Additionally, absence of RGS9-2 signaling pathway resulted in elevation of GluR1 expression at protein level (Fig. 19a), showing that RGS9 knockout mice may display enhanced synaptic plasticity compared to their wild type littermates. As expected, GluR2 protein expression was not altered in cell compartments either by morphine treatment or by RGS9 signaling pathway manipulations (Fig. 20). Moreover, GluR1 is supposed to be activated by phosphorylation at Ser831. Autophosphorylated form of CamKIIa is responsible for GluR1 activation, so it was very important to evaluate if morphine has any effect in phospho-CamKIIa protein expression. Indeed, morphine treatment and deletion of Rgs9 gene resulted in elevated protein levels of the phorphorylated form of CamKIIa in synaptosomes (Fig. 21b), but not in homogenates (Fig21a). On the other hand, no significant effect was observed in total CamKIIa protein expression either by morphine administration or by Rgs9 gene deletion in homogenates or synaptosomes (Fig. 21c and b respectively).

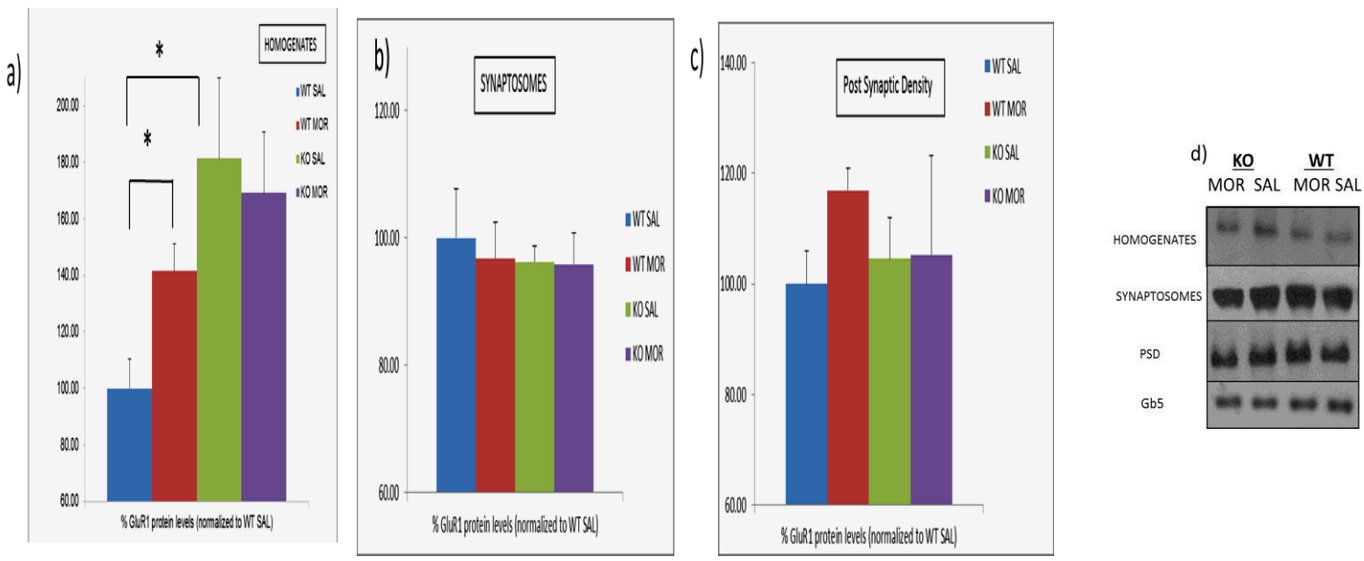


Figure 19: a) Elevated protein levels of total AMPA GluR1 subunit in RGS9 knockout mice in homogenates from cell fractionation. Moreover, there is an increasing effect of morphine administration in GluR1 expression levels in RGS9 wild type mice compared to saline-treated wild type mice (*p=0.0420 for treatment, *p=0.0282 for genotype, p=0.5305 for interaction) b) **GluR1 protein levels were not regulated by morphine administration in synaptosomes** (p=0.7987 for treatment, p=0.677 for genotype, p=0.7534 for interaction).**c) Morphine resulted in a slightly enhancement of GluR1**

protein levels in post synaptic density cell fractionation for RGS9 wild type mice compared to saline treated wild type littermates, although without a significant effect. On the other hand, morphine did not alter GluR1 expression in RGS9 knockout mice compared with their saline treated animals (p=0.4086 for treatment, p=0.7373 for genotype, p=0.4086 for interaction). **d) Representative western blots for GluR1 protein and Gb5 control.** Values expressed as average ± SEM (normalized to WT SAL) and analyzed using two-way ANOVA (WT: wild type, KO: knockout, SAL: saline, MOR: morphine) (n=5-6 per group).

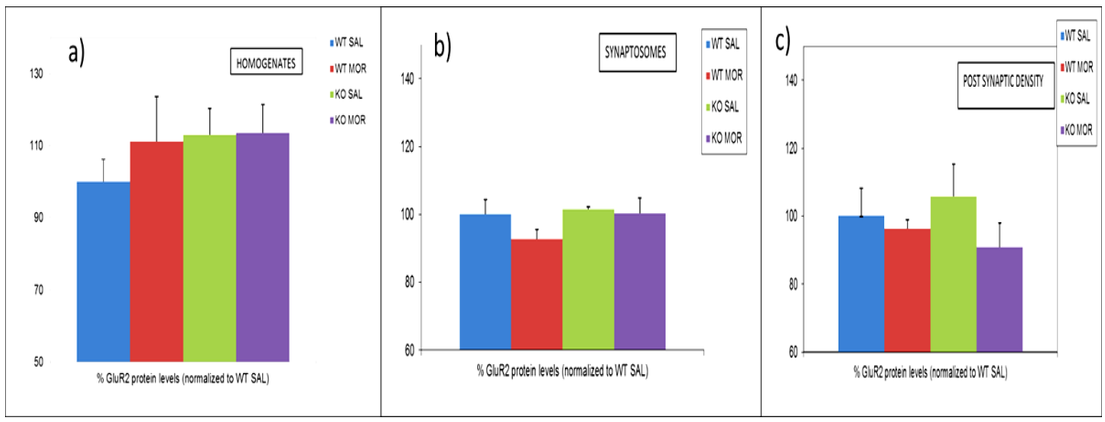


Figure 20: No detectable change in protein levels of total AMPA GluR2 subunit in either cell fractionation (a: p=0.5157 for treatment, p=0.3912 for genotype, p=0.5495 for interaction) (b: p=0.4017 for treatment, p=0.2164 for genotype, p=0.4017 for interaction) (b: p=0.2663 for treatment, p=0.9888 for genotype, p=0.4835 for interaction) **d) Representative western blots for GluR2 protein and Gb5 control.** Values expressed as average ± SEM (normalized to WT SAL) and analyzed using two-way ANOVA (WT: wild type, KO: knockout, SAL: saline, MOR: morphine) (n=5-6 per group).

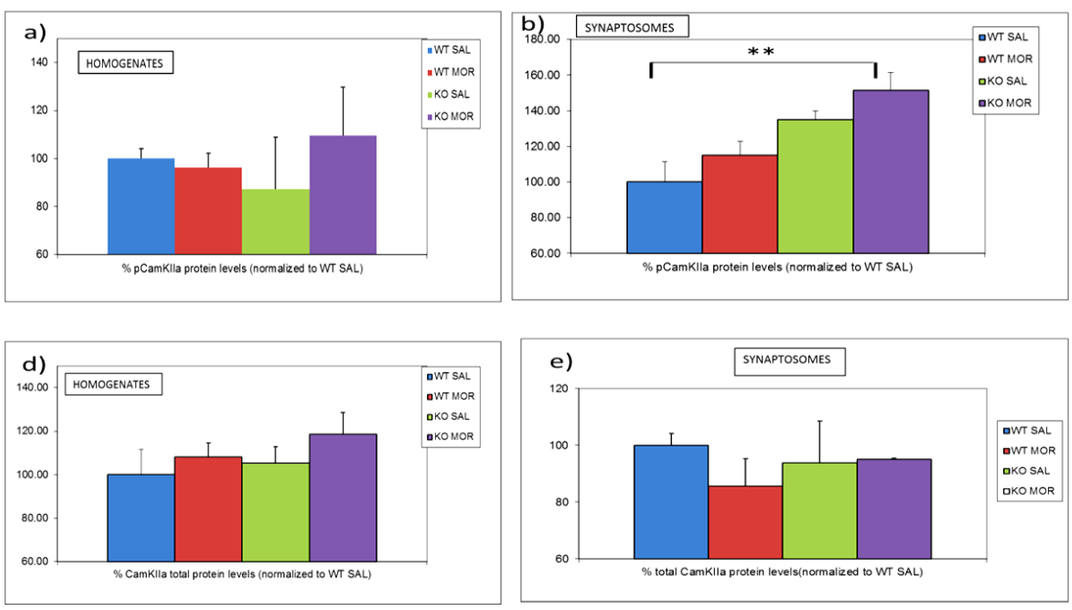
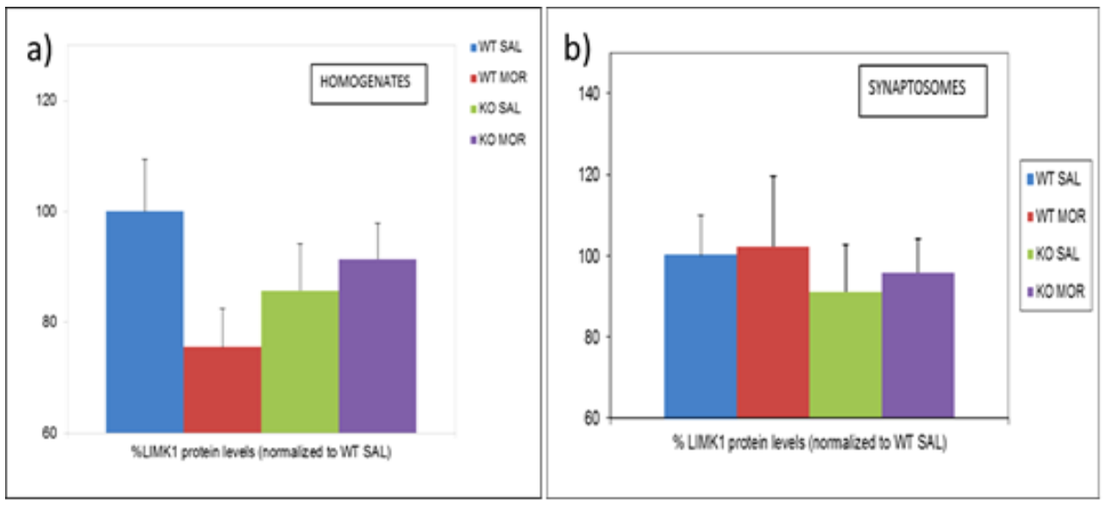


Figure 21: Phosphorylation of CamKII subunit at Thr867 residue is increased in RGS9 knockout mice injected with morphine compared with RGS9 wild type mice that were treated with saline (b: p=0.1711 for treatment, **p=0.0093 for genotype, p=0.826 for interaction) in synaptosomes, whereas no change of p-CamKII was observed in homogenates (a: p=0.6474 for treatment, p=0.9905 for genotype, p=0.5289 for interaction). Also, total CamKIIa expression was not altered in homogenates (d: p=0.2696 for treatment, p=0.4183 for genotype, p=0.7859 for interaction) and synaptosomes (e: p=0.4599 for treatment, p=0.849 for genotype, p=0.3883 for interaction) cell fractionation by morphine or Rgs9 gene deletion. d) Representative western blots for phospho-CamKII, total CamKIIa protein and Gb5 control. Values expressed as average ± SEM (normalized to WT SAL) and analyzed using two-way ANOVA (WT: wild type, KO: knockout, SAL: saline, MOR: morphine, n=3-5 per group).

3.3.2 REGULATION OF MOLECULES INVOLVED IN SPINE MORPHOLOGY BY MORPHINE IN RGS9 WILD TYPE AND KNOCKOUT MICE.

Moreover, proteins that participate in the determination of spine morphology, such as Rac1 and LimK1 were also examined by western blot for determining protein changes induced by morphine treatment or deletion of Rgs9 gene. It is well documented that enhanced activity of Rac1 correlates with spine enlargement. Similarly, Limk1 a downstream target of Rac1 is also positively associated with spine enlargement, since Limk1 is responsible for de-phosphorylation of cofilin, a protein involved in actin depolymerization. Regarding morphine treatment, Rac1 is not regulated in synaptosomes (Fig. 22d). Similarly, morphine is not responsible for LimK1 regulation in synaptosomes (Fig. 22b) and homogenates (Fig. 22a), although there is a slight reduction in Limk1 protein levels in this specific cell compartment by morphine administration in RGS9 wild type mice treated with morphine compared to their wild type littermates that were treated with saline.



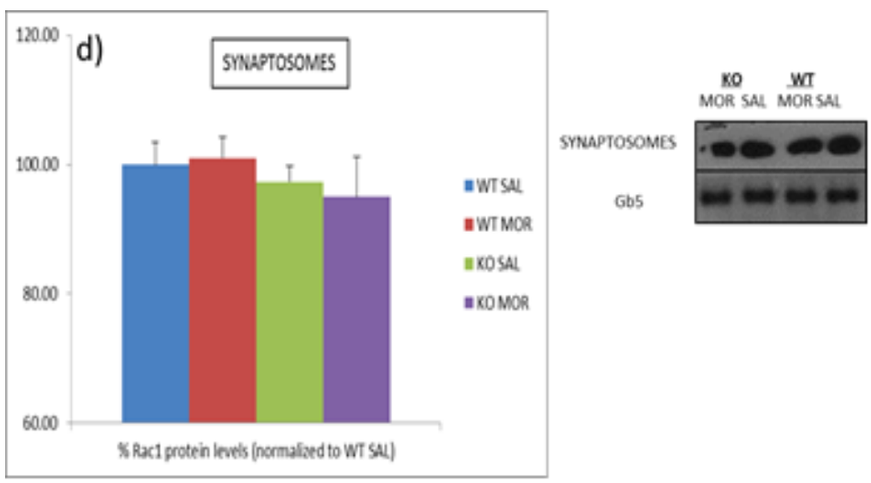


Figure 22: No detectable change in protein levels of Limk1 subunit in either cell fractionation (a: p=0.0711 for treatment, p=0.9125 for genotype, p=0.2583 for interaction) (b: p=0.7812 for treatment, p=0.7812 for genotype, p=0.9227 for interaction), although there is a reduction trend in homogenate cell fractionation in RGS9 WT mice treated with morphine compared with their saline injected wild type littermates (a). d) Representative western blots for Limk1 protein and Gb5 control. Also, Rac1 protein levels were not regulated by morphine in synaptosomes (d: p=0.8715 for treatment, p=0.3087 for genotype, p=0.692 for interaction). Values expressed as average ± SEM (normalized to WT SAL) and analyzed using two-way ANOVA (WT: wild type, KO: knockout, SAL: saline, MOR: morphine, n=3-5 per group)

4. DISCUSSION

Opiates are clinically used for the chronic pain treatment, because of their analgesic effects. Nowadays, the rate of chronic pain ocurance is elevated, so there is an increase rate of opiate dependence. Opioid addiction together with long-term psychostimulant drug abuse, are associated with altered synaptic plasticity and neuronal adaptations in various brain regions. Synaptic plasticity is often depicted by neuronal morphology changes and alterations in dendritic spine density. Thus, unraveling signaling mechanisms that underlie these molecular adaptations induced by addiction provide a novel step in identifying signaling pathways involved in persistent neuronal changes observed in the drug abuse actions.

In this study, RGS9-2 signaling has been shown to determine spine morphology and density in the dorsal striatum. In particular, this brain region is considered to be an extension of nucleus accumbens core and as such it has been shown to be involved in synaptic plasticity alterations regarding spine density. Specifically, amphetamine administration resulted in increased spine density, similar to the actions of other psychostimulants, such as cocaine. Moreover, this effect was only observed in distal dendrites of dorsal striatum {142}. On the contrary, opiates result in decreased spine density {85}. As shown here, morphine administration did not result in decreased total spine density in proximal dendrites. On the contrary, morphine actions in dorsal striatum regarding spine density appear to be mediated through RGS9 signaling. In particular, deletion of rgs9 gene resulted in decreased mushroom density in proximal and distal dendrites in mice that received morphine compared to control RGS9 knockout mice. This effect was also observed in stubby spine density in proximal dendrites, whereas thin spine density was unaffected in both types of dendrites. On the other hand, in distal dendrites stubby spine density was diminished with deletion of Rgs9 gene and this effect was enhanced by morphine administration, an effect also depicted in total spine density. These results also show that morphine has a preferential regulation towards spine subtype and spine dendritic location. The latter one may be explained by differential neuronal inputs that project onto proximal and distal dendrites. In particular, distal dendrites of medium spiny neurons of dorsal striatum are under regulation of extrinsic glutaminergic signaling, whereas proximal receive intrinsic inp{142}. The differential regulation of proximal and distal dendritic spines was also observed in the morphology of spines. More specifically, in proximal dendrites head mushroom diameter requires RGS9 signaling for morphine actions whereas neck mushroom diameter requires impaired RGS9 signaling. Moreover, thin and stubby head diameter was decreased by morphine administration, an effect that was not dependent on RGS9 signaling. In distal dendrites, mushroom and stubby head diameter remained unaffected, whereas thin head diameter was decreased with morphine only in the presence of RGS9 expression.

These neuronal adaptations of dendritic spine morphology also reflect expression changes in protein participating in the spine enlargement or in the neuronal activity. In particular, morphine leads to GluR1 upregulation in the cytoplasm an effect that is also observed in the VTA {79}. On the other hand, chronic morphine administration did not alter GluR1 mRNA expression

{143}. This discrepancy could be either due to post-transcriptional regulation of GluR1 or by different dose effect of morphine. Also, morphine treatment had a slight, but not significant decrease in GluR1 protein levels in post synaptic density. Taken in account that spine head enlargement is correlated with increased presence of GluR1 in the post-synaptic densities of dendrites {42}, mushroom head diameter signaling should be increased by morphine, since this type of spine is considered to be more mature spine subtype compared to thin and stubby spine subtypes. In fact, morphine is responsible for decreasing mushroom head diameter in proximal dendrites. This effect could be a result of homeostatic signaling events, compensating morphine actions in decreasing synaptic plasticity. Additionally, this effect could be explained by the experiments showing that the phosphorylated form of GluR1 at Ser831 is important for induction of LTP and thus induction of spine enlargement. Taken these into account, phospho-GluR1 levels could be down-regulated with morphine. On the other hand, there are reports demonstrating that this particular phosphorylation is not important for LTP induction {63} and thus for altering dendritic spines. Also, GluR1 was found to be elevated by deletion of Rgs9 gene, showing a possible role of RGS9-2 in synaptic plasticity since GluR1 levels are positively correlated with enhanced synaptic plasticity, but this observation needs to be examined further with immuno-histochemical and electrophysiology studies. Moreover, AMPA GluR2 subunit protein expression was not altered by morphine treatment, showing the preferential regulation of GluR1 subunit by morphine. On the other hand, phospho-CamKIIa protein levels were found to be increased in postsynaptic densities after deletion of Rgs9 and morphine treatment, whereas total CamKIIa expression remained unaffected, confirming that CamKIIa activity near post synaptic density is essential for GluR1 regulation and that RGS9-2 impaired signaling promote CamKIIa activation by morphine. Also, morphine leads to a slight downregulation of LimK1. Enhanced activity of LimK1 has been correlated with spine enlargement since it is responsible for phosphorylation of cofilin, a depolymerization protein. Indeed, decreased LimK1 protein expression by morphine could be correlated with decreased mushroom head diameter. On the other hand, Limk1 is not downregulated by morphine in the absence of RGS9-2 signaling, an effect that is also observed in mushroom head diameter, supporting that Rgs9 gene deletion is affecting morphine actions in downregulation of LimK1. Lastly, Rac1 an important mediator of dendritic spine morphology is not affected by morphine treatment, and is known to regulate Limk1, showing that other molecular mechanisms are involved in the spine morphology in morphine action. Together, these data illustrate a role of RGS9-2 in important changes in cellular and structural plasticity that mediate the long-lasting effects of opiates in neuronal populations of the dorsal striatum. Future studies are needed to delineate the plasticity that occurs in specific neuronal subtypes of medium spiny neurons. This type of investigation will shed more light in how opiates control molecular mechanisms involved in addiction.

5. REFERENCES

1. Koob GF, Le Moal M. (2001) Drug addiction, dysregulation of reward and allostasis. Neuropsychopharmacology 24: 97–129.
2. Koob GF, Le Moal M. (1997) Drug abuse: hedonic homeostatic dysregulation .Drug addiction, dysregulation of reward, and allostasis. Science. 278(5335):52-8.
3. Wise RA. (2002) Brain reward circuitry: insights from unsensed incentives. Neuron. 36(2):229-40.
4. Koob GF, Volkow ND. (2010) Neurocircuitry of addiction. Neuropsychopharmacology. 35(1):217-38.
5. Olds J, Milner (1954) Positive reinforcement produced by electrical stimulation of septal area and other regions of rat brain. J Comp Physiol Psychol. 47(6):419-27.
6. Bozarth MA. 1987. Intracranial self-administration procedures for the assessment of drug reinforcement. In Methods of assessing the reinforcing properties of abused drugs (ed. Bozarth MA), pp. 173–187. Springer-Verlag, New York.
7. Di Chiara G, Imperato A. (1988) Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats. Proc Natl Acad Sci U S A.85(14):5274-8.
8. Caine SB, Thomsen M, Gabriel KI, Berkowitz JS,Gold LH, Koob GF, Tonegawa S, Zhang J, Xu M. (2007) Lack of self-administration of cocaine in dopamine D1 receptor knock-out mice. J Neurosci. 27(48):13140-50.
9. Sellings LH, Clarke PB. (2003) Segregation of amphetamine reward and locomotor stimulation between nucleus accumbens medial shell and core. J. Neurosci. 23(15):6295- 303.
10. Johnson SW, North RA. (1992) Opioids excite dopamine neurons by hyperpolarization of local interneurons. J Neurosci. 12(2):483-8.
11. Xia Y, Driscoll JR, Wilbrecht L, Margolis EB, Fields HL, Hjelmstad GO. (2011) Nucleus accumbens medium spiny neurons target non-dopaminergic neurons in the ventral tegmental area. J Neurosci. 31(21):7811-6.
12. Jalabert M, Bourdy R, Courtin J, Veinante P, Manzoni OJ, Barrot M, Georges F. (2011) Neuronal circuits underlying acute morphine action on dopamine neurons.Proc Natl Acad Sci U S A. 108(39):16446-50.
13. Olmstead MC, Franklin KB. (1996) Differential effects of ventral striatal lesions on the conditioned place preference induced by morphine or amphetamine. Neuroscience. 71(3):701-8.
14. Nestler EJ. (1996).Under siege: the brain on opiates. Neuron 16, 897–900
15. Everitt BJ, Robbins TW. (2005) Neural systems of reinforcement for drug addiction: from actions to habits to compulsion. Nat Neurosci. 8(11): 1481-9
16. Volkow ND, Wang GJ, Telang F, Fowler JS, Logan J, Childress AR, Jayne M, Ma Y, Wong C.(2006) Cocaine cues and dopamine in dorsal striatum: mechanism of craving in cocaine addiction. JNeurosci. 26(24):6583-8.
17. Volkow ND, Wang GJ, Telang F, Fowler JS, Logan J, Childress AR, Jayne M, Ma Y, Wong C.(2008)Dopamine increases in striatum do not elicit craving in cocaine abusers unless they are coupled with cocaine cues. Neuroimage. 39(3):1266-73.
18. Schulteis G, Ahmed SH, Morse AC, Koob GF, Everitt BJ. (2000)Conditioning and opiate withdrawal. Nature. 2000 405(6790):1013-4.
19. Heinrichs SC, Menzaghi F, Schulteis G, Koob GF, Stinus L.(1995)Suppression of corticotropin-releasing factor in the amygdala attenuates aversive consequences of morphine withdrawal.Behav Pharmacol. 6(1):74-80.
20. Papaleo F, Kitchener P, Contarino A. (2007) Disruption of the CRF/CRF1 receptor stress system exacerbates the somatic signs of opiate withdrawal.Neuron. 53(4):577-89.
21. Zachariou V, Bolanos CA, Selley DE, Theobald D, Cassidy MP, Kelz MB, Shaw-Lutchman T, Berton O, Sim-Selley LJ, Dileone RJ, Kumar A, Nestler EJ.(2006) An essential role for DeltaFosB in the nucleus accumbens in morphine action. Nat Neurosci. 9(2):205-11.
22. Hyman SE. (2005) Addiction: a disease of learning and memory. Am J Psychiatry. 162(8):1414-22.
23. Lüscher C, Malenka RC. (2011) Drug-evoked synaptic plasticity in addiction: from molecular changes to circuit remodeling. Neuron. 69(4):650-63.
24. Russo SJ, Mazei-Robison MS, Ables JL, Nestler EJ. (2009) Neurotrophic factors and structural plasticity in addiction. Neuropharmacology. 56 Suppl 1:73-821.
25. . Ramón y Cajal, S. (1894) La fine structure des centres nerveux. Proc. R. Soc. Lond. 55, 444–468.
26. Ho VM, Lee JA, Martin KC. (2011) The cell biology of synaptic plasticity. Science. 2011 Nov 4;334(6056):623-8.
27. Rochefort NL, Konnerth A. (2012) Dendritic spines: from structure to in vivo function. EMBO Rep. 13(8):699-708.
28. Star EN, Kwiatkowski DJ, Murthy VN. (2002) Rapid turnover of actin in dendritic spines and its regulation by activity. Nat Neurosci. 5(3):239-46.
29. Honkura N, Matsuzaki M, Noguchi J, Ellis-Davies GC, Kasai H.(2008) The subspine organization of actin fibers regulates the structure and plasticity of dendritic spines. Neuron. 57(5):719-29.
30. Lamprecht R, Farb CR, Rodrigues SM, LeDoux JE.(2006) Fear conditioning drives profilin into amygdala dendritic spines. Nat Neurosci. 9(4):481-3.
31. Hotulainen P, Llano O, Smirnov S, Tanhuanpää K, Faix J, Rivera C,Lappalainen P.(2009) Defining mechanisms of actin polymerization and depolymerization during dendritic spine morphogenesis .J Cell Biol. 185(2):323-39.
32. Niwa R, Nagata-Ohashi K, Takeichi M, Mizuno K, Uemura T.(2002) Control of actin reorganization by Slingshot, a family of phosphatases that dephosphorylate ADF/cofilin. Cell. 108(2):233-46.
33. Penzes P, Rafalovich I.(2012) Regulation of the actin cytoskeleton in dendritic spines. Adv Exp Med 970:81-95.
34. Meng Y, Zhang Y, Tregoubov V, Janus C, Cruz L, Jackson M, Lu WY, MacDonald JF, Wang JY, Falls DL, Jia Z (2002) Abnormal spine morphology and enhanced LTP in LIMK-1 knockout mice. Neuron 35(1):121-33.

35. Sin WC, Haas K, Ruthazer ES, Cline HT. (2002) Dendrite growth increased by visual activity requires NMDA receptor and Rho GTPases. Nature 419(6906):475-80.
36. Luo L, Hensch TK, Ackerman L, Barbel S, Jan LY, Jan YN.(1996) Differential effects of the Rac GTPase on Purkinje cell axons and dendritic trunks and spines. Nature 379(6568):837-40.
37. Golden SA, Christoffel DJ, Heshmati M, Hodes GE, Magida J, Davis K, Cahill ME, Dias C, Ribeiro E, Ables JL, Kennedy PJ, Robison AJ, Gonzalez-Maeso J, Neve RL, Turecki G, Ghose S, Tamminga CA, Russo SJ. (2013) Epigenetic regulation of RAC1 induces synaptic remodeling in stress disorders and depression. Nat Med. 19(3):337-44.
38. Allen PB, Ouimet CC, Greengard P. (1997) Spinophilin, a novel protein phosphatase 1 binding protein localized to dendritic spines.Proc Natl Acad Sci U S A. 94(18):9956-61.
39. MacMillan LB, Bass MA, Cheng N, Howard EF, Tamura M, Strack S, Wadzinski BE, Colbran RJ.(1999)Brain actin-associated protein phosphatase 1 holoenzymes containing spinophilin, neurabin, and selected catalytic subunit isoforms.J Biol Chem.274(50):35845- 54.
40. Feng J, Yan Z, Ferreira A, Tomizawa K, Liauw JA, Zhuo M, Allen PB, Ouimet CC, Greengard P. (2000) Spinophilin regulates the formation and function of dendritic spines. Proc Natl Acad Sci U S A. 97(16):9287-92.
41. Buchsbaum RJ, Connolly BA, Feig LA. (2003) Regulation of p70 S6 kinase by complex formation between the Rac guanine nucleotide exchange factor (Rac-GEF) Tiam1 and the scaffold spinophilin.J Biol Chem. 278(21):18833-41.
42. Matsuzaki M, Ellis-Davies GC, Nemoto T, Miyashita Y, Iino M, Kasai H. (2001) Dendritic spine geometry is critical for AMPA receptor expression in hippocampal CA1 pyramidal neurons.Nat Neurosci. 4(11):1086-92
43. Park M, Penick EC, Edwards JG, Kauer JA, Ehlers MD. (2004) Recycling endosomes supply AMPA receptors for LTP. Science. 305(5692):1972-5.
44. Ju W, Morishita W, Tsui J, Gaietta G, Deerinck TJ, Adams SR, Garner CC, Tsien RY, Ellisman MH, Malenka RC.(2004) Activity-dependent regulation of dendritic synthesis and trafficking of AMPA receptors. Nat Neurosci. 7(3):244-53.
45. Hayashi Y, Shi SH, Esteban JA, Piccini A, Poncer JC, Malinow R. (2000) Driving AMPA receptors into synapses by LTP and CaMKII: requirement for GluR1 and PDZ domain interaction.Science. 287(5461):2262-7.
46. Seeburg PH. (1993) The TINS/TiPS Lecture. The molecular biology of mammalian glutamate receptor channels. Trends Neurosci. 16(9):359-65.
47. Makino H, Malinow R (2009) AMPA receptor incorporation into synapses during LTP: the role of lateral movement and exocytosis. Neuron. 64(3):381-90.
48. Malenka RC, Kauer JA, Zucker RS, Nicoll RA. (1988) Postsynaptic calcium is sufficient for potentiation of hippocampal synaptic transmission. Science. 242(4875):81-4.
49. Kauer JA, Malenka RC. (2007) Synaptic plasticity and addiction. Nat Rev Neurosci. 8(11):844-58
50. Asrican B, Lisman J, Otmakhov N.(2007) Synaptic strength of individual spines correlates with bound Ca2+-calmodulin-dependent kinase II.J Neurosci. (51):14007-11.
51. Silva AJ, Stevens CF, Tonegawa S, Wang Y. (1992) Deficient hippocampal long-term potentiation in alpha-calcium-calmodulin kinase II mutant mice. Science. 257(5067):201-6.
52. Lee, S.-J. R., Escobedo-Lozoya, Y., Szatmari, E. M., Yasuda, R. (2009) Activation of CaMKII in single dendritic spines during long-term potentiation. Nature 458: 299-304
53. Bayer KU, De Koninck P, Leonard AS, Hell JW, Schulman H.(2001) Interaction with the NMDA receptor locks CaMKII in an active conformation. Nature.411(6839):801-5.
54. Kiraly DD, Lemtiri-Chlieh F, Levine ES, Mains RE, Eipper BA. (2011) Kalirin binds the NR2B subunit of the NMDA receptor, altering its synaptic localization and function.J Neurosci. 31(35):12554-65.
55. Xie Z, Srivastava DP, Photowala H, Kai L, Cahill ME, Woolfrey KM, Shum CY, Surmeier DJ, Penzes P. (2007) Kalirin-7 controls activity-dependent structural and functional plasticity of dendritic spines.Neuron. 56(4):640-56.
56. Lisman JE, Zhabotinsky AM. (2001) A model of synaptic memory: a CaMKII/PP1 switch that potentiates transmission by organizing an AMPA receptor anchoring assembly.Neuron. 31(2):191-201.
57. Baucum AJ 2nd, Jalan-Sakrikar N, Jiao Y, Gustin RM, Carmody LC, Tabb DL, Ham AJ, Colbran RJ. (2010) Identification and validation of novel spinophilin-associated proteins in rodent striatum using an enhanced ex vivo shotgun proteomics approach.Mol Cell Proteomics. 9(6):1243-59.
58. Brown AM, Baucum AJ, Bass MA, Colbran RJ. (2008) Association of protein phosphatase 1 gamma 1 with spinophilin suppresses phosphatase activity in a Parkinson disease model.J Biol Chem. 283(21):14286-94.
59. Giese KP, Fedorov NB, Filipkowski RK, Silva AJ. (1998) Autophosphorylation at Thr286 of the alpha calcium-calmodulin kinase II in LTP and learning. Science. 279(5352):870-3.
60. Hojjati MR, van Woerden GM, Tyler WJ, Giese KP, Silva AJ, Pozzo-Miller L, Elgersma Y. (2007) Kinase activity is not required for alphaCaMKII-dependent presynaptic plasticity at CA3-CA1 synapses.Nat Neurosci. 10(9):1125-7. .
61. Kristensen AS, Jenkins MA, Banke TG, Schousboe A, Makino Y, Johnson RC, Huganir R, Traynelis SF. (2011) Mechanism of Ca2+/calmodulin-dependent kinase II regulation of AMPA receptor gating.Nat Neurosci.14(6):727-35.
62. Kielland A, Bochorishvili G, Corson J, Zhang L, Rosin DL, Heggelund P, Zhu JJ. (2009) Activity patterns govern synapse-specific AMPA receptor trafficking between deliverable and synaptic pools. Neuron. 62(1):84-101.
63. Opazo P, Labrecque S, Tigaret CM, Frouin A, Wiseman PW, De Koninck P, Choquet D. (2010) CaMKII triggers the diffusional trapping of surface AMPARs through phosphorylation of stargazin.Neuron. 67(2):239-52.
64. Kim CH, Takamiya K, Petralia RS, Sattler R, Yu S, Zhou W, Kalb R, Wenthold R, Huganir R. (2005) Persistent hippocampal CA1 LTP in mice lacking the C-terminal PDZ ligand of GluR1.Nat Neurosci. 8(8):985-7.
65. Granger AJ, Shi Y, Lu W, Cerpas M, Nicoll RA.(2013)LTP requires a reserve pool of glutamate receptors independent of subunit type.Nature.493(7433):495-500.

66. Hayashi Y, Shi SH, Esteban JA, Piccini A, Poncer JC, Malinow R. (2000) Driving AMPA receptors into synapses by LTP and CaMKII: requirement for GluR1 and PDZ domain interaction.Science. 287(5461):2262-7.
67. Kim E, Sheng M. (2004) PDZ domain proteins of synapses. Nat Rev Neurosci. 5(10):771-81.
68. Zhou Q, Homma KJ, Poo MM.(2004) Shrinkage of dendritic spines associated with long-term depression of hippocampal synapses.Neuron.2004 Dec 2;44(5):749-57.
69. Ehlers MD.(2000) Reinsertion or degradation of AMPA receptors determined by activity-dependent endocytic sorting.Neuron.28(2):511-25.
70. Lee HK, Takamiya K, Han JS, Man H, Kim CH, Rumbaugh G, Yu S, Ding L, He C, Petralia RS, Wenthold RJ, Gallagher M, Huganir RL.(2003) Phosphorylation of the AMPA receptor GluR1 subunit is required for synaptic plasticity and retention of spatial memory.Cell.112(5):631-43.
71. Morishita W, Connor JH, Xia H, Quinlan EM, Shenolikar S, Malenka RC. (2001) Regulation of synaptic strength by protein phosphatase 1. Neuron. 32(6):1133-48.
72. Westphal RS, Tavalin SJ, Lin JW, Alto NM, Fraser ID, Langeberg LK, Sheng M, Scott JD.(1999) Regulation of NMDA receptors by an associated phosphatase-kinase signaling complex.Science.285(5424):93-6.
73. Peineau S, Taghibiglou C, Bradley C, Wong TP, Liu L, Lu J, Lo E, Wu D, Saule E, Bouschet T, Matthews P, Isaac JT, Bortolotto ZA, Wang YT, Collingridge GL(2007) LTP inhibits LTD in the hippocampus via regulation of GSK3beta.Neuron.53(5):703-17.
74. Turrigiano GG,Nelson SB.(2004) Homeostatic plasticity in the developing nervous system.Nat Rev Neurosci.5(2):97-107.
75. Kasanetz F, Deroche-Gamonet V, Berson N, Balado E, Lafourcade M, Manzoni O, Piazza PV. (2010) Transition to addiction is associated with a persistent impairment in synaptic plasticity. Science. 328(5986):1709-12.
76. Saal D, Dong Y, Bonci A, Malenka RC. (2003) Drugs of abuse and stress trigger a common synaptic adaptation in dopamine neurons. Neuron. 37(4):577-82.
77. Ungless M.A.,Whistler J.L.,Malenka R. C., Bonci A.(2001). Single cocaine exposure in vivo induces long-termpotentiation in dopamine neurons. Nature 411, 583–587.
78. Carlezon WA Jr, Boundy VA, Haile CN, Lane SB, Kalb RG, Neve RL, Nestler EJ. (1997) Sensitization to morphine induced by viral-mediated gene transfer. Science 277(5327):812-4.
79. Fitzgerald LW, Ortiz J, Hamedani AG, Nestler EJ. (1996) Drugs of abuse and stress increase the expression of GluR1 and NMDAR1 glutamate receptor subunits in the rat ventral tegmental area: common adaptations among cross-sensitizing agents. J Neurosci.16(1):274-82.
80. Ding X, Liang J, Zheng X, Bai Y, Liu Z, Li Y, Xing X. (2013) Altered phosphorylation of GluA1 in the striatum is associated with locomotor sensitization induced by exposure to increasing doses of morphine.Eur J Pharmacol. 702(1-3):294-301.
81. Nugent FS, Penick EC, Kauer JA.(2007) Opioids block long-term potentiation of inhibitory synapses.Nature.446(7139):1086-90.
82. Cohen JY, Haesler S, Vong L, Lowell BB, Uchida N. (2012) Neuron-type-specific signals for reward and punishment in the ventral tegmental area.Nature.482(7383):85-8.
83. Russo SJ, Mazei-Robison MS, Ables JL, Nestler EJ. (2009) Neurotrophic factors and structural plasticity in addiction.Neuropharmacology. 56 Suppl 1:73-82.
84. Liao D, Lin H, Law PY, Loh HH. (2005) Mu-opioid receptors modulate the stability of dendritic spines. Proc Natl Acad Sci U S A 102(5):1725-30
85. Robinson TE, Gorny G, Savage VR, Kolb B.(2002) Widespread but regionally specific effects of experimenter- versus self-administered morphine on dendritic spines in the nucleus accumbens, hippocampus, and neocortex of adult rats.Synapse.46(4):271-9.
86. Russo SJ, Bolanos CA, Theobald DE, DeCarolis NA, Renthal W, Kumar A, Winstanley CA, Renthal NE, Wiley MD, Self DW, Russell DS, Neve RL, Eisch AJ,Nestler EJ.(2007) IRS2-Akt pathway in midbrain dopamine neurons regulates behavioral and cellular responses to opiates.Nat Neurosci.10(1):93-9.
87. Norrholm SD, Bibb JA, Nestler EJ, Ouimet CC, Taylor JR, Greengard P. (2003) Cocaine- induced proliferation of dendritic spines in nucleus accumbens is dependent on the activity of cyclin-dependent kinase-5.Neuroscience 116(1):19-22.
88. Robison AJ, Vialou V, Mazei-Robison M, Feng J, Kourrich S, Collins M, Wee S, Koob G, Turecki G, Neve R, Thomas M, Nestler EJ.(2013) Behavioral and structural responses to chronic cocaine require a feedforward loop involving ΔFosB and calcium/calmodulin- dependent protein kinase II in the nucleus accumbens shell.J Neurosci.33(10):4295-307.
89. White SL, Schmidt HD, Vassoler FM, Pierce RC. (2013). Acute cocaine increases phosphorylation of CaMKII and GluA1 in the dorsolateral striatum of drug naïve rats, but not cocaine-experienced rats.Neurosci Lett.537:71-6.
90. Miller EC, Zhang L, Dummer BW, Cariveau DR, Loh H, Law PY, Liao D. (2012) Differential modulation of drug-induced structural and functional plasticity of dendritic spines. Mol Pharmacol. 82(2):333-43.
91. Zachariou V, Bolanos CA, Selley DE, Theobald D, Cassidy MP, Kelz MB, Shaw-Lutchman T, Berton O, Sim-Selley LJ, Dileone RJ, Kumar A, Nestler EJ.(2006) An essential role for DeltaFosB in the nucleus accumbens in morphine action. Nat Neurosci. 9(2):205-11.
92. Maze I, Covington HE 3rd, Dietz DM, LaPlant Q, Renthal W, Russo SJ, Mechanic M, Mouzon E, Neve RL, Haggarty SJ, Ren Y, Sampath SC, Hurd YL, Greengard P, Tarakhovsky A, Schaefer A, Nestler EJ.(2010) Essential role of the histone methyltransferase G9a in cocaine-induced plasticity. Science. 327(5962):213-6.
93. Zheng H, Zeng Y, Chu J, Kam AY, Loh HH, Law PY. (2010) Modulations of NeuroD activity contributes to the differential effects of morphine and fentanyl on dendritic spine stability. J Neurosci.

30(24):8102-10.
94. Gaudillière B, Konishi Y, de la Iglesia N, Yao Gl, Bonni A. (2004) A CaMKII-NeuroD signaling pathway specifies dendritic morphogenesis. Neuron. 41(2):229-41.
95. Orefice LL, Waterhouse EG, Partridge JG, Lalchandani RR, Vicini S, Xu B. (2013) Distinct roles for somatically and dendritically synthesized brain-derived neurotrophic factor in morphogenesis of dendritic spines. J Neurosci. 33(28):11618-32.
96. Vigers AJ, Amin DS, Talley-Farnham T, Gorski JA, Xu B, Jones KR. (2012) Sustained expression of brain-derived neurotrophic factor is required for maintenance of dendritic spines and normal behavior. Neuroscience.212:1-18.
97. Koo JW, Mazei-Robison MS, Chaudhury D, Juarez B, LaPlant Q, Ferguson D, Feng J, Sun H, Scobie KN, Damez-Werno D, Crumiller M, Ohnishi YN, Ohnishi YH, Mouzon E, Dietz DM, Lobo MK, Neve RL, Russo SJ, Han MH, Nestler EJ. (2012) BDNF is a negative modulator of morphine action. Science 338(6103):124-8.
98. Horch HW, Krüttgen A, Portbury SD, Katz LC. (1999)Destabilization of cortical dendrites and spines by BDNF. Neuron 23(2):353-64.
99. Dietz DM, Sun H, Lobo MK, Cahill ME, Chadwick B, Gao V, Koo JW, Mazei-Robison MS, Dias C, Maze I, Damez-Werno D, Dietz KC, Scobie KN, Ferguson D,Christoffel D, Ohnishi Y, Hodes GE, Zheng Y, Neve RL, Hahn KM, Russo SJ, Nestler EJ. (2012) Rac1 is essential in cocaine-induced structural plasticity of nucleus accumbens neurons. Nat Neurosci. 15(6):891-6.
100. Badiani A, Belin D, Epstein D, Calu D, Shaham Y. (2011) Opiate versus psychostimulant addiction: the differences do matter. Nat Rev. Neurosci. 12(11):685-700.
101. Mazei-Robison MS, Nestler EJ. (2012) Opiate-induced molecular and cellular plasticity of ventral tegmental area and locus coeruleus catecholamine neurons. Cold Spring Harb. Perspect Med. 2(7):a012070.
102. Lagerström MC, Schiöth HB. (2008) Structural diversity of G protein-coupled receptors and significance for drug discovery. Nat Rev Drug Discov. 7(4):339-57.
103. Rosenbaum DM, Rasmussen SG, Kobilka BK. (2009). The structure and function of G-protein-coupled receptors. Nature. 459(7245):356-63.
104. Betke KM, Wells CA, Hamm HE. (2010) GPCR mediated regulation of synaptic transmission. Prog Neurobiol. 96(3):304-21.
105. Chen Y, Mestek A, Liu J, Hurley JA, Yu L. (1993) Molecular cloning and functional expression of a mu-opioid receptor from rat brain. Mol Pharmacol. 44(1):8-12.
106. Kieffer BL. (1995) Recent advances in molecular recognition and signal transduction of active peptides: receptors for opioid peptides. Cell Mol Neurobiol. 15(6):615-35.
107. Mansour A, Fox CA, Akil H, Watson SJ.(1995) Opioid-receptor mRNA expression in the rat CNS: anatomical and functional implications. Trends Neurosci. 18(1):22-9.
108. Moriwaki A, Wang JB, Svingos A, van Bockstaele E, Cheng P, Pickel V, Uhl GR. (1996) mu Opiate receptor immunoreactivity in rat central nervous system. Neurochem Res.21(11):1315-31
109. Georges F, Normand E, Bloch B, Le Moine C.(1998)Opioid receptor gene expression in the rat brain during ontogeny, with special reference to the mesostriatalsystem: an in situ hybridization study.Brain Res Dev Brain Res.109(2):187-99.
110. Wang H, Cuzon VC, Pickel VM. (2003)Postnatal development of mu-opioid receptors in the rat caudate-putamen nucleus parallels asymmetric synapse formation. Neuroscience. 118(3):695-708.
111. Matthes HW, Maldonado R, Simonin F, Valverde O, Slowe S, Kitchen I, Befort K, Dierich A, Le Meur M, Dollé P, Tzavara E, Hanoune J, Roques BP, Kieffer BL.(1996) Loss of morphine-induced analgesia, reward effect and withdrawal symptoms in mice lacking the mu-opioid-receptor gene. Nature.383(6603):819-23.
112. Nassirpour R, Bahima L, Lalive AL, Lüscher C, Luján R, Slesinger PA.(2010)Morphine- and CaMKII-dependent enhancement of GIRK channel signaling in hippocampal neurons.J Neurosci.30(40):13419-30.
113. Mitrovic I, Margeta-Mitrovic M, Bader S, Stoffel M, Jan LY, Basbaum AI.(2003) Contribution of GIRK2-mediated postsynaptic signaling to opiate and alpha 2-adrenergic analgesia and analgesic sex differences.Proc Natl Acad Sci U S A. 100(1):271-6.
114. Fajardo-Serrano A, Wydeven N, Young D, Watanabe M, Shigemoto R, Martemyanov KA, Wickman K, Luján R. (2013) Association of Rgs7/Gβ5 complexes with girk channels and GABAB receptors in hippocampal CA1 pyramidal neurons.Hippocampus [Epub ahead of print]
115. Xie K,Allen KL, Kourrich S, Colón-Saez J, Thomas MJ, Wickman K, Martemyanov KA.(2010)Gbeta5 recruits R7 RGS proteins to GIRK channels to regulate the timing of neuronal inhibitory signaling.Nat Neurosci.13(6):661-3.
116. Shenoy SK, McDonald PH, Kohout TA, Lefkowitz RJ.(2001)Regulation of receptor fate by ubiquitination of activated beta 2-adrenergic receptor and beta-arrestin.Science. 294(5545):1307-13.
117. Attramadal H, Arriza JL, Aoki C, Dawson TM, Codina J, Kwatra MM, Snyder SH, Caron MG, Lefkowitz RJ.(1992)Beta-arrestin2, a novel member of the arrestin/beta-arrestin gene family.J Biol Chem.267(25):17882-90.
118. Bohn LM, Lefkowitz RJ, Gainetdinov RR, Peppel K, Caron MG, Lin FT.(1999)Enhanced morphine analgesia in mice lacking beta-arrestin 2. Science.286(5449):2495-8
119. Gold SJ, Ni YG, Dohlman HG, Nestler EJ. (1997) Regulators of G-protein signaling (RGS) proteins: region-specific expression of nine subtypes in rat brain. J Neurosci.17(20):8024-37.
120. Zachariou V, Georgescu D, Sanchez N, Rahman Z, DiLeone R, Berton O, Neve RL, Sim- Selley LJ, Selley DE, Gold SJ, Nestler EJ. (2003) Essential role for RGS9 in opiate action Proc Natl Acad Sci U S A. 2003 Nov 11;100(23):13656-61. Epub 2003 Oct 31.
121. Han MH, Renthal W, Ring RH, Rahman Z, Psifogeorgou K, Howland D, Birnbaum S, Young K, Neve R, Nestler EJ, Zachariou V. (2010) Brain region specific actions of regulator of G protein signaling 4 oppose morphine reward and dependence but promote analgesia. Biol Psychiatry. 67(8):761-9.
122. Terzi D, Stergiou E, King SL, Zachariou V. (2009) Regulators of G protein signaling in neuropsychiatric disorders. Prog Mol Biol Transl Sci 86:299-333.
123. Stratinaki M, Varidaki A, Mitsi V, Ghose S, Magida J, Dias C, Russo SJ, Vialou V, Caldarone BJ,

Tamminga CA, Nestler EJ, Zachariou V. (2013) Regulator of G protein signaling 4 is a crucial modulator of antidepressant drug action in depression and neuropathic pain models. Proc Natl Acad Sci U S A. 110(20):8254-9.

124. Lerner TN, Kreitzer AC. (2012) RGS4 is required for dopaminergic control of striatal LTD and susceptibility to parkinsonian motor deficits. Neuron. 26;73(2):347-59.
125. Neubig RR, Siderovski DP. (2002) Regulators of G-protein signalling as new central nervous system drug targets. Nat Rev Drug Discov. 1(3):187-97.
126. Granneman JG, Zhai Y, Zhu Z, Bannon MJ, Burchett SA, Schmidt CJ, Andrade R, Cooper J. (1998) Molecular characterization of human and rat RGS 9L, a novel splice variant enriched in dopamine target regions, and chromosomal localization of the RGS 9 gene. Mol Pharmacol. 54(4):687-94.
127. Chen, C. K., P. Eversole-Cire, H. K. Zhang, V. Mancino, Y. J. Chen, W. He, T. G. Wensel, and M. I. Simon. (2003) Instability of GGL domain-containing RGS proteins in mice lacking the G protein β-subunit Gβ5. Proc. Natl. Acad. Sci. USA 1006604-6609.
128. Bouhamdan M, Michelhaugh SK, Calin-Jageman I, Ahern-Djamali S, Bannon MJ. (2000) Brain-specific RGS9-2 is localized to the nucleus via its unique proline-rich domain.Biochim Biophys Acta. May 3;1691(2-3):141-50.
129. Zhang K, Howes KA, He W, Bronson JD, Pettenati MJ, Chen C, Palczewski K, Wensel TG, Baehr W. Structure, alternative splicing, and expression of the human RGS9 gene.Gene. 240(1):23-34
130. Song JH, Waataja JJ, Martemyanov KA.(2006) Subcellular targeting of RGS9-2 is controlled by multiple molecular determinants on its membrane anchor, R7BP. J Biol Chem. 281(22):15361-9.
131. Anderson GR,Semenov A, Song JH, Martemyanov KA. (2007) The membrane anchor R7BP controls the proteolytic stability of the striatal specific RGS protein, RGS9-2.J Biol Chem. 282(7):4772-81.
132. Anderson GR,Lujan R, Semenov A, Pravetoni M, Posokhova EN, Song JH, Uversky V, Chen CK, Wickman K, Martemyanov KA. (2007) Expression and localization of RGS9-2/Gβ5/R7BP complex in vivo is set by dynamic control of its constitutive degradation by cellular cysteine proteases. J Neurosci. 27(51):14117-27.
133. Papachatzaki MM, Antal Z, Terzi D, Szücs P, Zachariou V, Antal M. (2011) RGS9-2 modulates nociceptive behaviour and opioid-mediated synaptic transmission in the spinal dorsal horn. Neurosci Lett. 501(1):31-4.
134. Rahman Z, Gold SJ, Potenza MN, Cowan CW, Ni YG, He W, Wensel TG, Nestler EJ. (1999) Cloning and characterization of RGS9-2: a striatal-enriched alternatively spliced product of the RGS9 gene.J Neurosci. 19(6):2016-26.
135. Psifogeorgou K, Papakosta P, Russo SJ, Neve RL, Kardassis D, Gold SJ, Zachariou V. (2007) RGS9-2 is a negative modulator of mu-opioid receptor function. J Neurochem.103(2):617-25.
136. Psifogeorgou K, Terzi D, Papachatzaki MM, Varidaki A, Ferguson D, Gold SJ, Zachariou V. (2011) A unique role of RGS9-2 in the striatum as a positive or negative regulator of opiate analgesia. J Neurosci.31(15):5617-24.
137. Cabrera-VeraTM,Hernandez S,Earls LR,Medkova M,Sundgren-Andersson AK, Surmeier DJ, Hamm HE. (2004)RGS9-2 modulates D2 dopamine receptor-mediated Ca2+ channel inhibition in rat striatal cholinergic interneurons.Proc Natl Acad Sci U S A.101(46):16339-44.
138. 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.(2003)RGS9 modulates dopamine signaling in the basal ganglia.Neuron.38(6):941-52.Zheng M, Cheong SY, Min C, Jin M, Cho DI, Kim KM.(2011)β-arrestin2 plays permissive roles in the inhibitory activities of RGS9-2 on G protein-coupled receptors by maintaining RGS9-2 in the open conformation.Mol Cell Biol.31(24):4887-901.
139. Zheng M, Cheong SY, Min C, Jin M, Cho DI, Kim KM.(2011) β-arrestin2 plays permissive roles in the inhibitory activities of RGS9-2 on G protein-coupled receptors by maintaining RGS9-2 in the open conformation.Mol Cell Biol.31(24):4887-901.
140. 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.50(10):813-6.
141. Gold SJ, Hoang CV, Potts BW, Porras G, Pioli E, Kim KW, Nadjar A, Qin C, LaHoste GJ, Li Q, Bioulac BH, Waugh JL, Gurevich E, Neve RL, Bezard E. (2007)RGS9-2 negatively modulates L-3,4-dihydroxyphenylalanine-induced dyskinesia in experimental Parkinson's disease.J Neurosci.27(52):14338-48.
142. Li Y, Kolb B, Robinson TE. (2003). The location of persistent amphetamine-induced changes in the density of dendritic spines on medium spiny neurons in the nucleus accumbens and caudate-putamen. Neuropsychopharmacology. 28(6):1082-5.
143. Sun H, Maze I, Dietz DM, Scobie KN, Kennedy PJ, Damez-Werno D, Neve RL, Zachariou V, Shen L, Nestler EJ. (2012) Morphine epigenomically regulates behavior through alterations in histone H3 lysine 9 dimethylation in the nucleus accumbens. J Neurosci. 32(48):17454-64