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Ο Ρόλος των RGS πρωτεϊνών στις δράσεις των οπιοειδών

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A Role of RGS proteins in Opiate Actions

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Στην οικογένειά μου…….

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Ευχαριστίες

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Περίληψη

Τα οποιοειδή αναλγητικά φάρμακα αποτελούν μια από τις πιο αποτελεσματικές θεραπείες σε περιπτώσεις οξέος πόνου, αλλά η χρόνια χρήση τους αντενδείκνυται λόγω της ανάπτυξης αναλγητικής αντοχής καθώς και φαινομένων εξάρτησης και εθισμού. Η κατανόηση των μοριακών μηχανισμών που ευθύνονται τόσο για τις αναλγητικές τους ιδιότητες, όσο και για την εμφάνιση των ανεπιθύμητων παρενεργειών, θα αποτελούσε σημαντική συνεισφορά στην βελτίωση της θεραπείας κατά του χρόνιου πόνου. Υπό αυτό το πρίσμα, σκοπό της εν λόγω διδακτορικής διατριβής αποτέλεσε η διερεύνηση του ρόλου των RGS πρωτεϊνών (ρυθμιστές της σηματοδότησης μέσω G πρωτεϊνών) στις δράσεις των οποιοειδών. Οι RGS πρωτεΐνες αποτελούν κύριο ρυθμιστή της σηματοδότησης μέσω υποδοχέων συνδεδεμένων με G-πρωτεΐνες (GPCR), επηρεάζοντας τόσο την διάρκεια όσο και την κατεύθυνση της σηματοδότησης δια της αλληλεπίδρασής τους με τις ετεροτριμερείς Gα υπομονάδες. Η παρούσα μελέτη επικεντρώθηκε σε δύο μέλη της RGS οικογένειας, που εμπλέκονται στην σηματοδότηση μέσω του μ υποδοχέα των οποιοειδών (MOPR), την RGS9-2 και την RGSz1. Συγκεκριμένα, η διατριβή αποτελείται από τρείς επιμέρους ερευνητικούς σκοπούς:

Πρώτο σκοπό αποτέλεσε η μελέτη του ρόλου της πρωτεΐνης RGS9-2 στις δράσεις της οξυκωδόνης με ή χωρίς την παρουσία χρόνιου πόνου. Η πρωτεΐνη RGS9-2 εκφράζεται κατά βάση στην περιοχή του ραβδωτού και είναι γνωστή η σημαντικότατη δράση της στην σηματοδότηση μέσω του MOPR. Αποτελεί αρνητικό ρυθμιστή τόσο των αναλγητικών όσο και των ανταμοιβικών δράσεων της μορφίνης, ενώ παράλληλα συμβάλλει στην ανάπτυξη αναλγητικής ανοχής. Αντιθέτως, φέρεται ως θετικός ρυθμιστής άλλων αγωνιστών του MOPR, όπως η μεθαδόνη και η φαιντανύλη. Εν προκειμένω διερευνήθηκε ο ρόλος της στις δράσης ενός ακόμη αγωνιστή του MOPR, ο οποίος παρουσιάζει εκθετικά αυξανόμενη χρήση καθώς και κατάχρηση τα τελευταία χρόνια, της οξυκωδόνης. Χρησιμοποιώντας διαγονιδιακά ποντίκια με έλλειψη του *Rgs9* γονιδίου (RGS9KO), παρατηρήθηκε ότι η RGS9-2 συμβάλλει στην εγκαθίδρυση των ανταμοιβικών δράσεων της οξυκωδόνης σε υγιή ζώα καθώς και σε ζώα που έχουν υποβληθεί σε μοντέλο χρόνιου νευροπαθητικού πόνου. Επιπλέον, ενώ η RGS9-2 δεν επηρεάζει τις άμεσες αναλγητικές δράσεις του φαρμάκου, παρεμποδίζει την ανάπτυξη αναλγητικής ανοχής. Σε γενικές γραμμές, τα ανωτέρω αναφερόμενα αποτελέσματα παρέχουν νέες πληροφορίες σχετικά με τους μοριακούς μηχανισμούς που διέπουν τις αναλγητικές και ανταμοιβικές δράσεις της οξυκωδόνης.

Ο δεύτερος ερευνητικός στόχος της διατριβής αφορά τον ρόλο της RGSz1 στις δράσεις των οποιοειδών. Η RGSz1 είναι μια μικρή RGS πρωτεΐνη, εκφραζόμενη κατά κύριο λόγο στον εγκέφαλο, η οποία εμφανίζει ιδιαίτερα υψηλή ειδικότητα για τις Gαz υπομονάδες και φέρεται να

εμπλέκεται στην ρύθμιση του MOPR καθώς και του υποδοχέα σεροτονίνης 1A (Htr1A). Με την χρήση γενετικά τροποποιημένων ποντικών με ολική ή μερική καταστολή έκφρασης της RGSz1, παρατηρήθηκε ότι οι αναλγητικές ιδιότητες διαφόρων αγωνιστών του MOPR αυξάνονται έπειτα από παρεμπόδιση της δράσης της πρωτεΐνης τόσο σε αρσενικά όσο και σε θηλυκά ποντίκια. Επιπλέον, σημειώθηκε καθυστέρηση στην ανάπτυξη αναλγητικής ανοχής στη μορφίνη, με ταυτόχρονη μείωση των ανταμοιβικών δράσεων του φαρμάκου. Πειράματα αλληλούχισης επόμενης γενιάς (RNA sequencing), σε συνδυασμό με βιοχημικά πειράματα προσδιορισμού επιπέδων έκφρασης καθώς και υποκυττάριου εντοπισμού πρωτεϊνών, ανέδειξαν τον μοριακό μηχανισμό μέσω του οποίου η RGSz1 στην περιϋδραγωγό φαιά ουσία (PAG) επηρεάζει την ανάπτυξη αναλγητικής ανοχής στην μορφίνη. Παρατηρήθηκε ότι η επανειλημμένη χορήγηση μορφίνης ενισχύει τη δράση της RGSz1 στο PAG, η οποία με τη σειρά της συμβάλλει στην καταστολή της σηματοδότησης μέσω του μονοπατιού Wnt/β-catenin προάγοντας την ανάπτυξη αναλγητικής ανοχής. Αντιστρόφως, παρεμπόδιση της δράσης της RGSz1 έχει ως αποτέλεσμα την διατήρηση σταθερότερων συμπλόκων Axin2-Gαz κοντά στην πλασματική μεμβράνη, γεγονός που οδηγεί σε ενεργοποίηση της β-catenin και αναστολή ανάπτυξης αναλγητικής ανοχής. Συνοψίζοντας, τα εν λόγω δεδομένα υποδεικνύουν την σημαντικότητα της στόχευσης των αλληλεπιδράσεων των RGS πρωτεϊνών, και συγκεκριμένα του συμπλόκου RGSz1-Gαz, για την βελτίωση της αναλγητικής δράσης των οποιοειδών χωρίς αύξηση της πιθανότητας ανάπτυξης εθισμού.

Τέλος, ο τρίτος ερευνητικός σκοπός της μελέτης επικεντρώθηκε στον ρόλο της RGSz1, τόσο στις συμπεριφορικές αποκρίσεις όσο και στις βιοχημικές προσαρμογές, σε ζωικά μοντέλα χρόνιου πόνου. Χρησιμοποιώντας ευρέως εδραιωμένα ζωικά μοντέλα χρόνιου πόνου, παρατηρήθηκε ότι αναστολή έκφρασης της RGSz1 στο PAG θηλυκών ποντικών, οδηγεί σε παρατεταμένες αλγαισθητικές αποκρίσεις σε περιπτώσεις φλεγμονώδους ή νευροπαθητικού πόνου. Επιπλέον, συνδυάζοντας βασικές τεχνικές μοριακής βιολογίας (Αλυσιδωτή αντίδραση πολυμεράσης σε πραγματικό χρόνο, ανοσοαποτύπωση) με πειράματα αλληλούχισης επόμενης γενιάς (RNA sequencing) διαπιστώθηκε ότι υπό καταστάσεις χρόνιου φλεγμονώδους πόνου επάγονται μοναδικές μεταγραφικές αλλαγές στο PAG των θηλυκών ποντικών, συμπεριλαμβανομένων αλλαγών στην έκφραση γονιδίων που σχετίζονται με παραγωγή και απελευθέρωση σεροτονίνης. Περεταίρω ανάλυση ανέδειξε ως ανώτερο ρυθμιστή των επαγόμενων προσαρμογών την σηματοδότηση μέσω του α-υποδοχέα των ιστογόνων (ERα). Τα ανωτέρω αποτελέσματα παρέχουν πληροφορίες σχετικά με τους φυλο-εξαρτώμενους ενδοκυττάριους μηχανισμούς που διέπουν τις αλγαισθητικές αποκρίσεις, τονίζοντας επιπλέον την χρησιμότητα της RGSz1 ως φαρμακευτικού στόχου για περιπτώσεις χρόνιου πόνου.

Abstract

Opioids are among the most effective medications used for the alleviation of severe pain conditions, but their long-term use is hindered by the development of analgesic tolerance, dependence and addiction. Understanding the molecular mechanisms underlying their analgesic effects and the adaptations leading to the development of the undesirable side-effects would be a significant contribution towards improving chronic pain treatment. Falling within this rational, the present thesis focused on examining the role of regulator of G-protein signaling (RGS) proteins in opiate actions. RGS proteins possess a wide and diverse family of proteins known to mediate G-protein coupled receptor (GPCR) signaling duration and direction, through their interaction with heterotrimeric Gα subunits. Here, we study the role of two RGS family members that modulate Mu opioid receptor (MOPR) mediated signaling, RGS9-2 and RGSz1. Particularly, the thesis is consisting of three specific aims:

Aim I examined if and how RGS9-2 modulates responses to oxycodone both in pain-free conditions and under chronic pain states. RGS9-2 is predominantly expressed in the striatum and it has been shown to exert a critical role on the MOPR signaling. It is a negative regulator of morphine analgesia and reward, while at the same time it promotes the development of morphine tolerance. In contrast, RGS9-2 appears as a positive modulator of other MOPR agonist, such as methadone and fentanyl. Here we investigate its role as a regulator of another MOPR agonist widely used and abused over the last years, oxycodone. Using transgenic mice lacking the *Rgs9* gene (RGS9KO), we observed that RGS9-2 positively regulates the rewarding effects of oxycodone both in pain-free states and in a model of neuropathic pain. Furthermore, although RGS9-2 does not affect the acute analgesic efficacy of the drug, it opposes the development of oxycodone tolerance. Overall, these results provide new information on the signal transduction mechanisms underlying the analgesic and rewarding actions of oxycodone.

Aim II is focused on the role of RGSz1 in opiate actions. RGSz1 is a small RGS protein primarily expressed in brain tissue, that exhibits high specificity for Gαz subunits and has been shown to act downstream of MOPR and serotonin receptor 1A (Htr1A). Using genetic mouse models for constitutive or conditional/brain region-targeted manipulations of RGSz1 expression, we demonstrated that the analgesic efficacy of several MOPR agonists is increased by preventing RGSz1 actions both in male and female mice. In addition, prevention of RGSz1 action delays the development of morphine tolerance while decreasing the sensitivity to rewarding and locomotor activating effects. Using next-generation RNA sequencing combined with further biochemical

assays examining protein expression and localization, we identified a key role of RGSz1 in the periaqueductal gray (PAG) in morphine tolerance. We show that chronic morphine administration promotes RGSz1 activity in the PAG, which in turn modulates transcription mediated by the Wnt/β-catenin signaling pathway and promotes analgesic tolerance to morphine. Conversely, prevention of RGSz1 action stabilizes Axin2-Gαz complexes near the membrane, promoting β-catenin activation, thereby delaying the development of analgesic tolerance. These data highlight that regulation of RGS complexes, particularly those involving RGSz1-Gαz, represent a promising target for optimizing the analgesic actions of opioids without increasing the risk of dependence or addiction.

Finally, aim III examined the role of RGSz1 in behavioral and biochemical adaptations to chronic pain. Using well established murine pain models, we show that deletion of the *Rgsz1* gene, as well as conditional knockdown of RGSz1 in the mouse PAG, lead to prolonged sensitized behaviors only in female mice undergoing inflammatory or neuropathic pain. In addition, by combining standard molecular biology techniques (RT-PCR, Western Blot) with next-generation RNA sequencing we showed that chronic inflammatory pain-like states promote a number of unique adaptations in the female PAG, including changes in the expression of molecules involved in serotonin synthesis and release. Further upstream regulator analysis, together with behavioral and biochemical data, revealed the involvement of estrogen receptor mediated signaling in nociceptive hypersensitivity. These findings provide insight to the sex-specific intracellular mechanisms underlying nociceptive responses under chronic pain conditions, and point to RGSz1-regulated pathways as promising targets for the treatment of chronic pain.

Introduction

Pain

Definition & Classification

The International Association of the Study of Pain (IASP) is defining pain as “an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage”, a definition initially established by Harold Merskey on 1994 (Merskey and Bogduk, 1994). Serving as an essential protective function alerting as against health threatening injuries, pain is not simply a sensory modality but rather a complicated integration of a variety of neural signals elaborately processed by the brain. As a result, each individual’s experience of the same stimulus depends largely on mood, attention, memories, and genetics making pain a subjective experience unique to the organism and the context and therefore hard to understand and control.

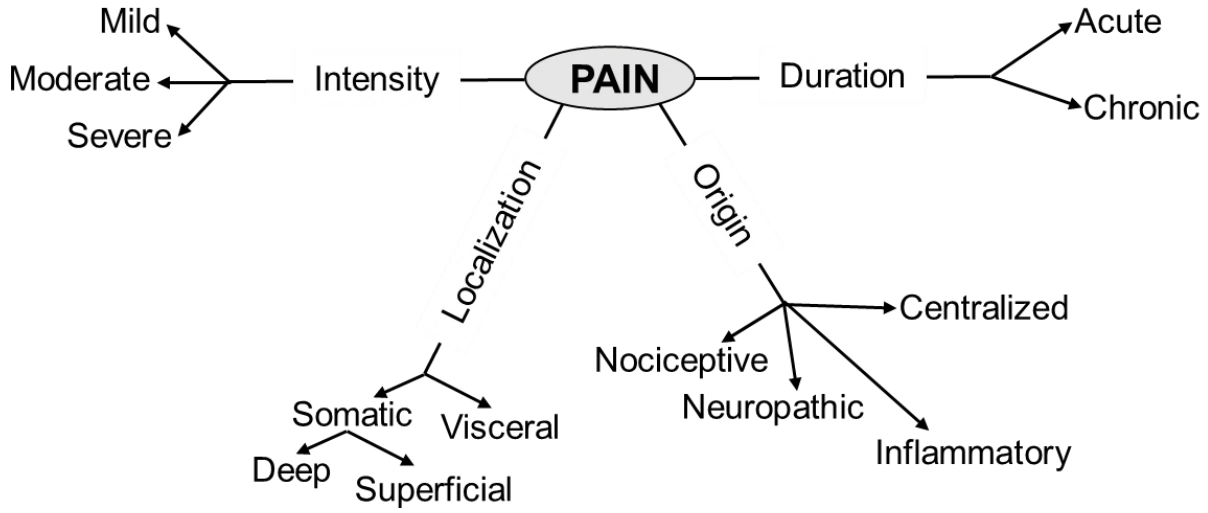


Figure A. Pain Classification. Pain is a complicated condition that largely depends on genetic and environmental factors and therefore classifying it in distinct categories becomes a demanding task. Some of the most widely accepted and used distinction characteristics are provided here. Based on intensity pain can be mild, moderate or severe. Based on the duration of the experience it can be considered either acute or chronic. Based on the localization of the nociceptive stimulus it can be somatic or visceral, with somatic pain further dividing into superficial or deep. Finally based on the origin of the stimulus pain can be nociceptive, neuropathic, inflammatory or centralized.

On the same basis, it becomes really difficult to classify pain conditions. A general and well-established categorization relies on pain duration. Acute pain (usually lasting up to a few days) is caused by injury to a specific part of the body, remains restricted to the injury site and it terminates right after healing. On the other side, chronic pain persists long after the peripheral stimulus has disappeared and sometimes even arises in the absence of any obvious pathological trigger. In terms of origin, pain can be divided in four distinct states: nociceptive pain is deriving from a localized noxious (mechanical) insult, inflammatory pain is caused by peripheral inflammation, neuropathic pain results as a consequence of sensory nerve damage, and dysfunctional or centralized pain has no detectable pathology. Finally, pain can also be classified based on localization to somatic or visceral and based on intensity to mild, moderate or severe (Fig. A).

Neuroanatomy & Pain Pathways

The pain signal travels from the site of the noxious stimulus, through several ascending tracts, to distinct structures in the brain. These structures then descend back down to the spinal cord in a feedback loop that influences the ascending information and subsequently alters pain perception (Basbaum and Fields, 1978; D'Mello and Dickenson, 2008; Millan, 2002; Perl, 2011; Schaible, 2007). Further information for the structures involved is provided below. For a schematic overview see figure B.

Noxious Stimuli Activate Nociceptors. Named after the Latin verb nocere (meaning "to hurt") and the English receptor, nociceptors are simply the free nerve endings of primary sensory neurons innervating peripheral organs and initiating the transmission of the pain signal to the brain. This specialized set of nerve fibers consists of unmyelinated C fibers and thinly myelinated Aδ fibers, which are distinct from myelinated tactile sensors (Aβ fibers) and proprioceptors. There are three main classes of nociceptors: thermal, mechanical and polymodal. Thermal and mechanical nociceptors, as their names imply, are activated by extremes in temperature and intense pressure, respectively. They are both peripheral endings of small-diameter thinly myelinated Aδ axons and they are responsible for the "fast-sharp" pain sensation that initially occurs after injury. On the other hand, polymodal nociceptors can be activated by high-intensity mechanical, chemical or thermal stimuli, are found at the ends of small-diameter unmyelinated C axons and transmit the "slow-dull" pain that follows the initial sharp pain. Noxious stimuli are converted to electrical activity by transient receptor potential–generating channels (TRP channels) and purinergic channels, and this electrical activity is amplified by sodium channels to elicit action potentials. The action potentials generated travel through the dorsal root ganglia

(DRG), where the cell bodies of the nociceptive sensory neurons are located, to the dorsal horn of the spinal cord where they transmit the signal onto second-order neurons mostly in the superficial laminae (I and II). Some processing and integration of the sensory signals occurs at this level, and the processed output from the spinal networks is carried through ascending pathways to distinct projection sites in the brain.

Ascending tracts. There are five major ascending pathways contributing to the central processing of nociceptive information: the spinothalamic tract, the spinoreticular tract, the spinomesencephalic or spinoparabrachial tract, the cervicothalamic tract and the spinohypothalamic tract. The spinothalamic tract consists of thermosensitive, nociception-specific and wide-dynamic-range neurons that initiate from laminae I, V, VI and VII of the dorsal horn and terminate in thalamic nuclei. The spinoreticular tract neurons project from laminae VII and VIII to both the reticular formation and the thalamus. The spinomesencephalic tract project from laminae I and V to the reticular formation, the periaqueductal gray (PAG) and the parabrachial nucleus. This tract is believed to contribute to the affective components of pain, mainly due to the known connection between the parabrachial nucleus and the amygdala regulation emotional states. The cervicothalamic tract connects laminae III and IV with midbrain nuclei and thalamus. Finally, the spinohypothalamic tract projects from laminae I, V and VIII to hypothalamic nuclei and it is thought to drive the neuroendocrine responses that accompany pain syndromes.

Descending tracts. There are several areas of endogenous pain control in the brain. Among them the most effective one on suppressing nociception appears to be the ventrolateral PAG (vlPAG). Stimulation of this particular area of the midbrain has profound analgesic results without affecting touch, pressure or temperature sensation. From the neurons located in vlPAG only a few project directly to the dorsal horn of the spinal cord, whereas the vast majority make excitatory connections with neurons in the rostroventral medulla (RVM). From the RVM serotonergic neurons project to the dorsal horn of the spinal cord where they form inhibitory connections with neurons in laminae I, II and V. Thus, stimulation of the RVM inhibits firing of dorsal horn neurons, including projection neurons of the spinothalamic tract. There is also a second major monoaminergic descending system suppressing the activity of nociceptive neurons in the dorsal horn. A noradrenergic system initiating from locus ceruleus and other nuclei in the medulla and pons projecting to laminae I and V.

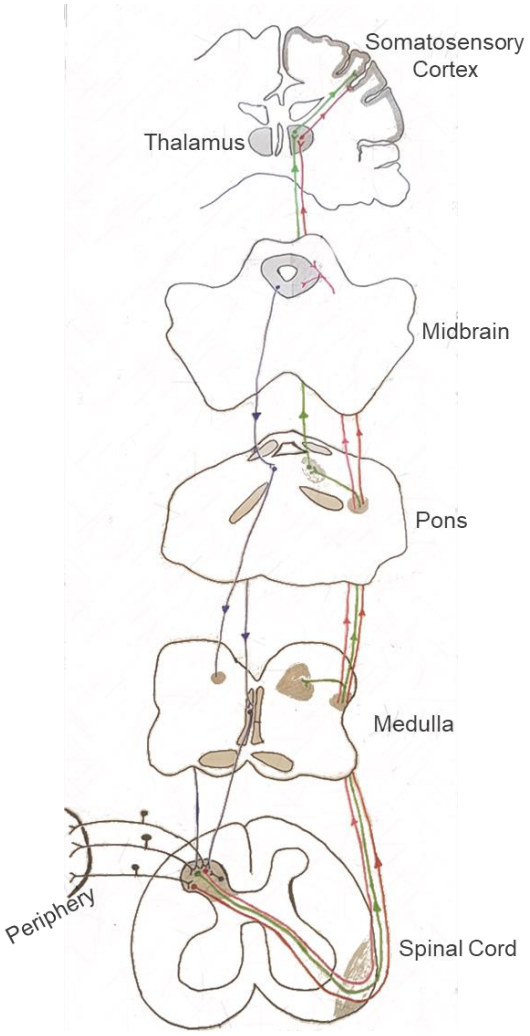


Figure B. A General Overview of Pain Pathways. Pain signals are transferred from the peripheral site of injury to the dorsal horn of the spinal cord through specialized nociceptive sensory neurons. Some processing and integration of the sensory signals occurs at this level, and the processed output from the spinal networks is carried through ascending pathways to distinct projection sites in the brain. The three main ascending tracts that have been widely studied over the years are depicted here. The spinothalamic pathway (shown in red) initiates from laminae I, V, VI and VII of the dorsal horn, passes through distinct nuclei in medulla and pons and terminates in thalamic nuclei. The spinoreticular pathway (shown in green) projects from laminae VII and VIII to both the reticular formation and the thalamus. Finally, the spinomesencephalic pathway (shown in pink) projects from laminae I and V to the reticular formation, the periaqueductal gray (PAG) and the parabrachial nucleus. From the thalamus sensory information is further transmitted to the somatosensory cortex. Except from these ascending pain-processing tracts there are also monoaminergic descending pain-modulating tracts (shown in blue) that originate from the PAG and trace back to the dorsal horn where they modulate neuronal excitability and integration of peripheral signals, therefore resulting in inhibition or in some cases facilitation of pain sensation.

Periaqueductal Gray (PAG). PAG constitutes the primary control center for descending pain modulation, with implication in ascending pain tracts as well. The first study reporting the involvement of PAG in pain dates back to 1937 (Magoun et al., 1937) with further evidence coming 20 years later from lesion studies revealing that lesions of the ventrolateral PAG (vlPAG) significantly attenuate pain perception (Melzack et al., 1958). In the following decades, numerous studies using a variety of different techniques identified ascending projections from spinal cord to PAG in monkey, cat, rabbit and rat (Blomqvist and Craig, 1991; Cliffer et al., 1991; Mantyh, 1982; Meller and Dennis, 1986; Wiberg et al., 1987; Yezierski, 1988). However, by far the most intense investigations of the PAG have focused on its role in descending pain inhibition, which was initially reported in 1969 when stimulation of the PAG was found to be analgesic in a degree that could

allow laparotomy in the absence of chemical anesthetics (Reynolds, 1969). A variety of studies indeed verified the existence of this stimulus-produced analgesia (SPA), that could also be recapitulated by local morphine injection, and further revealed that the mechanism underlying this phenomenon was inhibition of the dorsal horn neuron through PAG-RVM projections (Basbaum and Fields, 1978, 1984). Overall, the PAG serves as an integrator, collecting information from forebrain regions and the spinal cord and translating them to descending modulatory signals through complicated networks involving afferent projections, tonically-active GABAergic interneurons as well as efferent projections to brainstem nuclei.

Pathological chronic/persistent pain conditions

Although physiological pain is an important protective mechanism, it can take on a chronic disease character in pathological states such as diabetes, neuropathy, inflammation or cancer. Chronic pain is a major cause of human suffering worldwide (Breivik et al., 2006), especially because safe, specific and effective therapies have yet to be developed. A large body of research over the years has revealed that chronic pain is not simply an extension of acute pain signals but rather implicates distinct mechanisms. Transitioning from acute to chronic/pathological pain involves a variety of different activity-dependent changes throughout the nociceptive pathway (Kuner, 2010; Prescott et al., 2014; Sandkühler, 2009; Woolf, 2011). More details on the neurobiology of peripheral as well as central sensitization contributing to chronic pain transition are presented below.

Peripheral sensitization. In chronic pain states, especially in the case of peripheral inflammation, nociceptive afferents get sensitized. In other words, the peripheral ends of the sensory nerve fibers reduce their activation threshold and increase the magnitude of their responsiveness as a reaction to chemical mediators released at the site of injury. These mediators can be cytokines, chemokines, prostaglandins, neurotrophins, neuropeptides, proteases, protons or ATP released locally from nociceptors themselves as well as non-neuronal cells (keratinocytes, fibroblasts and immune cells) (Gold and Gebhart, 2010; Julius and Basbaum, 2001). By binding to specific receptors on nociceptors (tyrosine kinase receptors, G-protein coupled receptors, purinoceptors, acid-sensing ion channels and others) locally released chemical mediators modulate the activity of transduction proteins that control the excitability of nociceptor terminals both at the

transcriptional (by recruiting transcription factors) as well as at the post-translational level (for example, phosphorylation of TRP and sodium channels) (Julius and Basbaum, 2001).

Central sensitization. In contrast to peripheral sensitization, central sensitization implicates novel inputs that do not normally drive nociception (Woolf and Salter, 2000), which together with an enhanced function of central nervous system (CNS) neurons caused by increases in membrane excitability, synaptic efficacy, or a reduced inhibition, result in pain signals deriving in the absence of noxious stimuli or any peripheral pathology (Latremoliere and Woolf, 2009). The molecular and cellular mechanisms involved in the establishment of central sensitization at the level of the spinal cord have been widely studied over the last 3 decades. Among them NMDA receptors (Petrenko et al., 2003; Qu et al., 2009; Seltzer et al., 1991), mGlu receptors (Chen and Pan, 2005; Derjean et al., 2003; Dogrul et al., 2000; Goudet et al., 2008; Hu et al., 2007), AMPA receptors (Lu et al., 2008; Park et al., 2008), BDNF (Kerr et al., 1999; Lu et al., 2009; Mannion et al., 1999; Obata and Noguchi, 2006), Substance P (Abbadie et al., 1996; Lee and Kim, 2007), Nitric oxide (Chu et al., 2005; Tanabe et al., 2009) and bradykinin (Petcu et al., 2008) have all been shown to contribute to the development of central sensitization.

Murine chronic pain models. Animal models of pain/nociception have been used since the late 19th century and have been a crucial component on understanding pain processes. Numerous disease-specific paradigms have been developed in the effort to capture the heterogeneity of chronic pain conditions (see table 1). There are two major aspects in each model: the method of insult and the output behavioral assessment. The appropriate model in each case, whether it derives from an injury, the application of a chemical agent or milder manipulations, should lead to the nociceptive response by mimicking the mechanism underlying each particular clinical condition. Under the same rational, behavioral outcomes should resemble at least some of the main clinical symptoms associated with the disease.

Neuropathic Assays. The vast majority of neuropathic pain syndromes is caused due to partial damage of the nerves instead of complete transection. Similarly, animal models of neuropathic pain involve surgical transection or ligation of central or peripheral branches of the nerves innervating the hind-paw (Bennett and Xie, 1988; Decosterd and Woolf, 2000; Kim and Chung, 1992; Malmberg and Basbaum, 1998; Seltzer et al., 1990; Shields et al., 2003).

Inflammatory Assays. Most inflammatory pain models are generated by subcutaneous hind-paw or intra-joint injection of agents resulting on either direct activation of nociceptors by binding to TRP channels (carrageenan, formalin) (Iadarola et al., 1988; Tjølsen et al., 1992) or activation of the immune system locally at the site of injection (Complete Freund's Adjuvant) (Iadarola et al., 1988).

Painful Disease Assays. There are also attempts to directly model prevalent clinical pain syndromes by attempting to induce the disease, injury or physiologic state itself. Widely used examples are diabetic neuropathy (Wuarin-Bierman et al., 1987), HIV-induced neuropathy (Wallace et al., 2007), complex regional pain syndrome (Coderre et al., 2004), cancer pain – chemotherapeutic-induced neuropathy (Pacharinsak and Beitz, 2008), post-operative pain (Brennan et al., 1996; Duarte et al., 2005), as well as numerous visceral pain models (Berkley et al., 2007; Lantéri-Minet et al., 1995).

In terms of behavioral assessment several approaches have been followed to monitor both physical and affective components of pain and there is a strong controversy about the validity of some of these assays. Among them, well-established, typically measured behaviors involve: *Nociceptive/Nocifensive* responses, including spinal reflexes (withdrawal from the applied stimulus), spino-bulbospinal reflexes (jumping) or even simple innate behaviors (licking, biting). *Allodynia,* referring to a nociceptive response to a stimulus that would under normal conditions be innocuous (mechanical, tactile, cold). And *Hyperalgesia,* concerning exaggerated responses to a stimulus that normally would appear as subthreshold painful (the most commonly tested form is thermal hyperalgesia detected using the Hargrave's test).

Table 1. Murine Neuropathic & Inflammatory Pain Models

TYPE	MODEL	REFERENCE
Neuropathic Pain		
Peripheral Nervous System	Spared Nerve Injury (SNI)	(Decosterd and Woolf, 2000; Shields et al., 2003)
	Spinal Nerve Ligation (SNL)	(Kim and Chung, 1992)
	Chronic Constriction Injury (CCI)	(Bennett and Xie, 1988)
	Partial Nerve Ligation (PNL)	(Malmberg and Basbaum, 1998; Seltzer et al., 1990)
	Diabetic Neuropathy	(Wuarin-Bierman et al., 1987)
	Complex Regional Pain Syndrome (CRPS)	(Coderre et al., 2004)
	Dorsal Root Ganglion Compression	(Hu and Xing, 1998)
	HIV Neuropathy	(Wallace et al., 2007)
Central Nervous System	MASCIS Impactor model	(Young, 2002)
	Excitotoxic Spinal Cord Injury (ESCI)	(Yezierski et al., 1998)
Inflammatory Pain		
Hind-Paw	Complete Freund's Adjuvant (CFA)	(Iadarola et al., 1988)
	Formalin	(Hunskaar and Hole, 1987; Tjølsen et al., 1992)
	Carrageenan	(Iadarola et al., 1988)
	Ultraviolet-B-Induced Inflammation	(Bishop et al., 2007)
Arthritis	Knee Joint Injection	(Sluka and Westlund, 1993)
	Collagen-Induced Arthritis	(Brand et al., 2004)

Female prevalence for chronic pain conditions. Women are greatly overrepresented among patients with chronic pain (Berkley, 1997; Greenspan et al., 2007; Institute of Medicine (US) Committee on Understanding the Biology of Sex and Gender Differences, 2001; Mogil, 2012). The most prevalent chronic pain syndromes (low back pain, migraine, headache, osteoarthritis) are all marked female predominance and others found in both sexes (including fibromyalgia, cystitis, chronic fatigue syndrome) occur overwhelmingly more often in women (Fillingim et al., 2009). Furthermore, studies of laboratory pain sensitivity report lower thresholds for female rodents among a variety of different pain modalities (Mogil, 2012). The underlying mechanisms have yet to be determined, with increasing evidence supporting a role of gonadal hormones in pain regulation (Aloisi and Bonifazi, 2006; Craft et al., 2004; Kuba and Quinones-Jenab, 2005). There are several studies suggesting an antinociceptive effect of estrogen. Ovariectomized rats show exaggerated responses to formalin injection as well as thermal and mechanical stimuli (Ceccarelli et al., 2003; Pajot et al., 2003; Sanoja and Cervero, 2005), that are attenuated after estrogen replacement (Forman et al., 1989; Kuba et al., 2006; Ma et al., 2011). Estradiol also increases the pain threshold of ovariectomized rats in common acute nociception tests such as hot-plate and tail-flick (Craft et al., 2008; Stoffel et al., 2005). Furthermore, human studies propose a higher prevalence of chronic pain syndromes in postmenopausal women. Fibromyalgia peaks around menopause (Pamuk and Cakir, 2005) and postmenopausal women have been reported to be twice as likely to experience joint pain (Szoeke et al., 2008). Finally, the existence of estrogen receptors (ERα, ERβ, GPER1) in key areas of the pain pathway, such as DRG, superficial laminae of the dorsal horn, midbrain and brainstem nuclei (Pfaff and Keiner, 1973; Takanami et al., 2010; Vanderhorst et al., 2005), together with behavioral studies reporting altered nociceptive phenotypes in ERα and ERβ knock-out mice further support the implication of gonadal hormones in pain processing.

Treatment

Pain management strategies are mainly based on the World Health Organization (WHO) pain ladder (Organization, 1996) with slight modifications (Vargas-Schaffer, 2010). In general treatment begins with non-opioid medications, such as NSAIDs, progresses to weak opioids, and as a final solution moves to strong opioids, particularly for cancer pain. With an alternative in some chronic pain cases being the use of antidepressant medication (Mika et al., 2013).

NSAIDs and Acetaminophen. Non-Steroidal Anti-inflammatory Drugs (NSAIDs) such as aspirin and ibuprofen, as well as acetaminophen, act by inhibiting cyclooxygenase 1 and 2, the enzymes

catalyzing the synthesis of prostaglandins. Inhibiting prostaglandin synthesis results in the analgesic, anti-pyretic and anti-inflammatory properties of these drugs. The most commonly reported adverse effects are associated with hepatotoxicity and gastrointestinal symptoms.

Opioids. Opioids are the most potent among all available analgesics and compose the mainstay of chronic pain management (Furlan et al., 2010). They exert their analgesic properties by binding to opioid receptors in the central nervous system (CNS). Although they are among the most commonly used analgesics, their clinical use is limited by the development of analgesic tolerance, physical dependence and the risk of addiction. Detailed information on opioid use, different formulation and side effects are provided in the following chapter.

Antidepressants. Tricyclic antidepressants (TCAs) and selective serotonin re-uptake inhibitors (SSRIs) are also used as alternative for chronic pain treatment. The underlying mechanism of action is not fully understood but is believed to be mediated mainly through inhibition of neurotransmitter reuptake and direct modulation of ion channels in areas mediating descending pain modulation, as well as higher brain structures mediating the affective components of pain. Except from their analgesic role, antidepressants commonly serve as dual medication treating at the same time depressive symptoms that often co-exist with chronic pain.

Opioids

Opioid analgesics, as mentioned above, are the most efficacious drugs for the treatment of moderate to severe pain and represent the largest market share of prescription pain medications (Melnikova, 2010). Although opioids are effective pain relievers, they also produce a number of adverse side effects that can limit their clinical utility, including respiratory suppression, nausea and constipation. Moreover, long-term exposure to opioids is also associated with the development of analgesic tolerance, physical dependence, and addiction (Harris, 2008; Morgan and Christie, 2011). A major goal in opioid research is to understand the molecular and cellular mechanisms that give rise to opioid-induced physiological and behavioral responses and adaptations in order to develop improved analgesics that can selectively provide pain relief while reducing the onset of the unwanted side effects (Manglik et al., 2016; Skolnick and Volkow, 2016).

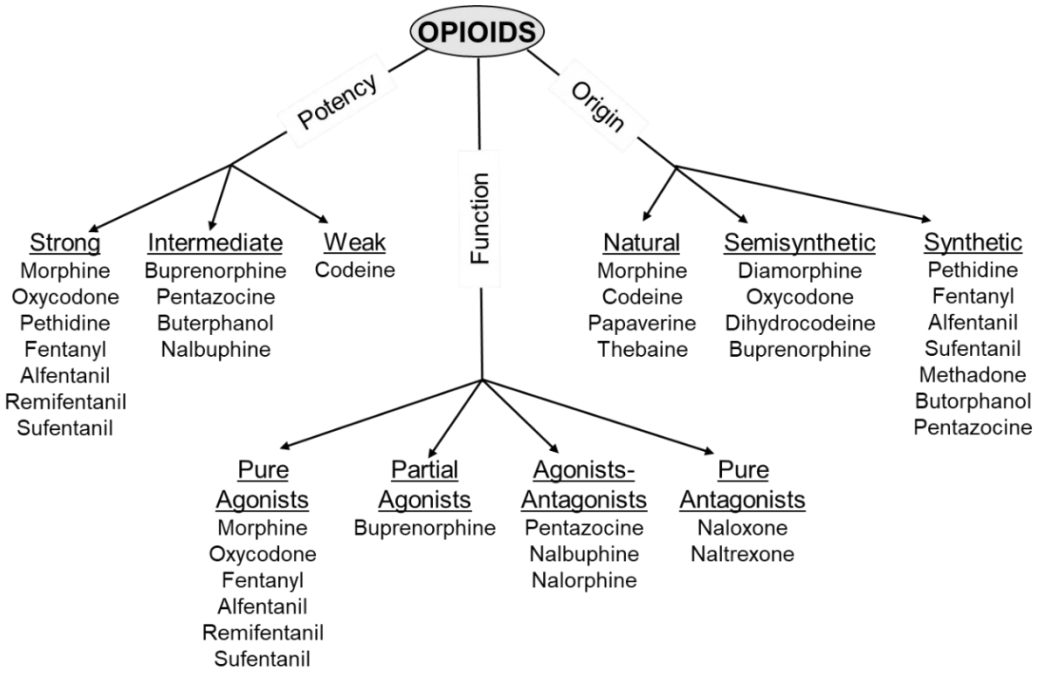


Figure C. Opioid Classification. Opioids can be classified based on their analgesic potency to strong, intermediate or weak analgesics. Another categorization focuses on the origin of each formulation, distinguishing between natural, semisynthetic and synthetic opioids. Finally, the efficacy towards the receptor can also be considered a factor of classification, leading to four distinct groups: pure agonists, partial agonists, agonists-antagonists and pure antagonists.

Mechanism of action

The μ opioid receptor (MOPR) belongs to the superfamily of G protein-coupled receptors (GPCRs) and it is the main opioid receptor subtype mediating the physiological actions of clinically used opioids (Kieffer, 1999). At the cellular level, the MOPR mediates opioid drug effects by coupling to heterotrimeric G proteins (Fig. C), particularly pertussis toxin-sensitive Gαi/o proteins, which act to inhibit adenylyl cyclases, modulate activity of certain ion channels, and signal through several second-messenger signal transduction cascades to promote signaling. As with most GPCRs, the extent and duration of agonist-induced MOPR signaling can be determined by several regulatory mechanisms including receptor desensitization, internalization, down-regulation, and resensitization. Upon agonist binding, MOPR rapidly gets phosphorylated by G protein-coupled receptor kinases (GRKs) or other second messenger-regulated kinases (including PKA and PKC). This may facilitate β-arrestin binding and the dampening of further coupling to G proteins, despite the continued presence of agonist (Ahn et al., 2003; Ferguson, 2001). Except from mediating receptor desensitization, β-arrestins are further contributing to

receptor internalization, which can lead to either down-regulation or resensitization events (Ferguson et al., 1996). Although all opioid analgesics act via MOPR, they possess different properties possibly due to the recruitment of distinct signaling pathways. Over the past several decades, numerous studies have demonstrated that not all GPCR agonists activate the same intracellular signaling pathways, even though they may be acting at the same receptor (Kenakin, 2011). It has been proposed that physical interactions between an agonist and a receptor impact the physical constraints of receptor conformation, which can result in a preferential or "biased" interaction with certain signaling components over others resulting to distinct property profiles (Kenakin, 2007, 2009; Manglik et al., 2016). Furthermore, the cellular environment (including the proteins expressed in close proximity with the receptor) can influence these interactions and thereby influence the signaling cascades triggered by a particular ligand. In this way, the same ligand can induce different signaling profiles in different cell types.

Pharmacokinetics

Except from the distinct intracellular mechanisms mentioned above, another really important factor that can affect opiate actions is their pharmacokinetic properties (Mercadante, 2015). Opioids are weak bases (pKa 6.5-8.7). In solution, they dissociate into ionised and unionised fractions, with the relative proportion of each fraction depending both on solution's pH and their pKa. The main difference between the two fractions is that the unionised form is more diffusible compared to the ionised one. In acidic environments (for example in the stomach) opioids appear highly ionised and therefore are poorly absorbed. Conversely, in alkaline environments (for example in the small intestine) they are predominantly unionised and therefore easily absorbed. Nevertheless, the extensive presystemic metabolism that takes place afterwards in the intestinal wall and liver, results in very pure oral bioavailability. Another important factor is lipid solubility. The higher the lipid solubility the quicker the onset of action. In general, drugs with high unionized fraction, high lipid solubility or low plasma protein binding abilities show larger volumes of distribution. Opioid metabolism occurs mainly in the liver, but extrahepatic metabolism is also important for some opioids. There is also wide variability in the duration of actions between different opioid formulations, that does not always depend on their plasma half-life. A clear example is that of morphine and fentanyl, with morphine having a shorter half-life but producing longer lasting analgesic effects, mainly because of its low lipid solubility and slow diffusion out of CNS tissues.

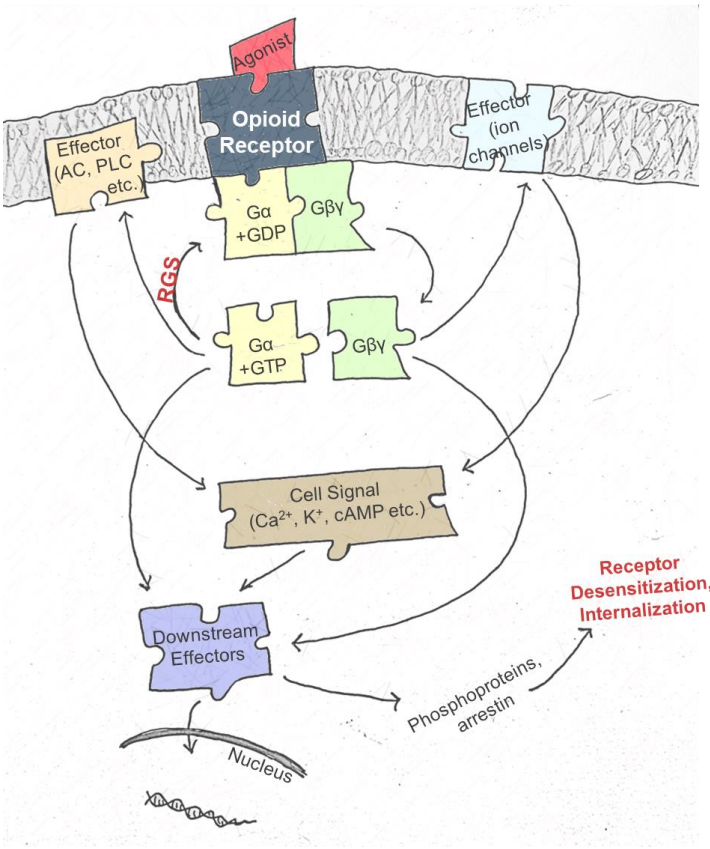


Figure D. Opioid Receptor Signaling / Regulation. Opioid receptors belong to the superfamily of G protein coupled receptors (GPCRs) and therefore signal through heterotrimeric G proteins. In brief, upon ligand binding on the receptor Gα subunits get phosphorylated, an event leading to their dissociation from the Gβγ complex and allowing both partners to interact with ion channels, kinases and other downstream effectors therefore triggering distinct signaling pathways. A key signaling event that has been associated with functional responses to opioids is receptor's phosphorylation by effector molecules (PLC, GRKs etc.) leading to receptor desensitization. Another key factor mediating signaling duration and direction is regulator of G protein signaling (RGS) proteins. RGS proteins associate with the activated Gα subunit and accelerate the hydrolysis of GTP to GDP and therefore the reassosiation of the Gα-Gβγ complex and the termination of signaling. Furthermore, depending on their enzymatic activity RGS proteins can also act as effector antagonist by binding to the Gα subunit and preventing the binding of downstream effector molecules.

Adverse Effects

Analgesic Tolerance. One of the main problems associated with opioid treatment is analgesic tolerance development. Tolerance develops when a given dose of an opioid produces a lower analgesic response and therefore a larger dose is required to maintain the original effect. Some degree of tolerance to analgesia appears to develop in most patients receiving long-term opioid analgesic therapy. The underlying mechanisms are still not fully understood, but rodent studies over the past decades have proposed multiple mechanisms at molecular, cellular and network levels that are contributing to tolerance development. Major mechanisms include those mediated by opioid receptor desensitization (Bohn et al., 2000) and internalization (Koch and Höllt, 2008; Whistler et al., 1999), glutamate receptors (Inoue et al., 2003; Trujillo and Akil, 1991;

Vekovischeva et al., 2001), chronic opioid-induced hyperalgesia (Ossipov et al., 2003; Vanderah et al., 2001), the cAMP-protein kinase A (PKA) pathway (Bie et al., 2005; Christie, 2008) and glial activation (Hutchinson et al., 2011; Johnston et al., 2004).

Addiction/Dependence. Another major drawback of opioid use is the fear of addiction. Addiction (also mentioned as psychological dependence) is a term used to describe a pattern of drug use characterized by a continued craving for an opioid that is manifested as compulsive drug seeking behavior leading to an overwhelming involvement with the use and procurement of the drug. Opioid dependence has become a problem of national concern, with prescription opioid misuse increasing over 3-fold in the USA since 1990. Despite the importance of various psycho-social factors contributing to the development and maintenance of addiction, it is essential to understand the biological mechanisms underlying such conditions. Numerous studies have contributed to the identification of molecular and cellular adaptations that occur in specific brain regions upon chronic opioid use leading to the development of addiction-associated behavioral abnormalities. These adaptations include altered neurotrophic signaling, epigenetic mechanisms driving alterations in gene expression and plasticity-mediated changes affecting neuronal function and synapse formation (Kalivas and Volkow, 2005; Nestler, 2013).

Other. Other side effects, that often limit the effectiveness and dosing of opioids, include respiratory depression, sedation, constipation, nausea, dizziness and vomiting. Among those, the most common appear to be constipation and nausea. Management for both of them can be really challenging, since in most cases tolerance does not develop. Side effects driven from the actions of MOPR in the gastrointestinal tract are the main reason for early discontinuation, under-dosing and insufficient analgesia.

Morphine

Morphine is a potent opiate analgesic drug that is used to relieve severe pain. In fact, it is considered to be the prototypical opioid. It was first isolated from opium poppy in 1804 by Friedrich Sertürner, and first commercially sold by Merck in 1827. Its mechanism of action appears to mimic endorphins, endogenous opioids responsible for analgesia. It binds to and activates the MOPR in the central nervous system leading to analgesia, sedation, euphoria, physical dependence, and respiratory depression. Morphine is a rapid-acting and very efficient analgesic, but its long-term use leads to analgesic tolerance much faster than other opioid narcotics.

Oxycodone

Oxycodone is a semisynthetic opioid used for the relief of moderate to severe pain. It was synthesized in 1917 from thebaine, an opioid alkaloid found in the Persian poppy, and one of the many alkaloids found in the opium poppy, in an attempt to improve the existing opioid analgesics. It is one of the drugs widely abused in the current opioid epidemic in the United States. It has been shown to be relatively selective for μ-opioid receptor with some rodent studies suggesting that it is also a partial agonist for κ-opioid receptor (Nielsen et al., 2007; Ross and Smith, 1997).

Regulators of G-protein Signaling (RGS)

G-protein Signaling

G proteins comprise a diverse family of proteins implicated in several cellular functions. Their name derives from their intrinsic GTPase activity. They are expressed in several tissues and play a central role in signal transduction mediating many cellular processes, such as membrane vesicle trafficking, cell growth, protein synthesis and neurotransmission (Zachariou et al., 2012). Mammalian G proteins are divided into heterotrimeric G proteins and small G proteins.

Heterotrimeric G proteins are involved in transmembrane signaling in the nervous system. They consist of three subunits: α, β and γ. Gα subunit has the ability to hydrolyze GTP, whereas β and γ subunits form a dimer (Gβγ) that separately activates different intracellular signaling cascades. Over 35 heterotrimeric G protein subunits exist, with unique distribution in the brain and peripheral tissues. Based on their structural and functional homology, they are divided into four main categories (Neubig and Siderovski, 2002):

$G\alpha_{i/o}$: implicated in inhibition of adenylyl cyclase

$G\alpha_{q}$: implicated in activation of phospholipase C (PLC)

$G\alpha_{s}$: implicated in stimulation of adenylyl cyclase

$G\alpha_{12/13}$: implicated in activation of guanine nucleotide exchange factor (Rho-GEF) proteins

In the resting state, Gα subunit is bound both to GDP and the Gβγ dimer. Upon ligand's binding and G-protein coupled receptor (GPCR) activation, a conformational change occurs which triggers the exchange of GDP to GTP and the dissociation of Gα subunit from the Gβγ complex. Therefore, both the activated Gα subunit and the Gβγ complex are free to interact with various

downstream effector molecules. Reassociation of Ga with Gβγ is triggered by the intrinsic GTPase activity of the Gα subunit. Furthermore, G protein subunits, when released from their G protein receptor interactions, can directly gate (i.e. open or close) specific ion channels mainly though interaction with the free Gβγ complex.

Small G proteins, on the other hand, are small molecules (around 21 kDa), homologous to the heterotrimeric Gα subunits that are found in the cytoplasm. They also possess GTPase activity, with the molecules triggering the conformational change that leads to GTP binding (GEFs) being various intracellular molecules, instead of membrane receptors. The most-known members of this superfamily are small Ras-GTPases.

G-protein Coupled Receptors (GPCR)

Heterotrimeric G proteins interact with GPCRs, also known as seven-transmembrane domain receptors (7TM receptors) and transduce signals to downstream signaling molecules resulting in the induction of cellular changes. GPCRs are the largest and most diverse group of eukaryotic membrane receptors acting as “sensors” of the extracellular environment. Upon ligand binding a conformational change occurs on the GPCR, which allows it to act as a guanine nucleotide exchange factor (GEF) and activate an associated G-protein by exchanging its bound GDP for a GTP. As described above, the Gα subunit-bound to GTP- can then dissociate from the βγ complex to further affect intracellular signaling proteins.

GPCRs are among the most important drug targets. Many of the medications used for pain relief, as well as neurological and neuropsychiatric disorders (including opioids, antidepressant and antiparkinsonian agents) mediate their actions via mechanisms involving modifications of GPCR function. In fact, GPCRs are involved in a variety of physiological processes such as vision (opsins), smell (olfactory receptors), immunity, behavioral and mood regulation, homeostasis modulation and many others. Opioid receptors (μ, δ, κ) are GPCRs coupled to the Gαq and Gαi/o subunits.

Regulator of G protein signaling (RGS) proteins

Regulators of G protein signaling (RGS), defined by the presence of a conserved 125 amino acid domain, comprise a diverse group of more than 40 proteins which modulate G protein signaling amplitude and duration. Via their conserved domain, RGS proteins function as GTPase-activating proteins (GAPs) for the Gα subunits of the heterotrimeric G proteins, accelerating the hydrolysis

of GTP to GDP and therefore the reassociation of Gα with the Gβγ complex and the termination of signaling (Fig D). Of course, based on their enzymatic activity RGS proteins can also act as effector antagonists by binding to the Gα subunit and preventing the binding of downstream effectors. Apart from their conserved RGS domain, RGS proteins show wide variability in terms of domain composition (Fig. E). Several members of the RGS family possess unique functions, attributed to their distinct pattern of expression, regulation and structure (Gerber et al., 2016; McCoy and Hepler, 2009). Most RGS are expressed in the brain, regulating essential physiological processes such as vision (Chen et al., 2000; Nishiguchi et al., 2004), working memory (Buckholtz et al., 2007) and locomotion (Rahman et al., 2003), while their dysfunction is associated with several neuropathological conditions such as addiction, mood disorders and schizophrenia (Hooks et al., 2008; Terzi et al., 2009).

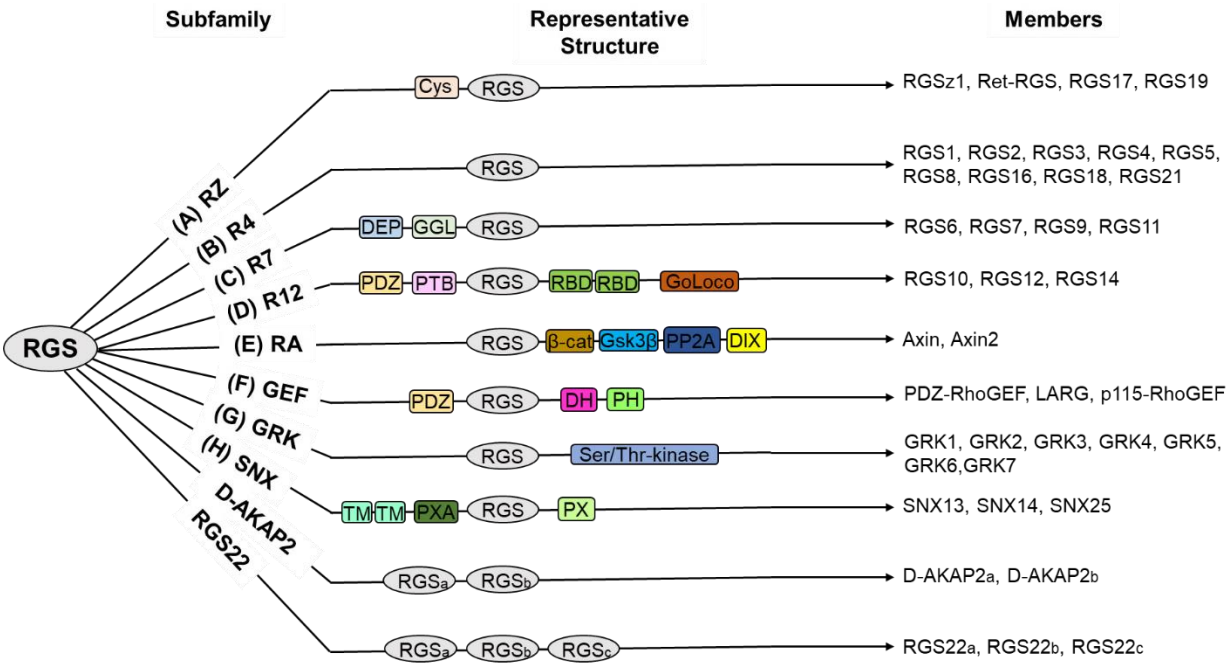


Figure E. Classification of Regulator of G-protein Signaling (RGS) proteins. RGS proteins are categorized into ten distinct subfamilies based on their domain composition.

RGS9-2

RGS9-2 is a member of the R7 subfamily of RGS proteins with a complicated structural composition. Except from the RGS domain, RGS9-2 contains another two distinct functional domains: a DEP domain, mediating its interaction with the anchor protein R7BP and therefore its membrane localization and a GGL domain that is binding to G-protein β5 leading to stabilization of the RGS9-2 protein (Chen et al., 2003). It is predominantly expressed in the striatum (Gold et al., 1997) and it has been shown to dynamically modulate the actions of several drugs, including psychostimulants (Rahman et al., 2003), opioids (Zachariou et al., 2003) and antidepressants (Mitsi et al., 2015). Regarding opiate actions, Rgs9-2 complexes have been shown to affect several aspects of morphine addiction, by negatively modulating reward and physical dependence, while at the same time affecting analgesia and the development of analgesic tolerance (Zachariou et al., 2003). These effects are mainly mediated through RGS9-2 complexes in the Nucleus Accumbens (NAc), a key region of the brain reward network (Gaspari et al., 2014; Psifogeorgou et al., 2011). Interestingly, the effect of RGS9-2 on opiate actions appear to be agonist-dependent, with different agonists leading to the recruitment of distinct RGS9-2 complexes and therefore opposing behavioral outcomes (Psifogeorgou et al., 2011). Based on these findings the first aim of the thesis was focused on the role of RGS9-2 on the actions of another opioid medication widely used and abused over the last years for the treatment of severe acute as well as chronic pain, oxycodone.

RGSz1

RGSz1, one of the products deriving from the RGS20 gene locus (Barker et al., 2001), is a member of the Rz subfamily (composed of GAIP [RGS19], RGSz1, RGSz2 [RGS17], and Ret-RGS) of RGS proteins and is predominantly expressed in the brain both in rodents as well as in humans (Glick et al., 1998; Larminie et al., 2004; Wang et al., 1998). In terms of structure, flanking the RGS box domain, RGSz1 possess an N-terminus with heavily palmitoylated cysteine string motif involved in membrane targeting (De Vries et al., 1996), and a short C-terminus of 12 aminoacid residues. RGSz1 is highly specific for Gαz subunits, accelerating the hydrolysis of GTP-bound Gαz more than 600-fold, but it may also modulate other Gαi subunits (Glick et al., 1998; Wang et al., 1998). There is both in vitro (Ajit et al., 2007; Ghavami et al., 2004) and in vivo (Creech et al., 2012; Garzón et al., 2004) evidence from published studies suggesting that RGSz1 is controlling the signaling events downstream of MOPR and serotonin receptor 1A (Htr1A). Given

the important role of both receptors in pain processing and analgesic responses the second and third aim of the thesis focused on the effect of RGSz1 on morphine actions and chronic pain states, respectively.

Materials & Methods

Animals and Drug treatments

All experiments were performed on adult (2-3 months old) male and female mice. Mice were kept on a 12-h dark/light cycle, group housed (2-5 per cage), with food and water available *ad libitum* according to the Animal Care and Use Committee of the Icahn School of Medicine at Mount Sinai. Global knock-out mouse lines for *Rgs9* (Zachariou et al., 2003) and *Rgsz1* were used. A floxed *Rgsz1* mouse line was used for cre recombinase-mediated generation of conditional knock-down in specific brain areas of adult mice. Opioids (oxycodone, morphine, fentanyl, methadone) were diluted in saline according to the desired dose and 100μl were injected subcutaneously (s.c.) or intraperitoneally (i.p.) depending on the paradigm used. Drug doses used for each experiment are stated in the figure legends. For all survival surgeries mice were anesthetized using freshly prepared tribromoethanol (avertin) anesthetic solution.

Stereotaxic surgeries

Mice were anesthetized and mounted on the stereotaxic frame. Skull was exposed through a precise incision revealing the anatomical landmarks. The coordinates of bregma and lambda were recorded and used to calculate the coordinates of the desired brain area based on the widely used mouse brain atlas (Paxinos and Franklin, 2004). 0.5μl of virus were injected per hemisphere using a 0.21mm thick Hamilton syringe. 4.0 silk sutures were used to close the incision and mice were given a recovery period of 2 weeks. Conditional deletion of *Rgsz1* was achieved by bilateral stereotaxic injections of the AAV2-CMV-CRE-EGFP (University of South Carolina Viral Core Facility) into the nucleus accumbens (NAc) or ventrolateral PAG (vlPAG) of RGSz1fl/fl mice. Control animals received injections of AAV2-CMV-EGFP vectors. Stereotaxic coordinates for viral vector injections were as follows: NAc (from bregma): AP, +1.6 mm; AL, +1.5 mm; and DV, -4.4 mm at 10^{o} from the midline; PAG (from lambda): AP, +0.6 mm; ML, +0.8 mm; and DV, -2.8 mm at 22^{o} from the midline. HSV-β-catenin (HSV-β-cat), HSV-β-catenin dominant negative (HSV-β-catDN), and HSV-GFP vectors were generated by R.L.N.(Dias et al., 2014).

Murine pain models

Spared Nerve Injury (SNI) model of neuropathic pain. Mice were anesthetized and the sciatic nerve was revealed by a mid-thigh level skin and muscle incision. With the help of a stereomicroscope the three branches of the sciatic nerve were identified, the common peroneal and sural branches were ligated with 6.0 silk sutures, transected and 1-2mm section of each nerve was removed leaving the tibial nerve intact (Mitsi et al., 2015; Shields et al., 2003; Stratinaki et al., 2013).

Complete Freund's Adjuvant (CFA) model of inflammatory pain. A solution of heat killed and dried *Mycobacterium tuberculosis* in oil (1 mg of Mycobacterium tuberculosis, 0.85 mL paraffin oil and 0.15 mL mannide monooleate per ml of solution) was purchased from Sigma-Aldrich and diluted 1:1 with saline producing water-in-oil emulsions of the immunogens. ~25μl of the diluted solution were injected in the left hind paw, leading to local inflammation that lasted up to 3 weeks (Latremoliere et al., 2015).

Formalin test of nociception. A 4% formalin solution, consisted of 1 ml of saturated formaldehyde (37-39 %) in water plus 9 ml saline, was freshly prepared and ~25μl were injected in the left hind paw (Tjølsen et al., 1992).

Ovariectomy

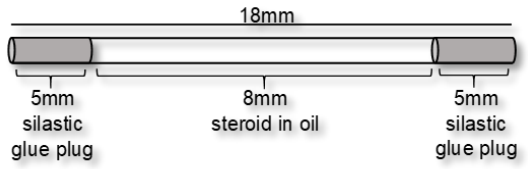
Mice were anesthetized and a small incision was made in each side of the body above the ovary. Each ovary was pulled through the body wall by carefully grasping the fat pad connected to it. Both ovaries were ligated with 6.0 silk suture and removed from the uterus and the fat pad. The remaining portion of the uterus and fat pad were replaced inside the body cavity and body wall and skin were closed via 4.0 silk suture (Maffucci et al., 2008). SHAM mice have only undergone general anesthesia.

Orchiectomy

Mice were anesthetized and a small single incision was made on the ventral side of the scrotum. Each testis was carefully pulled through the incision by grasping the fat pad connected to it. Both testicles were ligated with 6.0 silk suture and removed. The remaining content of the testicular sac was replaced inside the body cavity and body wall and skin were closed via 4.0 silk suture (Idris, 2012). SHAM mice have only undergone general anesthesia.

Subcutaneous implants

18mm silastic capsules were custom made and filled with 25µl of 25µg/ml β-estradiol or cholesterol solution in oil as shown below:



A small skin incision was made in the dorsal thoracic area, capsules were implanted subcutaneously and the incision was closed using wound clips.

Behavioral Tests

Locomotor sensitization. Mice received a saline injection (s.c.) and were habituated to the locomotor apparatus (Med Associates, Inc.) for 30 min each day for three consecutive days. Then, oxycodone or morphine were administered for several days and ambulatory activity was monitored each day immediately after the injection of the drug for a period of 30 min (Charlton et al., 2008).

Conditioned Place Preference (CPP). An unbiased place conditioning procedure was performed using a two-chamber place conditioning system (Med Associates, Inc., VT) (Gaspari et al., 2014; Zachariou et al., 2003). Briefly, after monitoring baseline preferences for 20 min on day 1, animals were conditioned to the drug-paired or saline-paired side for 45 min on alternate days. After six conditioning sessions, animals were tested for 20 min, and the preference was determined as the time spent in the drug-paired compartment after conditioning minus the time spent at baseline. The CPP score is defined as the amount of time spent in the drug-paired side at test day minus the amount spent at the same compartment at baseline.

Opiate withdrawal paradigm. Mice were injected intraperitoneally (i.p.) with escalating drug doses every 12 hours for four consecutive days (day 1: 20 mg/kg; day 2: 40 mg/kg; day 3: 60 mg/kg; day 4: 80 mg/kg). On the fifth day, mice were injected with 80 mg/kg in the morning and 3 hours after drug administration withdrawal was precipitated using naloxone hydrochloride (1 mg/kg, s.c.; Sigma, MO. Withdrawal signs (jumps, wet-dog shakes, tremor, diarrhea, and weight loss) were monitored for 30 min after naloxone administration. Data are expressed as

percentages of the wild-type control. For tremor and ptosis, we monitored the presence of signs at the beginning of each 5-min interval during the monitoring period (Gaspari et al., 2014; Zachariou et al., 2003).

Hot plate test. Analgesia was measured using a 52^{o}C hot plate apparatus (IITC Life Sciences, CA) as previously described (Zachariou et al., 2003). Animals were habituated in the testing room for 1 hour and the baseline latencies to jump or lick the hind paw were measured. Morphine was administered subcutaneously (s.c.) and 30 min later, mice were placed on the hot plate and the latencies to jump or lick the hind paw were monitored. A cutoff time of 40 seconds was used to avoid tissue damage and inflammation. For tolerance studies, this procedure was repeated for four to eight consecutive days using a high dose of the drug. Data are expressed as percentages of maximal possible effect [MPE = (latency – baseline)/(cutoff – baseline)].

Von Frey test for mechanical allodynia. For the assessment of mechanical allodynia, we used Von Frey testing (Mitsi et al., 2015; Shields et al., 2003) with ascending forces expressed in grams. Each filament was applied five times in a row against the lateral area of the paw. Hind paw withdrawal or licking induced by the filament was defined as a positive allodynia response. A positive response in three out of five repetitive stimuli was defined as the allodynia threshold. For the tolerance studies, mice were injected subcutaneously for 17 consecutive days with a low oxycodone dose and allodynia was assessed 1 hour after the injection.

Hargrave's test for thermal hyperalgesia. Mice were placed in Plexiglas boxes on top of a glass surface and the latency to withdraw the injured (CFA-injected) hind paw was measured after a high-intensity heat beam (30%) was applied to the mid-plantar area (IITC Life Sciences). Two measurements were obtained with a 10-min interval and the average was defined as the thermal nociceptive threshold. A cutoff time of 20 s was used to avoid tissue damage (Terzi et al., 2014).

Cold plate test. Nocifensive behavior was assessed at 0 ◦C (Colburn et al., 2007) using IITC hot/cold – plate. Mice were placed on the cold surface and total number of jumps and hind paw flicking/licking were measured for a period of 5 minutes.

Formalin test. ~25μl of 4% formaldehyde were injected into the plantar surface of the left hind paw. Mice were immediately placed into Plexiglas boxes and the duration of nocifensive behaviors (licking, flicking) within an hour after the injection was evaluated.

Genotyping

All mice used in this study derived from homozygote breedings. The genotype of each mouse line was verified by polymerase chain reaction (PCR) using the proper primers based on the design strategy of each transgenic line (for RGS9-2 see Zachariou et al. 2003; for RGSz1 see figure 6). Primer sequences and PCR protocols are provided below:

RGS9-2

RGS9-2 Genotyping Primers	
P1	5'-GTA ACA GCT GCT GTT CCA AAA TCG-3'
P2	5'-TGC ATT CTG ACT CCC GTC TCT-3'
P3	5'-CGG CGA GGA TCT CGT CGT GAC CCA-3'

RGS9-2 WT PCR MasterMix x1	
Reagents	volume (µl)
ddH2O	15.5
10x Buffer	2.5
dNTPs (2mM)	2.5
MgCl2 (25mM)	1.25
P1	1
P2	1
Taq polymerase	0.25
DNA template	1
Product size: 484 bp	

RGS9-2 KO PCR MasterMix x1	
Reagents	volume (µl)
ddH2O	15.5
10x Buffer	2.5
dNTPs (2mM)	2.5
MgCl2 (25mM)	1.25
P1	1
P3	1
Taq polymerase	0.25
DNA template	1
Product size: 700 bp	

RGS9-2 PCR Program		
Step	Temp.	Duration
1	95˚C	5 min
2	95˚C	30 sec
3	60˚C	30 sec
4	72˚C	1 min
5	go to step 2 35x	
6	72˚C	7 min
7	4˚C	for ever

RGSz1

RGSz1 Genotyping Primers	
P1	5'-GAC AGT AAG CCC AAG GTC AAG TCT-3'
P2	5'-CAC AAG ATA GAT GCC TGC AAA TGG C-3'
P3	5'-ATT CTG TTC GCA GCT CTT CC-3'

RGSz1 WT/FL PCR MasterMix x1	
Reagents	volume (µl)
ddH2O	15.5
10x Buffer	2.5
dNTPs (2mM)	2.5
MgCl2 (25mM)	1.25
P1	1
P2	1
Taq polymerase	0.25
DNA template	1
Product size: 598 bp (WT), 685 bp (FL)	

RGSz1 KO PCR MasterMix x1	
Reagents	volume (µl)
ddH2O	15.5
10x Buffer	2.5
dNTPs (2mM)	2.5
MgCl2 (25mM)	1.25
P1	1
P3	1
Taq polymerase	0.25
DNA template	1
Product size: 349 bp	

RGSz1 PCR Program		
Step	Temp.	Duration
1	95˚C	5 min
2	95˚C	30 sec
3	60˚C	30 sec
4	72˚C	1 min
5	go to step 2 35x	
6	72˚C	7 min
7	4˚C	for ever

Immunofluorescence

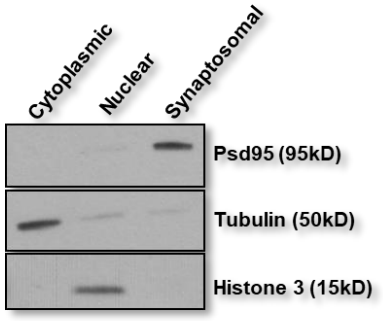
Mice were anesthetized with chloral hydrate and then perfused for 3 min with phosphate buffered saline (PBS) followed by 12 min with 4% paraformaldehyde (PFA). Brains were stored in 4% PFA overnight and then transferred to 30% sucrose plus 0.02% sodium azide. For immunofluorescence studies, 40μm brain sections were obtained with a microtome (Leica). To identify EGFP expression, the slices were dehydrated and mounted, and the viral expression pattern was visualized using a fluorescence microscope (ZEISS AXIO). For cell type specific labeling a free-floating staining approach was obtained. Brain slices were incubated overnight with primary antibody at 4^{o}C on horizontal shaker, washed 3x with PBS, incubated with secondary antibody for 3 hours at room temperature on horizontal shaker, washed again 3x with PBS and mounted. The primary antibodies used were a mouse anti-NeuN (#MAB377; Millipore), a rabbit anti-GFAP (#12389; Cell Signaling) and a rabbit anti-Iba1 (#019-19741; Wako).

Immunoblotting, subcellular fractionation and co-Immunoprecipitation (co-IP) Assays

Nucleus Accumbens (NAc), Dorsal Striatum (DS) and Periaqueductal Gray (PAG) tissue samples were rapidly dissected using a 14-gauge syringe needle from 1mm thick coronal sections of mouse brain. For NAc and DS bilateral punches from a single coronal section were obtained. For PAG each sample consisted of two punches deriving from two consecutive midbrain coronal sections.

Western blot was performed as previously described (Zachariou et al., 2003). In brief, samples were sonicated in 1% SDS containing phosphatase, protease and proteasome inhibitors (Sigma). Protein concentration was determined by lowry quantification and 20-30μg of protein were loaded per sample on 8-12% SDS-PAGE gels based on the molecular weight of the examined protein. After electrophoresis, samples were transferred to nitrocellulose membrane (Biorad), incubated with blocking solution (3% milk in PBS-Tween) for 1 hour in room temperature on horizontal shaker, washed with PBS-Tween and incubated overnight with primary antibody at 4^{o}C on horizontal shaker. The following day, membranes were incubated for one hour with peroxidase-conjugated secondary antibody at room temperature on horizontal shaker, washed with PBS-Tween and developed using the enhanced chemiluminescence (ECL) method. The optical density of each protein band was quantified using the Image J software and data for each sample were calculated as the optical density ratio of protein of interest/loading control or IP/total lysates in the case of co-IP assays. Results are presented as % of the control group.

For subcellular fractionation, samples were homogenized using a pestle in 50 µl of hypotonic cell lysis buffer (HCLB) plus phosphatase, protease and proteasome inhibitors, incubated on ice for 45 min, and centrifuged at 2,000 rpm at 4ºC for 10 min, and supernatants were collected as the crude cytoplasmic fractions. The remaining nuclear pellets were washed twice with 100 µl HCLB plus inhibitors, resuspended in 25 µl nuclear lysis buffer with inhibitors, incubated for 2 h on ice, and centrifuged at 13,200 rpm at 4ºC for 5 min, and the supernatants were collected as the crude nuclear fractions. To obtain the synaptosomal fractions, the crude cytoplasmic fractions were centrifuged twice to remove the remaining nuclei (2,000 rpm at 4ºC for 10 min). The supernatants were collected and centrifuged at 10,000 rpm at 4ºC for 10 min. Supernatants were collected as the cytoplasmic fractions and the synaptosomal pellets were resuspended in 20 µl HCLB. Fractionation specificity and efficiency were verified by immunoblotting against specific markers for each compartment as shown below:



Co-IPs for MOPR-Gαz were performed using whole lysate samples as previously reported (Psifogeorgou et al., 2011). In brief, samples were sonicated in 50ul RIPA buffer containing phosphatase, protease and proteasome inhibitors, centrifuged for 30 min at 13,000 rpm and supernatant was precleared with 10 µl of G agarose beads (Roche) by a 2-hour incubation at 4°C on circular rotator. Immunoprecipitation was performed by incubating the precleared supernatant with primary antibody of interest overnight at 4°C on circular rotator and precipitating using 10µl of G agarose beads. For co-IPs with Axin2, 1 µl of antibody was added to each synaptosomal fraction (co-IP with anti-GFP was used as the negative control), and samples were incubated overnight at 4°C on a circular rotator. Each sample was then incubated for an additional 3 h with 10 µl anti-rabbit Dynabeads (Thermo Fisher Scientific). Co-immunoprecipitated proteins were washed three times with 700 µl blocking solution using a magnetic stand and then resuspended in 10 µl RIPA buffer.

The following rabbit antibodies were used: anti-RGS9-2 (Psifogeorgou et al., 2011), anti-RGSz1 (provided by Elliott Ross), anti-Gαz (#3904; Cell Signaling), anti-Gαi3 (Lilly Jiang, UTSW Medical Center), anti-Gαo (#3975; Cell Signaling), anti-Gαq (Paul Sternweis, UTSW Medical Center), anti-MOPR (#24216; Immunostar), anti-phospho-β-catenin (ser675, #4176; Cell Signaling), anti-β-catenin (#9562; Cell Signaling), anti-phospho-GSK3β (ser9 #9336; Cell Signaling), anti-GSK3β (#9315; Cell Signaling), anti-Axin2 (#ab32197; Abcam), anti-GADPH (#5174; Cell Signaling), anti-β-actin (#4967; Cell Signaling), anti-psd95 (#2507; Cell Signaling),anti-H3 (#4499; Cell Signaling) and anti-GFP (#ab290; Abcam). For estrogen receptor studies a mouse anti-ERα was used (#ab32063; abcam). Mouse anti-β-tubulin III (#T8578; Sigma) or GADPH or β-actin antibodies were used as the loading control.

RNA sequencing (RNA-seq) and Real Time PCR (RT-PCR)

Four biological replicates per group were used for the RNA-seq studies. PAG punches from two animals were pooled per biological replicate (four 14-gauge punches per sample). Total RNA was isolated with TRIzol and the integrity was confirmed with an Agilent 2100 Bioanalyzer (Mitsi et al., 2015). mRNA-Seq libraries were prepared using a TruSeq RNA sample preparation kit v2 (Illumina). Sequencing was performed using the Illumina HiSeq2000 apparatus. Read alignment, read counting, and differential analysis were performed using TopHat2 (Kim et al., 2013), HTSeq (Anders et al., 2015) , and voom-limma R package (Law et al., 2014), respectively. A threshold of p-value<0.05 and $-0.5>\log_2(\text{fold change})>0.5$ was set in order to obtain the gene list used for further bioinformatic analysis. qPCR was performed using SYBR green and analyzed using the ΔΔCt method. All primer sets used are provided at the table below:

RT-PCR PRIMER SETS		
Gene	**Forward primer 5'→3'**	**Reverse primer 5'→3'**
Ddc	GGCGACGATTTCGCTCTTTG	TCATTGGAGCCCTTTAGCCG
Gch1	CAAGTTCAAGAGCGCCTCAC	TTCATTTTCTGCACGCCTCG
Pdyn	GCCCTCTAATGTTATGGCGGA	AGGGAGCAAATCAGGGGGTT
Bdkrb1	CCTCCCAACATCACCTCCTG	GTCGCCAAGGCAAAAGGAAG
Wnt9a	TGCTTTCCTCTACGCCATCT	CCCAGGAACTCCTTGACAAA
Cxcl12	ACCTCGGTGTCCTCTTGCT	TCAGCCGTGCAACAATCTGAA
Ccr1	ATACTCTGGAAACACAGACTCAC	AGTTGTGGGGTAGGCTTCTG
Il1r1	ACTTGAGGAGGCAGTTTTCGT	TACATACGTCAATCTCCAGCGAC
Cxcr4	AGGCGTTTGGTGCTCCG	CAGGGTTCCTTGTTGGAGTCA
Tph2	GAAAAACCTCCCCCTGCTGA	GTCTCTTGGGCTCAGGTAGC
Npy	GGACTGACCCTCGCTCTATC	CTTCAAGCCTTGTTCTGGGG
Gabre	TCTCACGGCTTTGGACTTCT	CAAACACTTCCTGCTGCTGT
Rgsz1	AAACTAAGGCACACTCTGGACG	GACAGTGAGGCAAGAACAGGT
Gαq	TTTGTCGTGAGGTCTGCTTG	CACTGAGCGGTGACTTTTGA
Gαz	CTCCAGCCGTGCTTAGAAAC	CACACCCTCCCCTCTAGTGA
Gαi1	TCAGAGCGGAAGAAGTGGAT	ATATCGTGAGGGGGCTCTTT
MOR	GGCTCCTGGCTCAACTTGTCCCAC	GGTGCAGAGGGTGAAGATACTGGTGAAC
DOR	GGACACGGCGGCGCCATG	GTTGTAGTAGTCAATGGAG
KOR	ATCAGCGATCTGGAGCT	GCAAGGAGCATTCAATGAC
Slc6a4	GACATCAGGAGGGGCGTATG	AGAAACAGAGGGCTGATGGC
Esr2	ATGTCCACCCGCTAGGCATT	AAGGGTAGGATGGACTGGCT
Esr1	AAAGGCGGCATACGGAAAGA	TAGATCATGGGCGGTTCAGC

Chromatin Immunoprecipitation (ChIP)

Ten 14-gauge PAG punches (tissue pooled from 5 individual animals) were incubated for 12 minutes at room temperature on circular rotator in 1% formaldehyde in PBS in order to fix the DNA with the associated proteins. After fixation 2 M glycine was added for 5 min. Samples were washed 5x with cold PBS plus protease and proteasome inhibitors and then homogenized using a pestle in cell lysis buffer plus inhibitors. Homogenates were incubated for 15 minutes at 4°C on circular rotator and then centrifuged for 5 minutes at 2700xg. Nuclear pellets well resuspended in nuclear lysis buffer, incubated for 10 minutes at 4°C on circular rotator and sonicated using bioruptor (2 rounds of 10 cycles, 30sec on/30sec off, high power). DNA concentration and shearing efficiency were examined using Agilent 2100 Bioanalyzer. Conjugated magnetic beads were used to IP ERα with the ChIP-validated ERα antibody (#ab32063; abcam) overnight in blocking solution (0.5% BSA in PBS). A control reaction using ChIP grade antibody against GFP was also performed for each sample. The IP reaction was collected using a magnetic rack, washed, and both the input chromatin and the immunoprecipitated DNA were reverse cross-linked at 65°C overnight. The DNA was then purified with RNase, proteinase K and the Qiagen PCR purification kit. RT–PCR was used to quantify differential binding on the genomic DNA. The primer

sets used are provided at the table below. Samples are expressed as log_2(fold change) normalized to the GFP immunoprecipitated control.

PRIMER SETS USED FOR ChIP		
Locus	**Forward primer 5'→3'**	**Reverse primer 5'→3'**
Rgsz1 promoter	GTCTGAGTCTGCTGTGGACC	ACAGCCGAGTGACTCACAAA
Rgsz1 gene body	GAAGGGTGCTTTCAGAGCAAG	ATGGAATCTGGCCTCCTTGA

Bioinformatic analysis

Heatmaps were generated using GENE-E (https://software.broadinstitute.org/GENE-E/). Venn diagrams were generated using VennPlex Version 1.0.0.2 (NIH) and pathway analysis was conducted using Ingenuity Pathway Analysis (IPA) from Qiagen. GO analysis was conducted using DAVID (Huang et al., 2009).

Statistics

For the experiments monitoring behavior of the same group of mice over time (Fig. 1a-b, 4a, 4c, 6c-d, 7b, 8a, 8c, 11e, 14a-b, 14d-e, 14g-j, 15d, 17a and 17c-d), we used two-way repeated-measures ANOVAs followed by Bonferroni's post hoc tests. For two-factor designs (Fig. 2b, 3a, 5b-g, 6a, 7a, 11c-d, 13a-c, 17b and 17h), we used two-way ANOVAs followed by Bonferroni's post hoc tests. For data containing a single independent variable (Fig. 1c-d, 2a, 3a inset, 3b, 4b, 5a, 6a inset, 8b, 8d, 12b, 14c, 14f, 15c and 17e-g) we used unpaired two-tailed t tests. For the data in graphs depicting multiple individual single-factor comparisons (Fig. 6b, 7c, 8e, 10c, 15a-b and 16e-h), we used multiple t tests. Correlation analysis (Fig. 13d) was contacted using Spearman's rho. For the ERα ChIP data one sample t-test against the GFP control value (0) was used. Error bars are depicting ±SEM. F and t values for each data set are provided in the figure legends.

Results

I. RGS9-2 Modulates Responses to Oxycodone in Pain-Free and Chronic Pain States

RGS9-2 modulates the locomotor sensitizing actions of oxycodone.

When administered repeatedly, drugs of abuse produce a progressive enhancement of locomotor response (locomotor sensitization). We used the RGS9KO line to investigate the behavioral consequences of *Rgs9* deletion in an oxycodone locomotor sensitization paradigm. As shown in Figure 1a, KO of the *Rgs9* gene does not affect basal locomotor activity nor the acute response to a high oxycodone dose (3mg/kg subcutaneously). However, RGS9KO mice are less sensitive than RGS9WT controls to the locomotor-sensitizing actions of oxycodone. We next used western blot analysis to determine if repeated oxycodone administration affects the expression of RGS9-2 in the NAc and DS. C57BL/6 mice were habituated to the locomotor chamber for 3 consecutive days, followed by 3 additional days of oxycodone treatment (2 mg/kg, subcutaneously). On day 3, NAc and DS tissues were collected 2 h after drug administration (Fig. 1b). Western blot analysis of these samples revealed that this treatment leads to the downregulation of RGS9-2 protein levels both in NAc and DS (Fig. 1c and d).

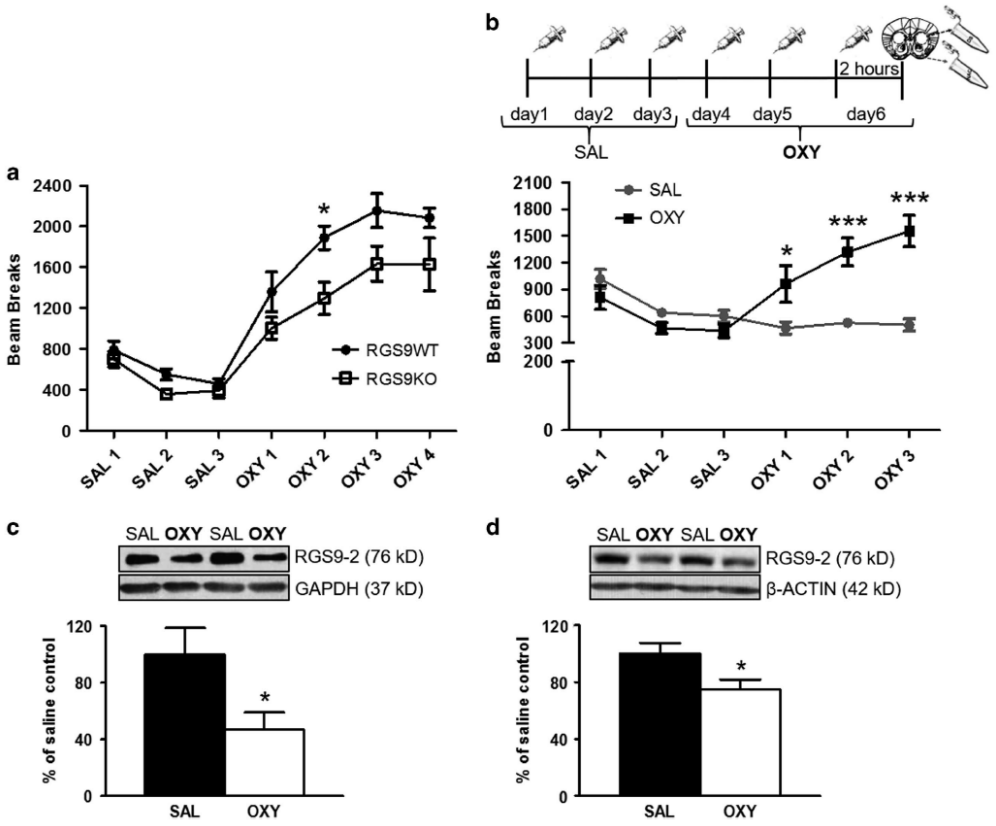


Figure 1. Regulator of G-protein signaling 9-2 (RGS9-2) modulates the locomotor sensitizing actions of oxycodone. (a) RGS9-knockout (RGS9KO) mice are less sensitive to the locomotor sensitizing actions of oxycodone compared to their RGS9-wildtype (RGS9WT) controls [dose: 3mg/kg, two-way ANOVA repeated-measures followed by Bonferroni posthoc test, $F(1,48) = 8.22$, *$p<0.05$]. **(b)** The schematic on top shows the experimental design for tissue collection for our western blot analysis studies. The graph shows the locomotor responses of the cohort used for western blot analysis. Mice were habituated to the locomotor chamber for 3 consecutive days followed by 3 additional days of oxycodone administration [dose: 2mg/kg, two-way ANOVA repeated measures followed by Bonferroni posthoc test, $F(1,40) = 4.93$, *$p<0.05$, ***$p<0.001$]. One day 3, nucleus accumbens (NAc) and dorsal striatum (DS) tissues were collected 2 hours after drug administration. Western blot analysis reveals that RGS9-2 is downregulated in the NAc **(c)** and DS **(d)** of mice treated for 3 consecutive days with 2mg/kg oxycodone (OXY) compared with saline (SAL)-treated controls [t-test, $t(19) = 2.43$, *$p<0.05$ for NAc and $t(9) = 2.43$, *$p<0.05$ for DS]. GAPDH:glyceraldehyde 3-phosphate dehydrogenase.

RGS9-2 acts as a positive modulator of oxycodone in the conditioned place preference (CPP) paradigm.

We next investigated the role of RGS9-2 in the rewarding actions of oxycodone. Our earlier work revealed a negative modulatory role of RGS9-2 in the rewarding actions of morphine (Gaspari et al., 2014; Zachariou et al., 2003). Here our data (Fig. 2a) suggest that RGS9-2 acts as a positive modulator of oxycodone actions in the CPP paradigm, as RGS9KO mice are less sensitive to the rewarding actions of this drug (1 mg/kg, subcutaneously). We also investigated if RGS9-2 contributes to the rewarding actions of oxycodone under chronic pain conditions. We used the SNI model of neuropathic pain, and 2 weeks after the induction of nerve injury [when the animals have developed pain-like behaviors, but not depression-like behaviors; (Terzi et al., 2014)], we conditioned RGS9WT and RGS9KO mice to oxycodone. The neuropathic pain-like state decreased sensitivity to oxycodone place preference in RGS9WT mice, as they did not respond to the 1mg/kg dose that conditions CPP in the pain-free state. RGS9KO mice did not respond to this dose either. When a higher dose was used, we found a similar phenotype to that observed under pain-free states: RGS9WT mice develop a significant place preference, while the RGS9KO show no significant preference to this dose of the drug (Fig. 2b; 2 mg/kg: RGS9WT—initial preference of the drug side = 427± 28, conditioned preference = 647 ±30, t(10) = 5.95, p = 0.0001; RGS9KO—initial preference = 490± 35, conditioned preference = 560± 22, t(8) =1.96, p = 0.085). At a higher dose (3 mg/kg) SNI groups of both RGS9WT and RGS9KO mice develop CPP to oxycodone (Fig. 2b).

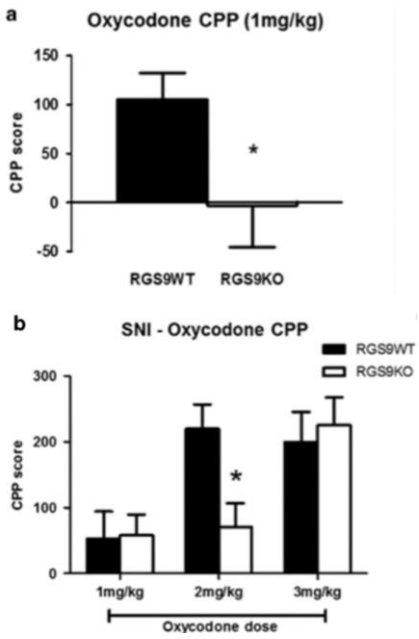


Figure 2. Regulator of G-protein signaling 9-2 (RGS9-2) modulates the rewarding actions of oxycodone. RGS9-2 acts as a positive modulator of oxycodone in the conditioned place preference (CPP) paradigm. (a) RGS9-knockout (RGS9KO) mice are less sensitive to the rewarding actions of oxycodone [dose:1mg/kg, t-test, t(17) = 2.25, *p<0.05]. **(b)** Using the spared nerve injury (SNI) model of neuropathic pain, we demonstrate that RGS9WT and RGS9KO mice do not develop place preference to a low dose of oxycodone (1mg/kg). At a higher dose (2mg/kg), only RGS9WT mice develop preference, whereas both genotypes condition place preference at 3mg/kg [two-way ANOVA followed by Bonferroni post hoc test, F(1,16) = 5.51, *p<0.05].

RGS9-2 does not affect the acute analgesic actions of oxycodone.

Except from the effect on the rewarding actions of the drug, we also investigated the role of RGS9-2 in the analgesic effects of oxycodone. We used the hot-plate assay to assess the consequences of *Rgs9* gene KO on the acute analgesic actions of oxycodone. Our previous work demonstrated that RGS9KO mice show increased responses to morphine in the hot-plate test (Zachariou et al., 2003). Here we found that KO of *Rgs9* does not affect the acute analgesic effects of oxycodone in the 52°C hot-plate assay (Fig. 3a). The top panel in Figure 3a shows that consistent with our earlier findings (Psifogeorgou et al., 2011; Zachariou et al., 2003), RGS9KO mice are more sensitive to the analgesic effect of morphine (15 mg/kg). Western blot analysis showed that acute oxycodone treatment (15 mg/kg) has no effect on RGS9-2 expression in the NAc of C57Bl/6 mice (Fig. 3b, NAc tissue dissected 2 h post oxycodone treatment).

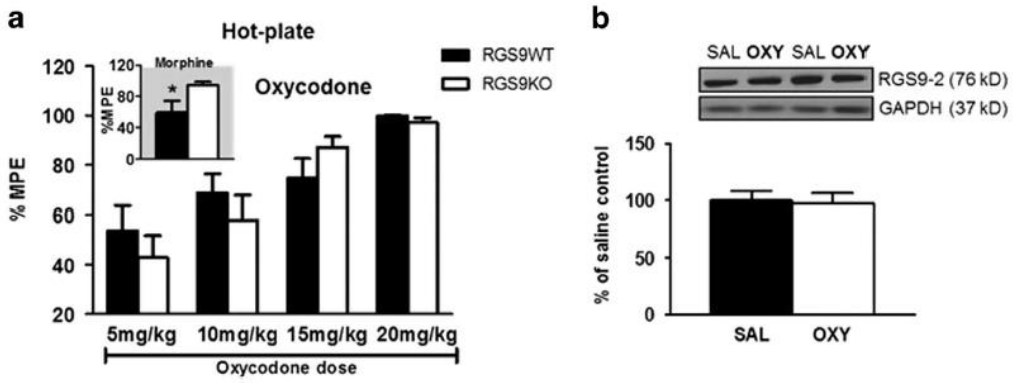


Figure 3. Regulator G-protein signaling 9-2 (RGS9-2) does not affect the acute analgesic actions of oxycodone. (a) Ablation of the *Rgs9* gene does not affect the acute analgesic effects of oxycodone in the hot-plate assay [each dose corresponds to a separate group of animals, two-way ANOVA followed by Bonferroni post hoc tests, $F(1,74) = 0.16$, $p>0.05$]. Upper panel: RGS9-knockout (RGS9KO) mice are more sensitive to the analgesic actions of morphine [dose: 15mg/kg, t-test, $t(7) = 2$, $^*p<0.05$]. **(b)** Acute administration of a high dose of oxycodone (15mg/kg) did not have any effect on RGS9-2 protein levels in the nucleus accumbens (NAc) [t-test, $t(19) = 0.23$, $p>0.05$]. OXY: oxycodone, SAL: saline.

RGS9-2 acts as a negative modulator of oxycodone tolerance.

The next set of experiments investigated the role of RGS9-2 in the development of tolerance to the analgesic effects of oxycodone in the hot plate assay. We found that RGS9-2 has a protective role towards the development of oxycodone tolerance, as RGS9KO mice become tolerant to the analgesic effects of the drug earlier than RGS9WT animals (Fig. 4a). As shown on the top panel and consistent with our earlier findings, RGS9-2 has the opposite role in the modulation of morphine tolerance, as RGS9KO mice respond to repeated morphine treatment at 4 days, while their WT controls show analgesic tolerance. Furthermore, protein levels of RGS9-2 are upregulated in the NAc of mice tolerant to oxycodone (Fig. 4b). The hot-plate test is a measure of acute analgesia, whereas oxycodone is often prescribed for chronic pain conditions. To determine if RGS9-2 affects the development of oxycodone tolerance under chronic pain states, we used the SNI paradigm and monitored responses to the antiallodynic effects of oxycodone in the Von Frey assay for mechanical allodynia. Our earlier work has shown that RGS9-2 affects Von Frey responses the first week after nerve injury, but by day 10, mechanical allodynia levels are not different from those observed in RGS9WT mice (Terzi et al., 2014). Here we started the treatment with oxycodone on day 15, when mechanical allodynia levels were identical between genotypes. The drug was administered once a day for 17 consecutive days, and Von Frey responses were monitored 1 hour after drug injection. Consistent with our findings using the hot-plate assay, while the antiallodynic effects of oxycodone are maintained in RGS9WT mice throughout the monitoring period, RGS9KO mice show decreased analgesic response to oxycodone by day 12 (Fig. 4c).

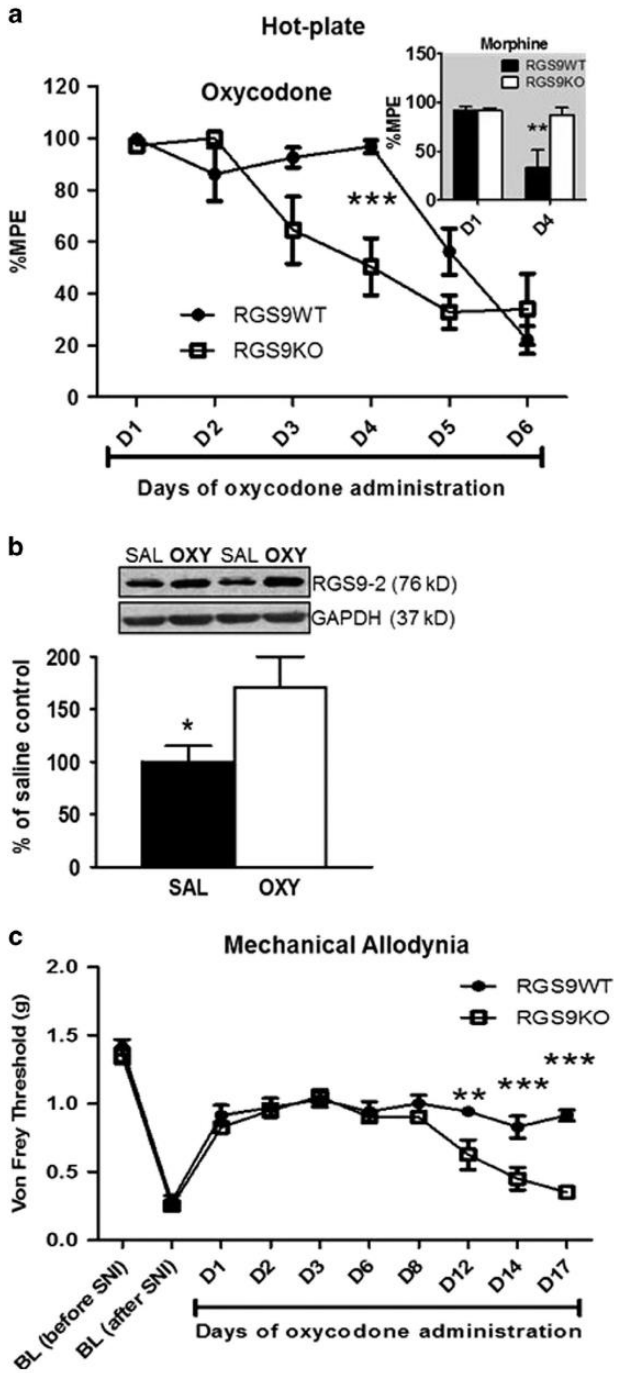


Figure 4. Regulator of G-protein signaling 9-2 (RGS9-2) acts as a negative modulator of oxycodone tolerance. (a) RGS9-knockout (RGS9KO) mice become tolerant to the analgesic effects of the drug earlier than their wildtype controls [dose: 20mg/kg, two-way ANOVA repeated-measures followed by Bonferroni post hoc test, $F(1,45) = 3.77$, ***$p<0.001$]. Upper panel: The opposite phenotype is observed when morphine is used in the hot-plate assay, as RGS9KO mice respond to morphine at a time point their wildtype controls show analgesic tolerance [dose: 20mg/kg, two-way ANOVA repeated-measures followed by Bonferroni post hoc test, $F(1,7) = 9.25$, **$p<0.01$]. **(b)** Furthermore, protein levels of RGS9-2 are upregulated in the nucleus accumbens (NAc) of mice tolerant to oxycodone [t-test, $t(28) = 2.14$, *$p<0.05$]. **(c)** RGS9KO mice experiencing neuropathic pain become tolerant to the antiallodynic effects of the drug earlier than their wildtype controls. Oxycodone treatment was initiated on day 15 after spared nerve injury (SNI) surgery, when mechanical allodynia levels were identical between genotypes. The drug was administered once a day for 17 consecutive days, and Von Frey responses were monitored 1 hour after drug injection [dose: 3mg/kg, two-way ANOVA repeated-measures followed by Bonferroni post hoc test, $F(1,117) = 10.49$, **$p<0.01$, ***$p<0.001$].

II. Suppression of RGSz1 function optimizes the actions of opioid analgesics by mechanisms that involve the Wnt/β-catenin pathway

Regulation of RGSz1 by chronic morphine.

We used western blot analysis to monitor the impact of chronic morphine on RGSz1 expression in the PAG. We applied a 4-day hot plate morphine tolerance paradigm, and monitored analgesic responses for 4 consecutive days, in groups of mice treated with morphine (15mg/kg). RGSz1 protein levels in the PAG were up-regulated 2 hours after the last morphine injection compared to saline-treated controls (Fig. 5a). No RGSz1 protein was detected in the RGSz1KO mice. Notably, morphine treatment up-regulated Gαz in the PAG in both RGSz1WT and RGSz1KO mice (Fig. 5b), but levels of other Gα subunits expressed in the PAG (Gαi3, Gαo, Gαq) were not altered (Fig. 5c–e). To further evaluate the association between RGSz1 and Gαz complexes in the PAG, we performed co-IP assays and found that morphine acutely (within 30 min) promoted the dissociation of Gαz from MOPRs (Fig. 5f). However, chronic morphine treatment induced the converse and promoted MOPR-Gαz complex formation (Fig. 5g).

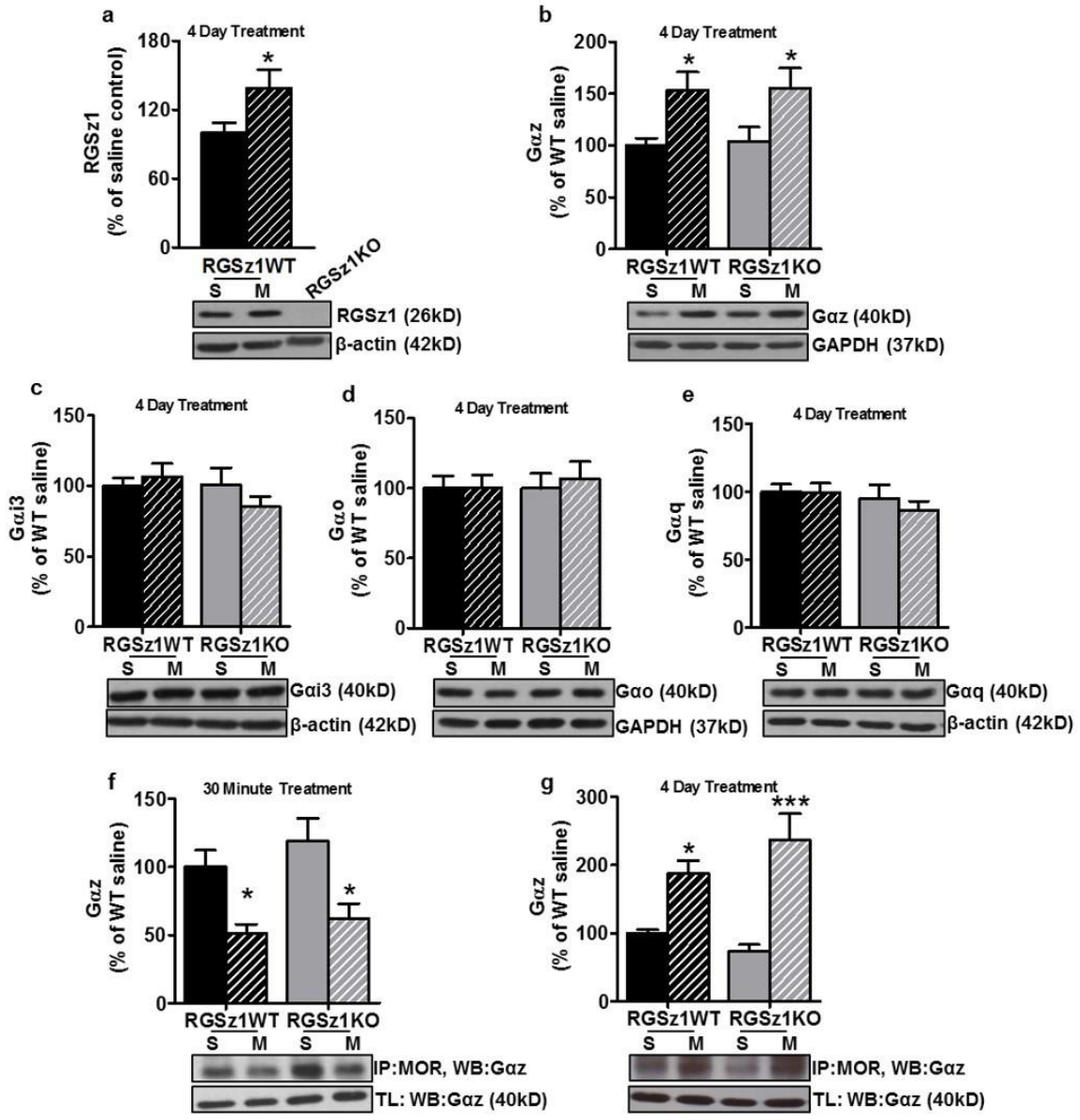


Figure 5. Morphine regulates RGSz1 and Gαz in the PAG. (a) RGSz1 protein levels were elevated in the PAG of RGSz1WT mice treated for four consecutive days with morphine [15 mg/kg; n = 16/group; t test, t(30) = 2.144; *p<0.05]. Chronic morphine also increased the levels of Gαz in the PAG **(b)**, which occurred in both RGSz1WT and RGSz1KO mice [15mg/kg; n = 11-12 per group; two-way ANOVA followed by Bonferroni post hoc test; F(1,42) = 11.94; *p<0.05)], but not the levels of Gαi3 [F(1,42) = 0.247] **(c)**, Gαo [F(1,42) = 0.124] **(d)**, or Gαq [F(1,43) = 0.366 (dose: 15mg/kg; n = 11-12 per group; two-way ANOVA followed by Bonferroni post hoc test; p>0.05 for all three subunits). **(f)** Co-IP analysis revealed that Gαz dissociated from MOPRs 30 min after an acute morphine injection [15 mg/kg; n = 3–4/group; two-way ANOVA followed by Bonferroni's post hoc test, F(1,11) = 17.84; *p<0.05]. **(g)** However, chronic treatment with morphine for four consecutive days promoted MOPR-Gαz associations measured 30 min after the last injection [15 mg/kg; n = 4 per group; two-way ANOVA followed by Bonferroni's post hoc test, F(1,12) = 32.94; *p<0.05, ***p<0.001). S: saline, M: morphine, TL: total lysate

RGSz1 knockout enhances the analgesic efficacy of MOPR agonists and delays morphine tolerance.

We next examined the role of RGSz1 in the analgesic actions of morphine and other MOPR agonists. In the hot plate test, male RGSz1KO mice show enhanced analgesia at lower doses of morphine compared with the responses of RGSz1WT controls (Fig. 6a). This phenotype was also observed in female RGSz1KO mice (Fig. 7a, inset). The sensitivity to the analgesic effects of fentanyl and methadone was also increased in RGSz1KO mice compared with that in their RGSz1WT controls (Fig. 6b). We repeated the hot plate assay for eight consecutive days to assess the impact of RGSz1 on the development of morphine analgesic tolerance. By day 3 RGSz1WT mice show reduced responses to morphine, due to the development of tolerance. Analgesic responses were significantly different between genotypes, indicating that tolerance to morphine treatment (15 mg/kg, s.c) is delayed in RGSz1KO mice (Fig. 6c). This delayed tolerance phenotype was also observed in female RGSz1KO mice (Fig 6c, inset). We similarly assessed morphine tolerance under inflammatory pain conditions. Thermal hind paw hyperalgesia after intraplantar injections of CFA was assessed by the Hargreaves test (Hargreaves et al., 1988). Similar to our observations under pain-free states, while morphine analgesia was reduced in RGSz1WT mice after three days of treatment, it was maintained in RGSz1KO mice throughout the testing period (Fig. 6d).

Interestingly, while RGSz1 knockout enhanced analgesic responses to morphine, it decreased the rewarding effects. Specifically, RGSz1KO mice showed a lower preference for the morphine-paired side in the conditioned place preference paradigm compared to their RGSz1WT controls (Fig. 7a). Importantly, RGSz1KO mice show normal responses to other reward-related behaviors, such as sucrose preference [sucrose consumption (% of total amount consumed): RGSz1WT = 60.4±6.2, RGSz1KO = 69.7±6.2]. Furthermore, the emergence of locomotor sensitization to repeated morphine administration was suppressed in RGSz1KO mice (Fig. 7b). In terms of addiction-like behaviors, as assessed using the withdrawal paradigm, RGSz1KO mice so no overall difference in symptomatology compared to their RGSz1WT controls except from a higher number of jumps (Fig.7c), a phenotype consistent in the case of female mice as well (Fig. 7c inset).

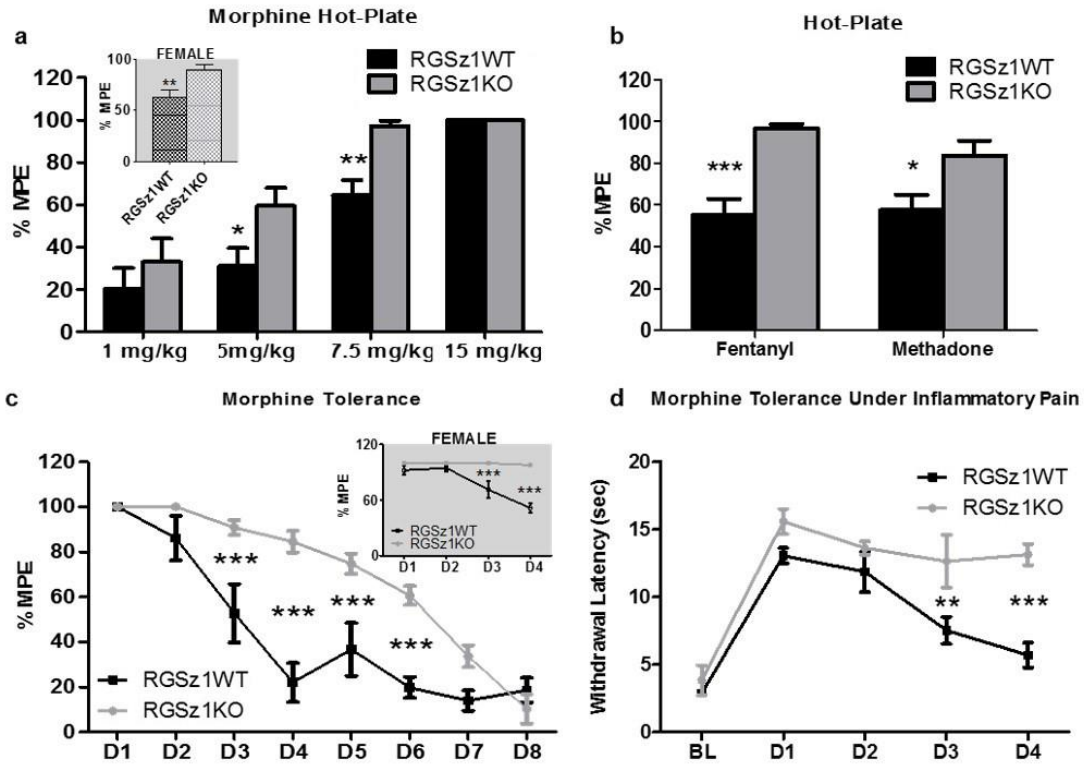


Figure 6. A Role of RGSz1 in morphine-induced analgesia. (a) Analgesic responses to morphine are enhanced in RGSz1KO compared with those in RGSz1WT controls [each dose was tested in a separate group of mice; n = 8–10/group; two-way ANOVA followed by Bonferroni's post hoc test, $F(1,64) = 9.28$; *p<0.05, **p<0.01]. A similar phenotype was observed in female mice [inset; dose, 7.5 mg/kg; n = 10–13/group; t test, $t(21) = 2.885$; **p<0.001]. **(b)** RGSz1KO mice show increased analgesic responses compared with those in the RGSz1WT mice to fentanyl [0.125 mg/kg; n = 9–11 per genotype; t test; $t(18) = 4.677$; ***p<0.001] and methadone [5 mg/kg; n = 11–12/group; t test, $t(21) = 2.519$; *p<0.05]. **(c)** The development of tolerance to 15 mg/kg morphine was delayed in male [n = 7/group; two-way ANOVA repeated-measures followed by Bonferroni's post hoc test, $F(1,12) = 20.99$; ***p<0.001] and female [inset; n = 6–7/group; two-way ANOVA repeated-measures followed by Bonferroni's post hoc test, $F(1,11) = 22.89$; ***p<0.001] RGSz1KO mice. **(d)** Delayed development of morphine tolerance was also observed under inflammatory pain states. Withdrawal latencies of hind paws with CFA-induced inflammation were significantly higher in RGSz1KO mice after three and four days, demonstrating that morphine (3 mg/kg) analgesia was maintained [n = 4–5/group; two-way ANOVA repeated-measures followed by Bonferroni's post hoc test, $F(1,7) = 18.59$; **p<0.01, ***p<0.001]. MPE: maximum possible effect.

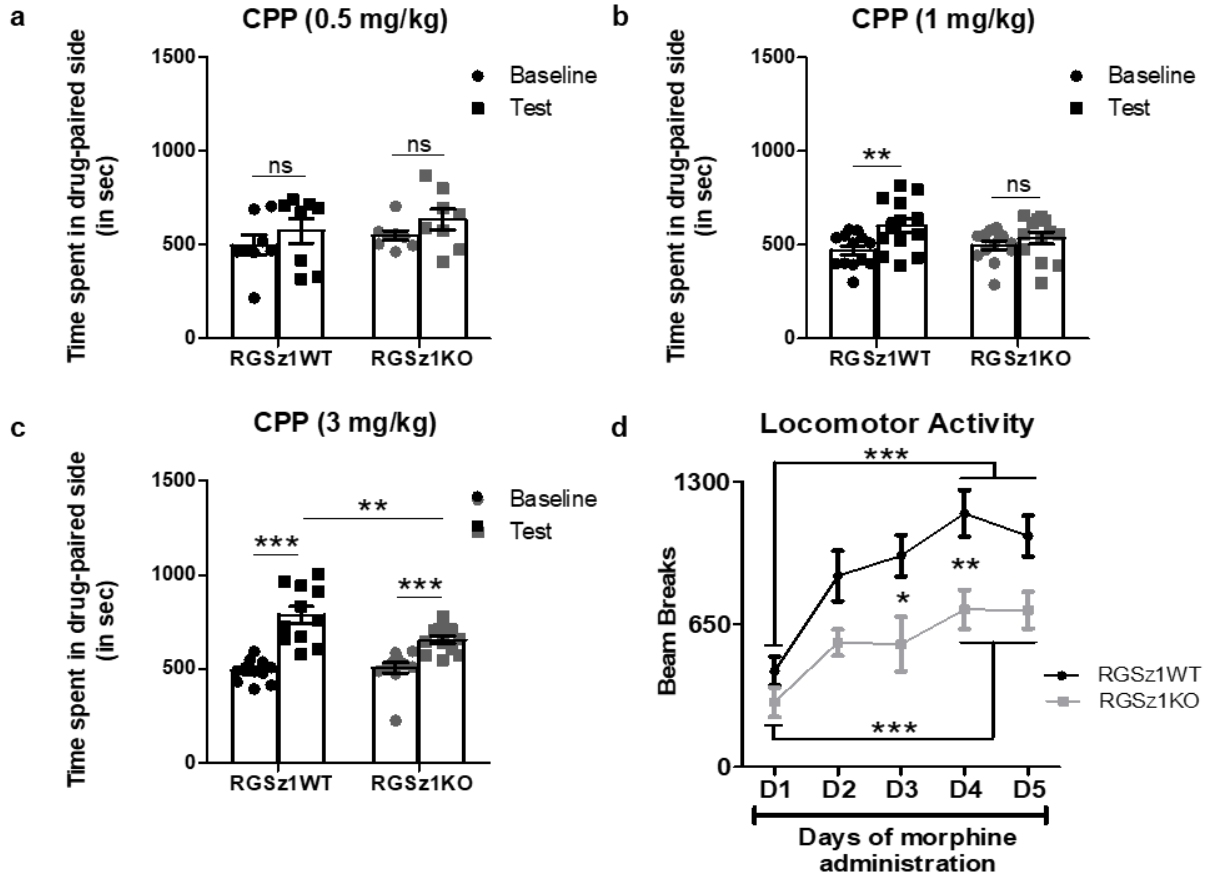


Figure 7. RGSz1 modulates reward-like behaviors. (a–c) Knockout of RGSz1 affects the rewarding effect of low morphine doses. RGSz1KO mice did not show conditioned place preference with 1 mg/kg and spent less time in the drug-paired side when 3 mg/kg of morphine was used [two-way repeated-measures ANOVA followed by Bonferroni's post hoc test; 0.5 mg: n = 8/group, $F(1,14) = 1.12$; 1 mg: n = 13/group, $F(1,24) = 0.33$, $^{**}P<0.01$; 3 mg: n = 11–12/group, $F(1,21) = 2,94$, $^{**}P<0.01$, $^{***}P<0.001$]. **(d)** Furthermore, RGSz1KO male mice showed lower locomotor activity in response to repeated morphine administrations than RGSz1WT control mice [10 mg/kg; n = 9–10/group; two-way ANOVA repeated-measures followed by Bonferroni's post hoc test, $F(1,17) = 11.16$; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$].

RGSz1 in PAG neurons modulates analgesic tolerance.

We hypothesized that RGSz1 modulates the analgesic and rewarding effects of MOPR agonists via actions in distinct brain regions. To test this, we used viral approaches to conditionally knockdown RGSz1 in specific brain regions of adult mice. On the basis of our initial Western blot analysis, we targeted the PAG. AAV2-CMV-CRE-GFP or an AAV2-CMV-GFP control virus was injected into the vlPAG of RGSz1fl/fl mice. Two weeks later, when maximal viral expression is achieved, mice were tested in the morphine tolerance paradigm. Knockdown of RGSz1 in the vlPAG significantly delayed the development of morphine tolerance (Fig 8a). Infection of the vlPAG with AAV2-CMV-CRE-GFP resulted in 50% reduction of RGSz1 protein levels (Fig 8b). As shown in Figure 8b and d and consistent with previous reports (Taymans et al., 2007), the virus infects only neuronal cells and not astrocytes. Interestingly, knockdown of RGSz1 in the NAc did not affect the development of analgesic tolerance (Fig 8c). Similar to vlPAG, infection of the NAc with AAV2-CMV-CRE-GFP resulted in an average reduction of RGSz1 protein levels around 50% (Fig 8d). Notably, knockdown of RGSz1 in the vlPAG of male mice does not affect sensitivity to low morphine doses in the hot plate assay [Hot plate latencies (%MPE) for 12mg/kg morphine: AAV2-CMV-GFP = 50.3±6.7 and AAV2-CMV-CRE-GFP = 44.2±8.4]. Furthermore, the actions of RGSz1 in the vlPAG do not affect the development of physical dependence. After treating mice for 5 days with escalating doses of morphine, withdrawal was precipitated with naloxone hydrochloride (1 mg/kg, s.c.). We immediately began monitoring several somatic withdrawal symptoms in AAV2-CMV-CRE injected RGSz1fl/fl mice and their AAV2-CMV-GFP injected controls, including jumps, wet-dog shakes, tremor, ptosis, diarrhea, and weight loss for a period of 30 min. As shown in Figure 9e, withdrawal symptoms were similar between the two groups of mice. Thus, RGSz1 actions in the vlPAG promote the development of analgesic tolerance without impacting the development of physical dependence.

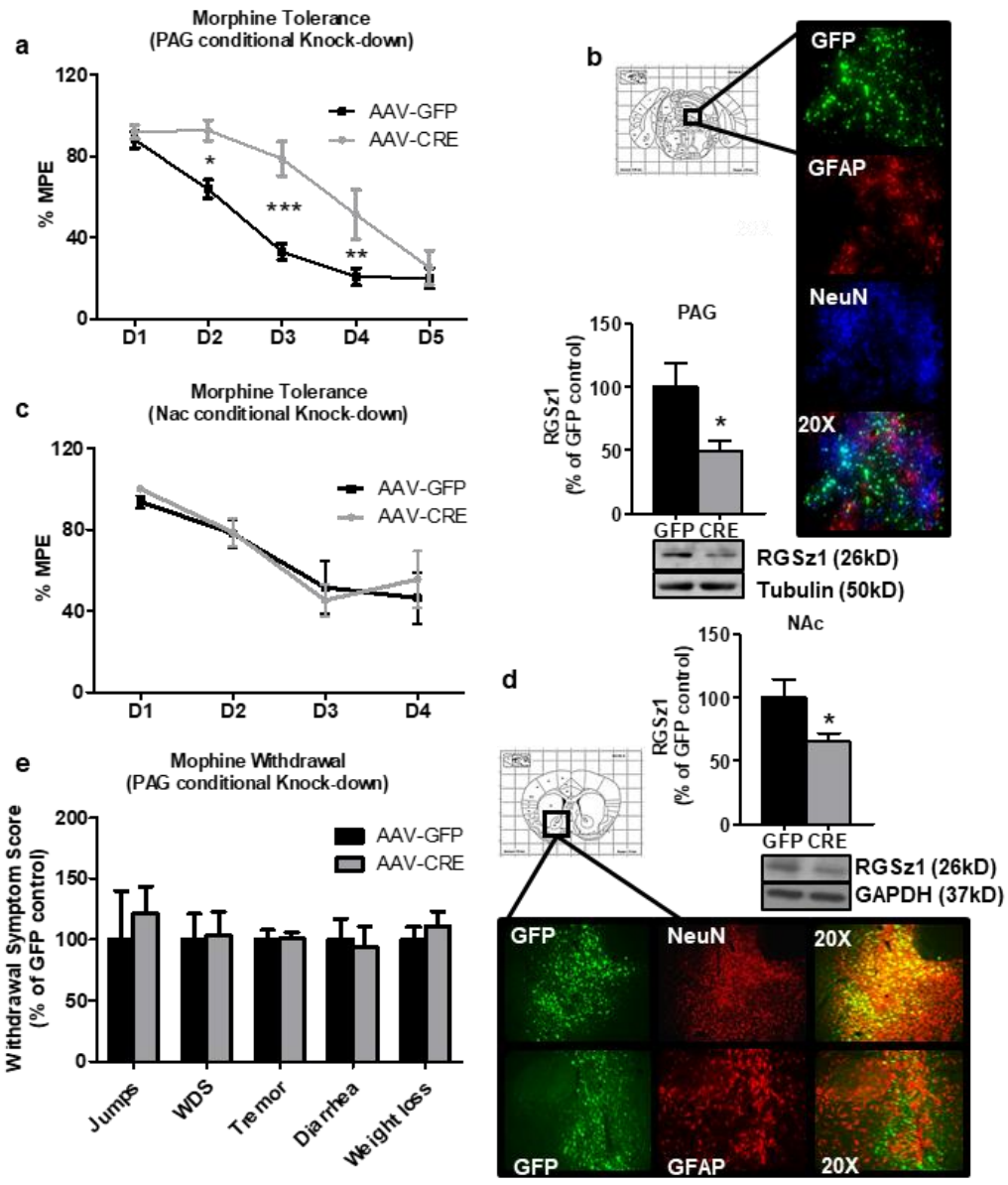


Figure 8. Rgsz1 knockdown in PAG neurons suppresses morphine tolerance. (a) Conditional knockdown of Rgsz1 in neurons in the vlPAG of RGSz1fl/fl mice by stereotaxic infection of AAV2-CMV-CRE vectors maintained the analgesic effects of morphine in the hot plate assay over several days [15 mg/kg; n = 7/group; two-way repeated-measures ANOVA followed by Bonferroni's post hoc test, $F(1,12) = 11.34$; *p<0.05, **p<0.01, ***p<0.001]. **(b)** Western blot analysis verifying RGSz1 down-regulation in the PAG of CRE virus-infected mice [n = 4–5/group; t test, $t(7) = 2.663$; *p<0.05] and verification of AAV2 viral vector distribution in the PAG. AAV2 vectors infect neurons (as indicated by NeuN staining) and not astrocytes (as indicated by GFAP staining). **(c)** The effect of Rgsz1 knockdown on the development of morphine tolerance was region specific, and no effect was observed when neurons of the NAc were targeted [15 mg/kg; n = 6–7/group; two-way repeated-measures ANOVA followed by Bonferroni's post hoc test, $F(1,11) = 0.08$]. **(d)** Western blot analysis verifying RGSz1 down-regulation in the NAc of CRE virus-infected mice [n = 9-10/group; t test, $t(17) = 2.12$; *p<0.05] and verification of AAV2 viral vector distribution in the NAc. **(e)** Knockdown of RGSz1 in the vlPAG does not affect the intensity of naloxone precipitated morphine withdrawal symptoms (n=9-10/group; multiple t-tests).

Morphine distinctly alters gene expression patterns in the PAG of RGSz1KO and RGSz1WT mice.

Repeated exposure to opioids promotes long-term adaptations in gene expression and neuronal plasticity (Robison and Nestler, 2011; Russo et al., 2010). We applied next-generation RNA-Seq to understand the impact of morphine tolerance and the influence of RGSz1 on gene expression in the PAG. Tissue from the PAG was collected from mice on day 4 of the morphine hot plate assay, when analgesic tolerance emerges in RGSz1WT mice (Fig. 9a). After pooling tissue from two animals per sample, RNA from naïve and morphine-treated RGSz1WT and RGSz1KO mice was analyzed. A heatmap analysis comparing treated versus naïve mice for each genotype shows that morphine tolerance altered gene expression patterns in RGSz1WT mice, but that a much larger number of genes was affected in the RGSz1KO group (Fig 9b). The numbers of genes regulated by morphine in each genotype are shown in a Venn diagram in Figure 9c. Our findings reveal that only ¼ of the genes regulated by morphine are common between genotypes, whereas ¾ of the genes with altered expression are unique to RGSz1WT and RGSz1KO mice (Fig. 9c). A gene ontology analysis indicated that the majority of genes regulated by chronic morphine in the PAG of RGSz1WT and RGSz1KO mice are associated with transcription and cell proliferation/differentiation, whereas genes associated with ion/transmembrane transport, cell adhesion, and behavior are also altered in RGSz1KO mice. We further investigated this observation using Ingenuity Pathway Analysis (IPA), and indeed, distinct pathways were affected in the PAG of each genotype, with Wnt/β-catenin signaling as the top pathway affected in RGSz1WT mice ($p<0.001$) and serotonin receptor signaling targeted in RGSz1KO mice ($p<0.001$) (Fig. 10a). Importantly, pathway analysis of the preexisting gene expression adaptations in the PAG of RGSz1KO naïve mice does not reveal alterations in any of the above stated pathways. Figure 10b summarizes the changes in several components for each of these pathways after morphine treatment. Differential gene expression patterns in RGSz1WT and RGSz1KO mice were validated by qPCR in separate groups (Fig. 10c).

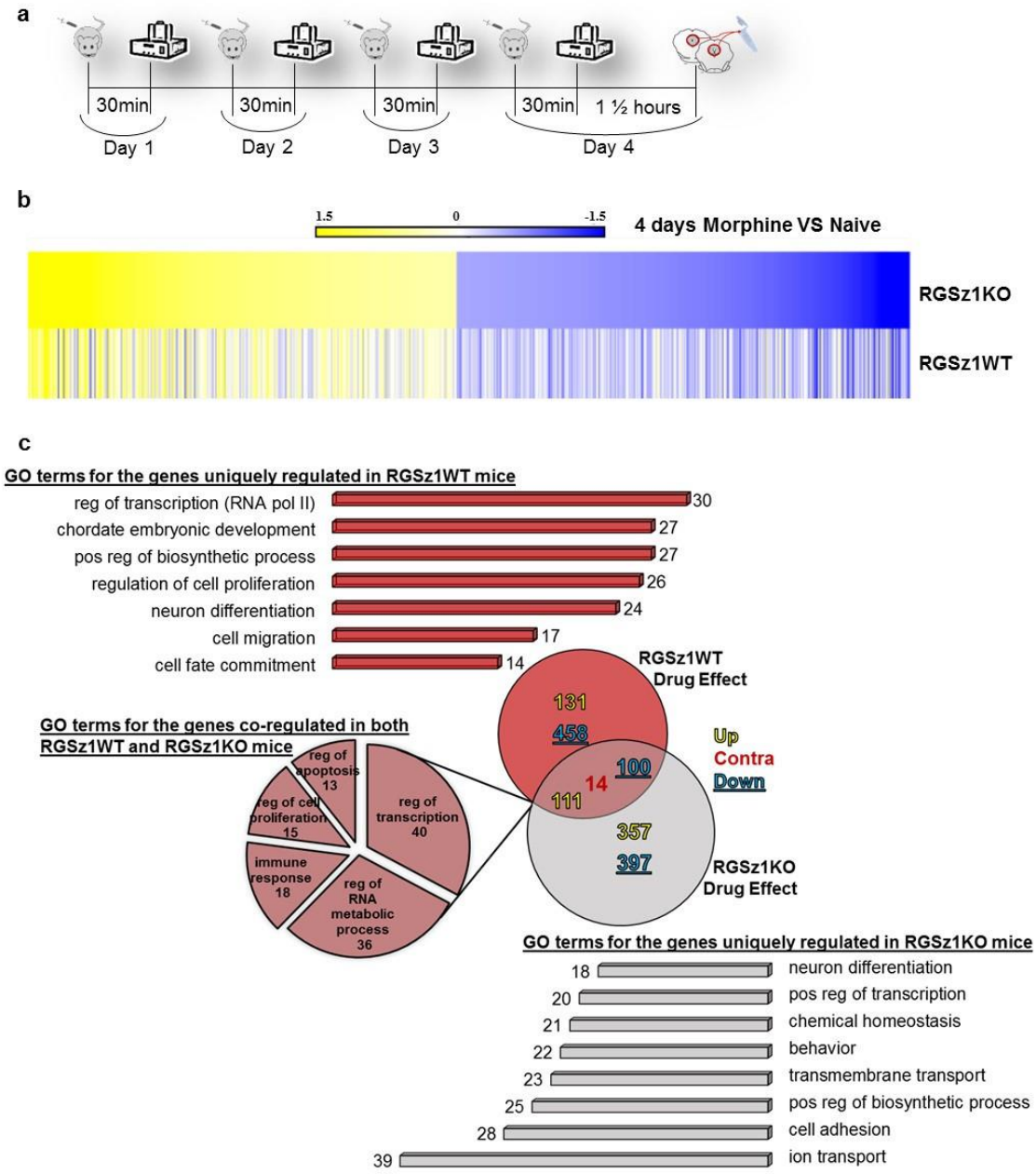


Figure 9. RNA-Seq of PAG tissue from morphine-treated RGSz1WT and RGSz1KO mice. (a) Experimental timeline showing that mice were treated for four consecutive days with 15 mg/kg morphine and tested in the hot-plate assay to evaluate analgesic responses. On day 4 (when analgesic tolerance emerges in RGSz1WT mice) PAG tissue was collected 2 h after morphine administration. **(b)** Heatmap analysis showing the effect of drug treatment on gene expression in RGSz1WT and RGSz1KO mice. The overall patterns of gene expression regulation were similar between the two genotypes, but a much larger group of genes was affected in the PAG of RGSz1KO mice. **(c)** Venn diagram depicting the actual numbers of genes affected in each group and their direction of regulation and associated Gene ontology (GO) categories. Pie chart depicts results of a GO analysis of the 225 genes regulated by morphine in the PAG of both genotypes. Bars graphs show GO of genes regulated by morphine in the PAG of RGSz1WT (red) and RGSz1KO (light gray) mice.

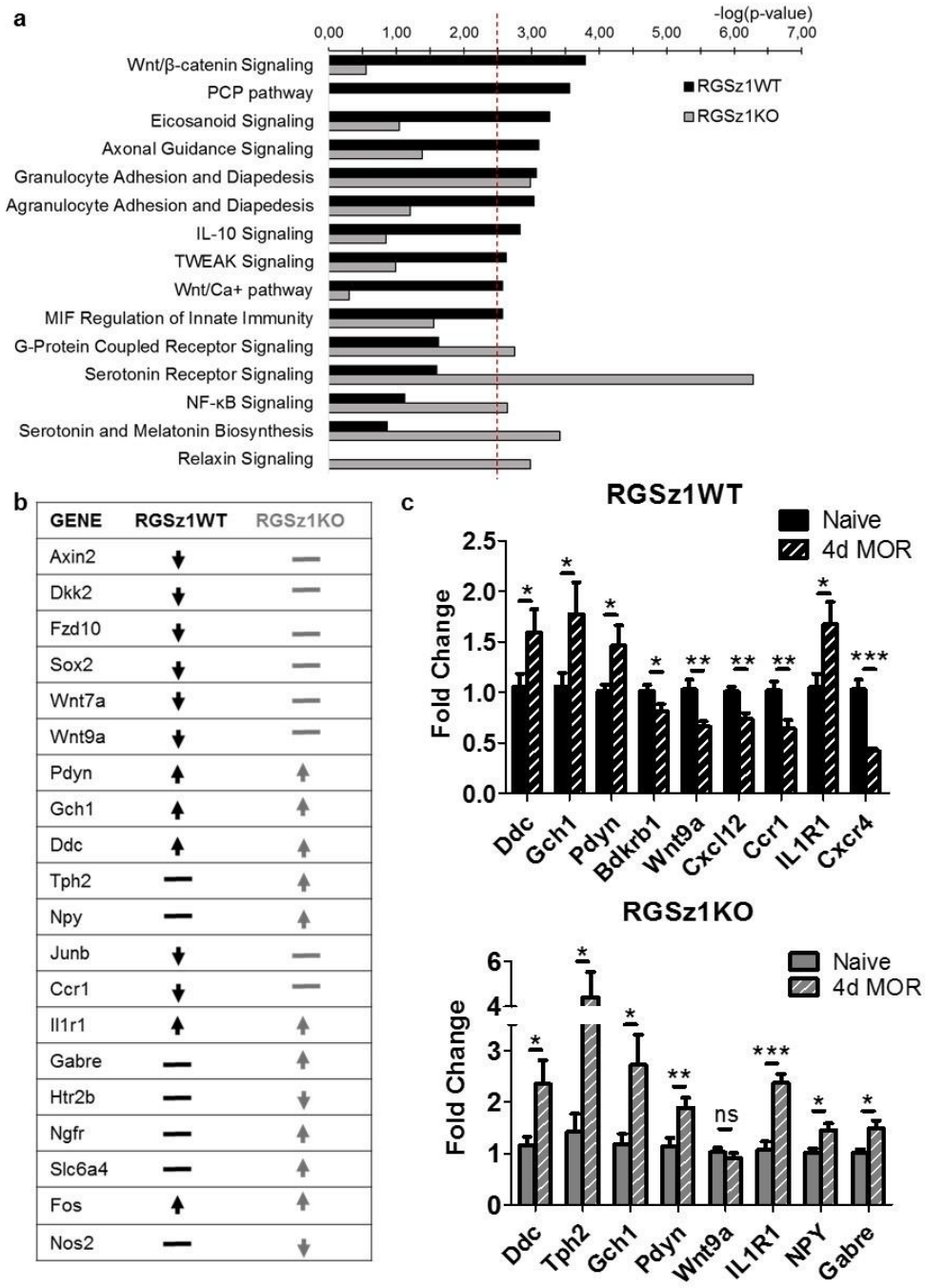


Figure 10. Chronic morphine affects different intracellular pathways in the PAG of RGSz1KO and RGSz1WT mice. (a) IPA analysis of the genes regulated by chronic morphine revealed distinct canonical pathways were affected in RGSz1WT and RGSz1KO mice (cut-off: p<0.003). **(b)** Selected genes in the canonical Wnt/β-catenin signaling or inflammatory response-related cascades and G-protein coupled/serotonin receptor signaling pathways and the direction of their regulation. **(c)** qPCR validation of a subset of genes in a separate cohort of animals by genotype [n = 7–8/group; t tests, RGSz1WT: Ddc, t(13) = 2.135; Gch1, t(13) = 2.2; Pdyn, t(13) = 2.27; Bdkrb1, t(14) = 2.154; Wnt9a, t(14) = 3.4; Cxcl12, t(14) = 3.69; Ccr1, t(12) = 2.99; Il1r1, t(14) = 2.44; Cxcr4, t(14) = 6.08; RGSz1KO: Ddc, t(13) = 2.29; Tph2, t(13) = 2.4, Gch1, t(13) = 2.36; Pdyn, t(13) = 2.9; Wnt9a, t(14) = 0.87; Il1r1, t(12) = 5.49; NPY, t(14) = 2.86; Gabre, t(14) = 2.98; *p<0.05, **p<0.01, ***p<0.001]. ns: not significant.

Wnt/β-catenin signaling is essential for analgesic responses to morphine.

Several key molecules in the Wnt/β-catenin signaling pathway were down-regulated by chronic morphine treatment in the PAG of RGSz1WT but not in RGSz1KO mice (shown in blue in Fig. 11a). Furthermore, narrowing our lists of differentially regulated genes to known β-catenin targets (Dias et al., 2014) revealed that those genes in RGSz1WT mice were primarily down-regulated (Fig. 11b). We hypothesized that analgesic responses to morphine are maintained in RGSz1KO mice because β-catenin remains active, whereas signaling in this pathway is suppressed in RGSz1WT mice. Western blotting confirmed this hypothesis, as higher levels of active p-β-catenin (ser675) and inactive p-GSK3β (ser9) were observed in the PAG of RGSz1KO mice after 4 days of morphine treatment (Fig. 11c and d). Furthermore, antagonizing the activity of β-catenin in the vlPAG of RGSz1KO mice by expressing a dominant-negative form of the protein induced tolerance similar to that seen in RGSz1WT controls, whereas overexpressing β-catenin itself in the vlPAG of RGSz1WT mice, for a period overlapping with morphine treatment, was not sufficient to prevent tolerance (Fig. 11e, for viral validation Fig. 12). We therefore hypothesized that other components of the Wnt/β-catenin are necessary for obtaining the prolonged analgesic response phenotype observed in RGSz1KO mice. In the absence of stimulation, β-catenin is bound to Axin 2 in the cytoplasm, which targets it to proteasomal degradation (Clevers, 2006). Wnt signaling releases β-catenin for translocation to the nucleus. Because Axin2 contains an RGS domain that has been shown to interact with Gα subunits (Castellone et al., 2005; Egger-Adam and Katanaev, 2010; Stemmle et al., 2006), we hypothesized that an interaction of Axin2 with Gαz indirectly affects Wnt/β-catenin signaling. This hypothesis was confirmed by our next set of biochemical assays. Although the total levels of Axin2 in the PAG were not affected by repeated morphine administration (Fig. 13a), the interaction with Gαz in the synaptosomal fraction was significantly decreased in RGSz1WT mice as indicated by co-precipitation of Gαz with Axin2 (Fig. 13b). However, this interaction was maintained in RGSz1KO mice that do not exhibit morphine tolerance (Fig 13b). The dissociation of Axin2 from Gαz was accompanied by higher levels of Axin2 in the nuclear fraction of the samples as shown in Fig 13c. Furthermore, correlation analysis reveals a significant negative association between the amount of Axin2 bound to Gαz in the synaptosomal fraction and the amount of Axin2 present in the nuclear fraction (Fig. 13d). Translocation of Axin2 to the nucleus was previously shown to block β-catenin-mediated transcription (Rennoll et al., 2014).

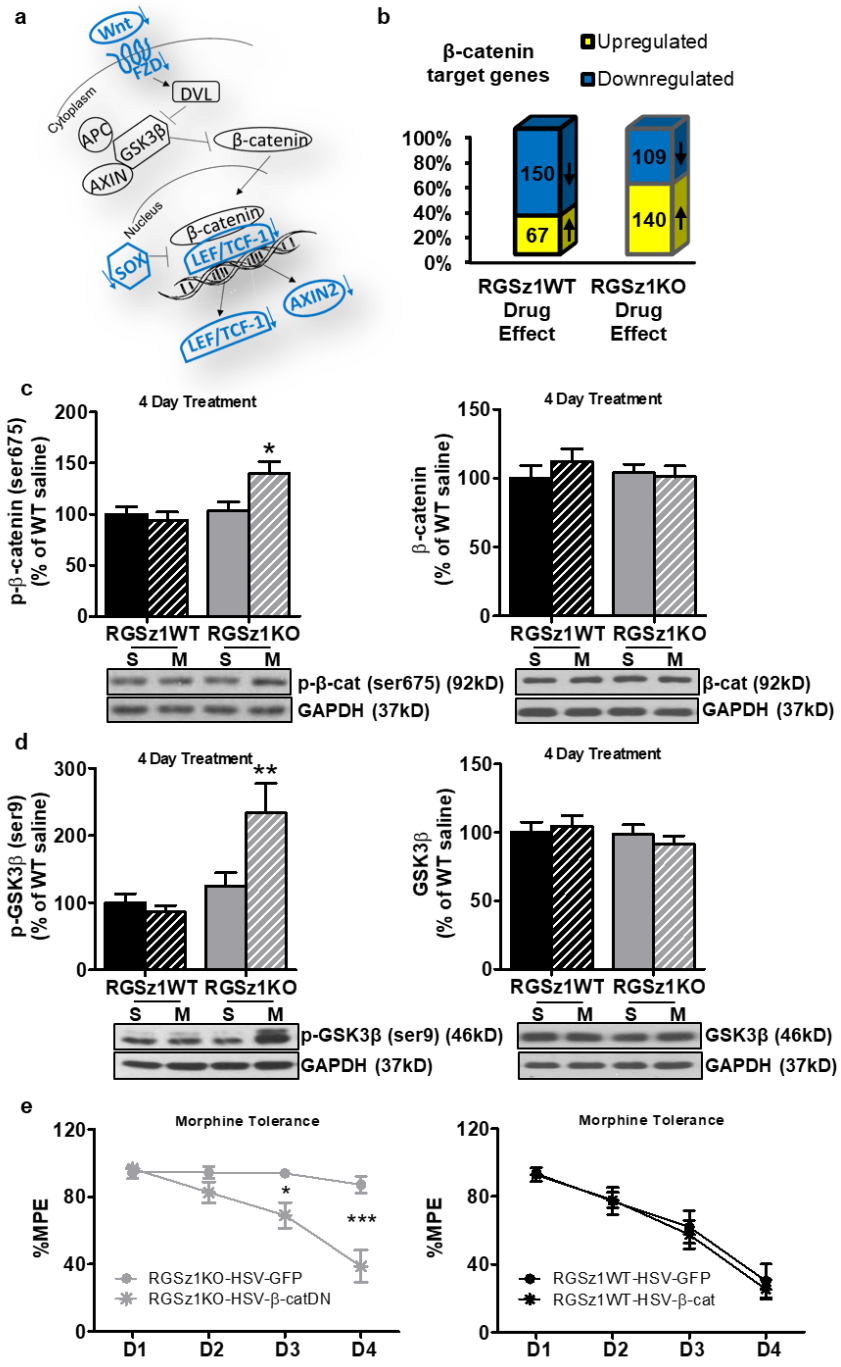


Figure 11. RGSz1 modulates the activity of the Wnt/β-catenin signaling pathway. **(a)** Schematic depiction of Wnt/β-catenin signaling pathway components (in blue) that are affected by chronic morphine in the PAG of RGSz1WT mice only. **(b)** Bar graph showing the subset of genes that are known β-catenin targets and the direction of their regulation after chronic morphine administration. Notably, the majority of genes in the RGSz1WT group were downregulated. **(c)** Western blot analysis revealed regulation of active phospho-β-catenin (ser675) only in the PAG of RGSz1KO mice after four consecutive days of morphine administration [15 mg/kg; n = 14–15/group; two-way ANOVA followed by Bonferroni's post hoc test, $F(1,53) = 7.37$; *$p<0.05$], with total β-catenin levels remaining unaffected [n = 11–12/group; two-way ANOVA followed by Bonferroni's post hoc test, $F(1,40) = 0.18$]. **(d)** Levels of inactive phospho-GSK3β (ser9) were also increased in the PAG of morphine-treated RGSz1KO mice [15 mg/kg; two-way ANOVA followed by Bonferroni's post hoc test; for p-GSK3β: n = 14–15/group; $F(1,53) = 11.65$, **$p<0.01$; for total GSK3β: n = 11–12/group; $F(1,40) = 1.004$]. **(e)** Antagonizing the activity of β-catenin in the vlPAG by overexpressing a dominant-negative form in RGSz1KO mice led to the development of analgesic tolerance to morphine [15 mg/kg; n = 7 for RGSz1KO-HSV-GFP and 12 for RGSz1KO-HSV-β-catDN; two-way repeated-measures ANOVA followed by Bonferroni's post hoc test, $F(1,51) = 9.9$; *$p<0.05$, ***$p<0.001$). However, overexpressing β-catenin in the PAG of RGSz1WT mice did not prevent the development of analgesic tolerance [15 mg/kg; n = 9 for RGSz1WT-HSV-GFP and 10 for RGSz1WT-HSV-β-cat; two-way repeated-measures ANOVA followed by Bonferroni's post hoc test, $F(1,51) = 0.14$]. S: saline, M: morphine.

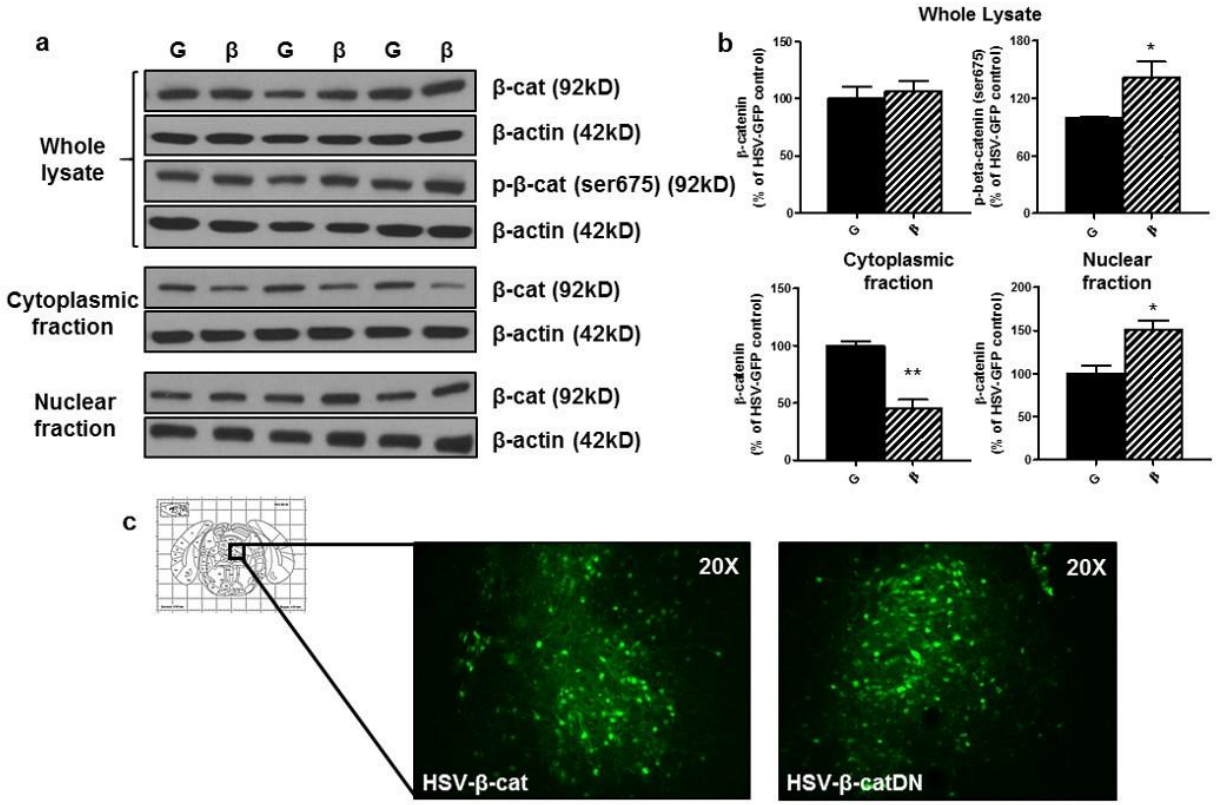


Figure 12. Validation of HSV-β-catenin viruses. (a) Western blot verifying the higher levels of active phospho-β-catenin (ser675), accompanied by higher levels of β-catenin in the nucleus and lower at the cytoplasm, in the PAG of HSV-β-catenin infected mice compared to their HSV-GFP injected controls. **(b)** Quantification of western blots [t test; total β-cat whole lysate: $t(6) = 0.48$, phosphor β-cat (ser675) whole lysate: $t(6) = 2.46$, total β-cat cytoplasmic fraction: $t(4) = 6.17$, total β-cat nuclear fraction: $t(4) = 3.68$; *$p<0.05$, **$p<0.01$]. **(c)** Verification of HSV viral vector distribution in the PAG.

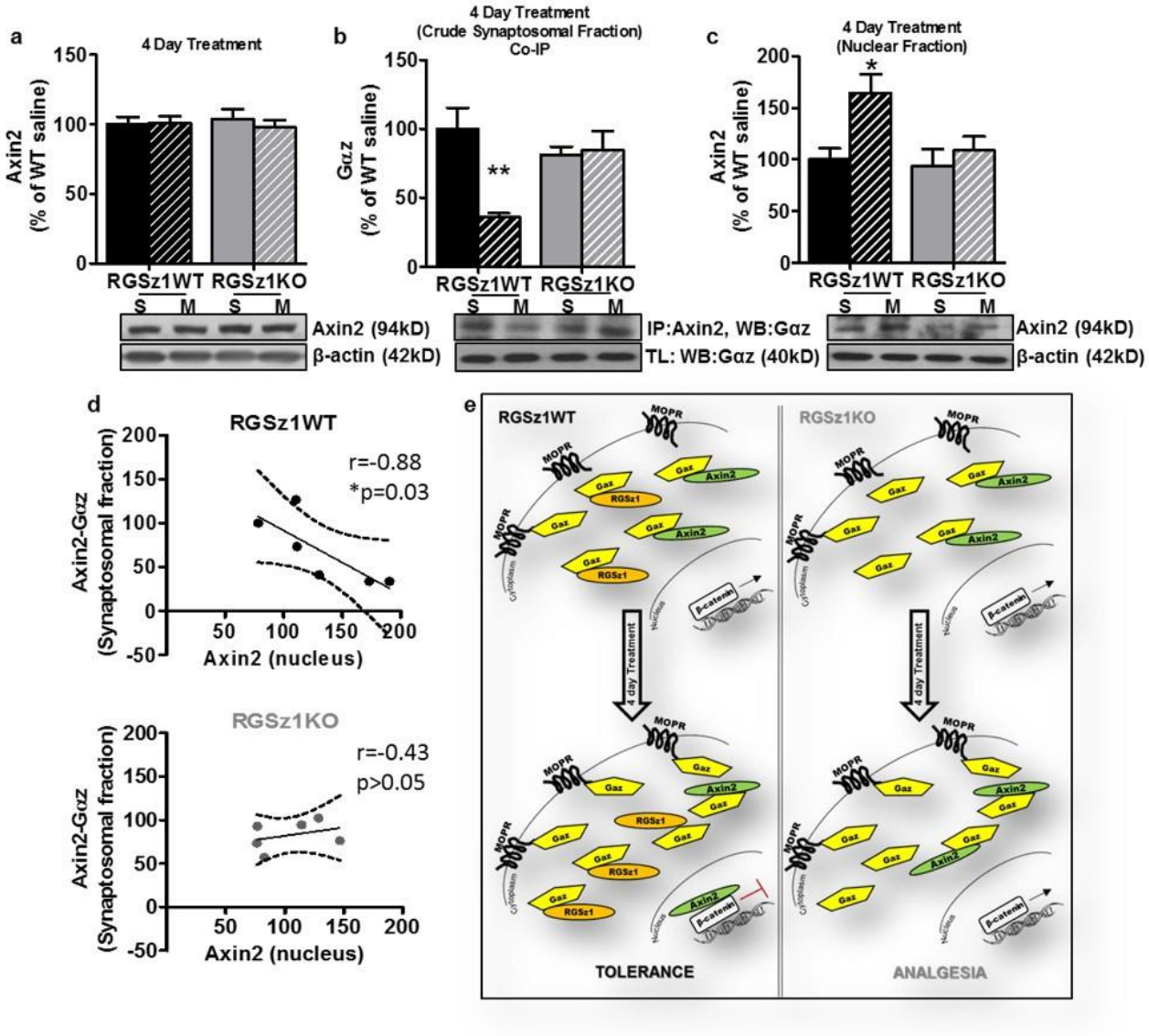


Figure 13. RGSz1 knockdown prevents dissociation of Axin2-Gαz complexes after chronic morphine. (a) Protein levels of Axin2 in the PAG are not affected by morphine treatment in RGSz1WT or RGSz1KO mice [15 mg/kg; n = 12/group; two-way ANOVA followed by Bonferroni's post hoc test, $F(1,44) = 0.19$]. **(b)** Co-IP assays on synaptosomal fractions revealed a reduced association of Axin2 with Gαz in RGSz1WT but not in RGSz1KO mice after repeated morphine administration [15 mg/kg; n = 3/group; two-way ANOVA followed by Bonferroni's post hoc test; $F(1,8) = 7.6$; $^{**}p<0.01$]. **(c)** Morphine tolerance in RGSz1WT also led to higher levels of Axin2 in the nucleus [15 mg/kg; n = 3/group; two-way ANOVA followed by Bonferroni's post hoc test; $F(1,8) = 7$; $^{*}p<0.05$]. **(d)** Correlation analysis reveals a negative association between the amount of Axin2 bound to Gαz in the synaptosomal fraction and the amount of Axin2 in the nuclear fraction of the same sample for RGSz1WT but not RGSz1KO mice. **(e)** Schematic depicting working hypothesis. In the PAG of tolerant mice higher levels of RGSz1 and stabilized complexes between MOPR and Gαz lead to reduction of Axin2-Gαz interaction, thereby enabling the translocation of Axin2 to the nucleus and silencing of β-catenin-mediated transcription. In the absence of RGSz1, Gαz-Axin2 complexes are not affected by repeated morphine administration and Axin2 is maintained in the cytoplasm. S: saline, M: morphine, TL: total lysate

III. A Role of RGSz1 Modulating Sensitized Responses in murine Chronic Pain models

RGSz1 modulates sensitized responses in murine chronic pain models in a sex-specific manner.

In order to examine the role of RGSz1 in chronic pain conditions we subjected RGSz1KO mice and their RGSz1WT controls in several established murine pain models and monitored development of sensitized responses over time. In the SNI model of neuropathic pain female RGSz1KO mice showed prolonged thermal hyperalgesia (Fig. 14a) and more intense mechanical (Fig. 14b) as well as cold (Fig. 14c) allodynia. Interestingly, this is not the case for RGSz1KO male mice that behave identically to their RGSz1WT controls in all three behaviors tested (Fig. 14d-f). A similar sex-dependent phenotype is observed using the CFA model of inflammatory pain, with RGSz1KO female mice exhibiting prolonged thermal hyperalgesia (Fig. 14g) and more intense and protracted mechanical allodynia (Fig. 14h), when male RGSz1KO mice recover from thermal hyperalgesia in a comparable rate to their RGSz1WT controls (Fig. 14j). We also used the formalin test, which is a model of sub-chronic pain. In this test the nociceptive responses are separated into two distinct phases. The acute phase refers to the first 10minutes after formalin injection and is believed to be driven mainly by peripheral signals and ascending pathway recruitment. The second phase (30-50 minutes after the hind paw injection) is supposed to implicate descending pain-modulating pathways as well. In this paradigm RGSz1KO female mice showed significantly higher nociceptive responses in the second phase of the test compared to their RGSz1WT control mice (Fig. 14i).

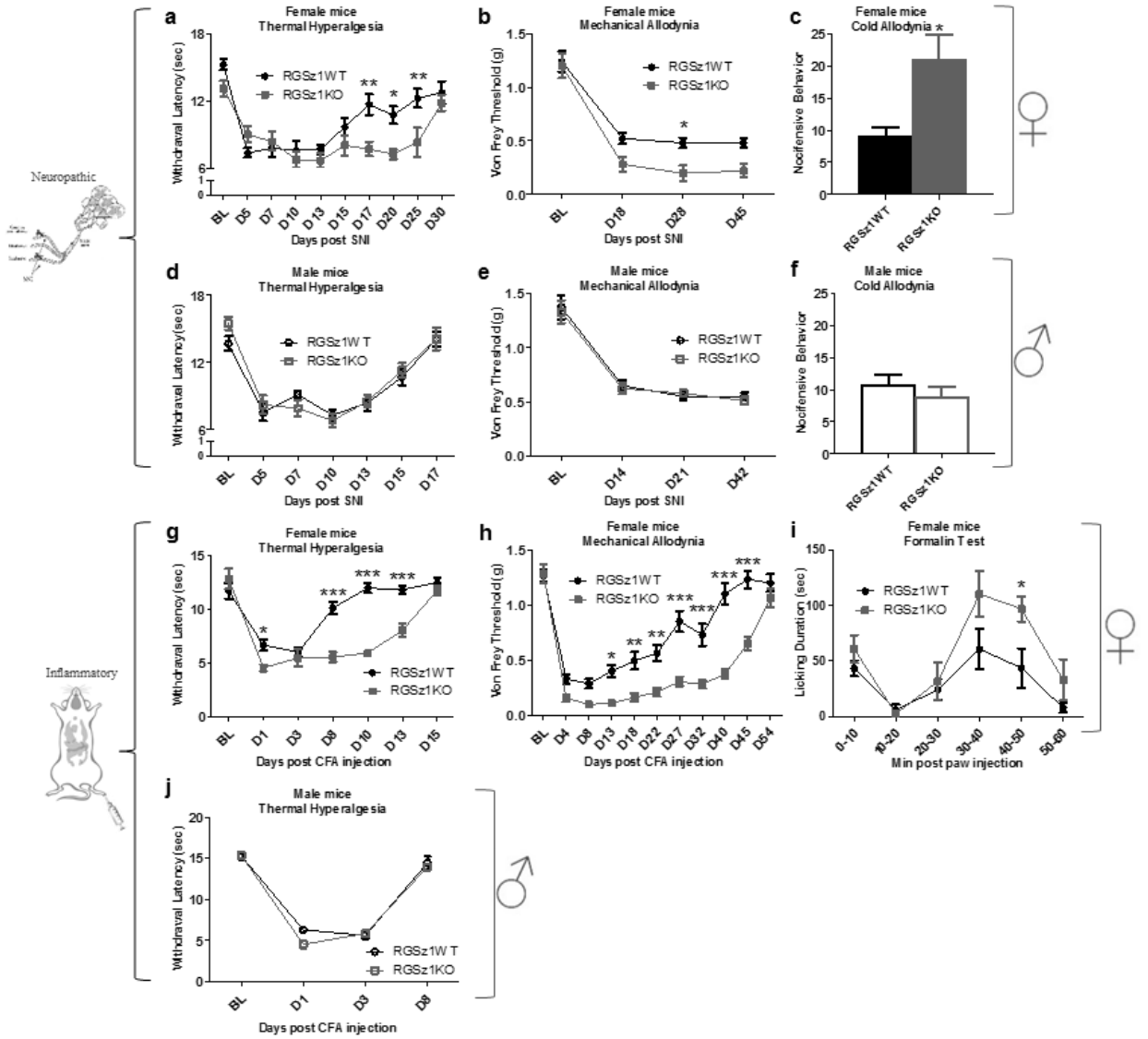


Figure 14. RGSz1 modulates sensitized responses in murine chronic pain models in a sex-specific manner. In the Spared Nerve Injury (SNI) model of neuropathic pain female RGSz1KO mice show **(a)** prolonged thermal hyperalgesia [n = 10/group; two-way repeated-measures ANOVA followed by Bonferroni's post hoc test, $F(9,162) = 12.34$; *p<0.05, **p<0.01], **(b)** more intense mechanical allodynia [n = 4-5/group; two-way repeated-measures ANOVA followed by Bonferroni's post hoc test, $F(3,21) = 12.89$; *p<0.05], **(c)** as well as more intense cold allodynia [n = 10/group; t-test, $t(18) = 2.78$; *p<0.05]. When the same paradigm was used in male mice no significant difference between genotypes was observed **(d-f)** [thermal hyperalgesia: n = 9/per group; two-way repeated-measures ANOVA followed by Bonferroni's post hoc test, $F(6,96) = 0.46$; mechanical allodynia: n = 8-9/per group; two-way repeated-measures ANOVA followed by Bonferroni's post hoc test, $F(3,45) = 0.17$; cold allodynia: n = 9/group; t-test, $t(16) = 0.76$; p>0.05 for all three behaviors tested]. Using the Complete Freund's Adjuvant (CFA) model of inflammatory pain a similar phenotype was revealed with RGSz1KO female mice showing **(g)** prolonged thermal hyperalgesia [n = 9-11/per group; two-way repeated-measures ANOVA followed by Bonferroni's post hoc test, $F(6,108) = 88.82$; *p<0.05, ***p<0.001] and **(h)** more intense and prolonged mechanical allodynia [n = 12/per group; two-way repeated-measures ANOVA followed by Bonferroni's post hoc test, $F(10,220) = 57.87$; *p<0.05, **p<0.01,***p<0.001], whereas **(j)** RGSz1KO male mice have no significant difference compared to their RGSz1WT controls [n = 9-13/per group; two-way repeated-measures ANOVA followed by Bonferroni's post hoc test, $F(3,60) = 1.35$; p>0.05]. Furthermore, **(i)** in the formalin test of inflammatory pain female RGSz1KO mice show higher nociceptive responses in the second/chronic phase [n = 6/per group; two-way repeated-measures ANOVA followed by Bonferroni's post hoc test, $F(5,50) = 8$; *p<0.05].

The effect of RGSz1 on CFA-induced thermal hyperalgesia is mediated through neurons in the vlPAG of female mice.

Real-time PCR analysis of PAG tissue derived from mice undergoing CFA-induced peripheral inflammation revealed a female-specific upregulation of RGSz1 mRNA three days after CFA injection (Fig. 15a and b). Consistently, higher protein levels of RGSz1 were also detected at the same timepoint using western blot (Fig. 15c). Taking in account the prolonged sensitized responses observed in RGSz1KO female mice, we hypothesized that RGSz1 actions in the PAG contribute to this sensory hypersensitivity phenotype observed. By the use of stereotaxic surgery, we injected a Cre recombinase expressing virus (AAV2-CMV-CRE-EGFP) or a GFP expressing control virus (AAV2-CMV-EGFP) in the vlPAG of RGSz1fl/fl female mice, waited 2 weeks for the mice to recover and for the virus to reach each maximal expression and then subjected the mice in the CFA model of inflammatory pain. Cre-injected female mice showed prolonged thermal hyperalgesia, a phenotype similar to that observed with global RGSz1KO mice (Fig. 15d). Viral distribution was examined by fluorescent microscopy confirming infection of vlPAG from rostral till caudal sections (Fig. 15e) and the ability of AAV2 serotype to infect only neuronal populations, as previously observed (Taymans et al., 2007), was further verified by immunostaining for neuronal (NeuN), astrocytic (GFAP) and microglial (Iba1) markers (Fig. 15f).

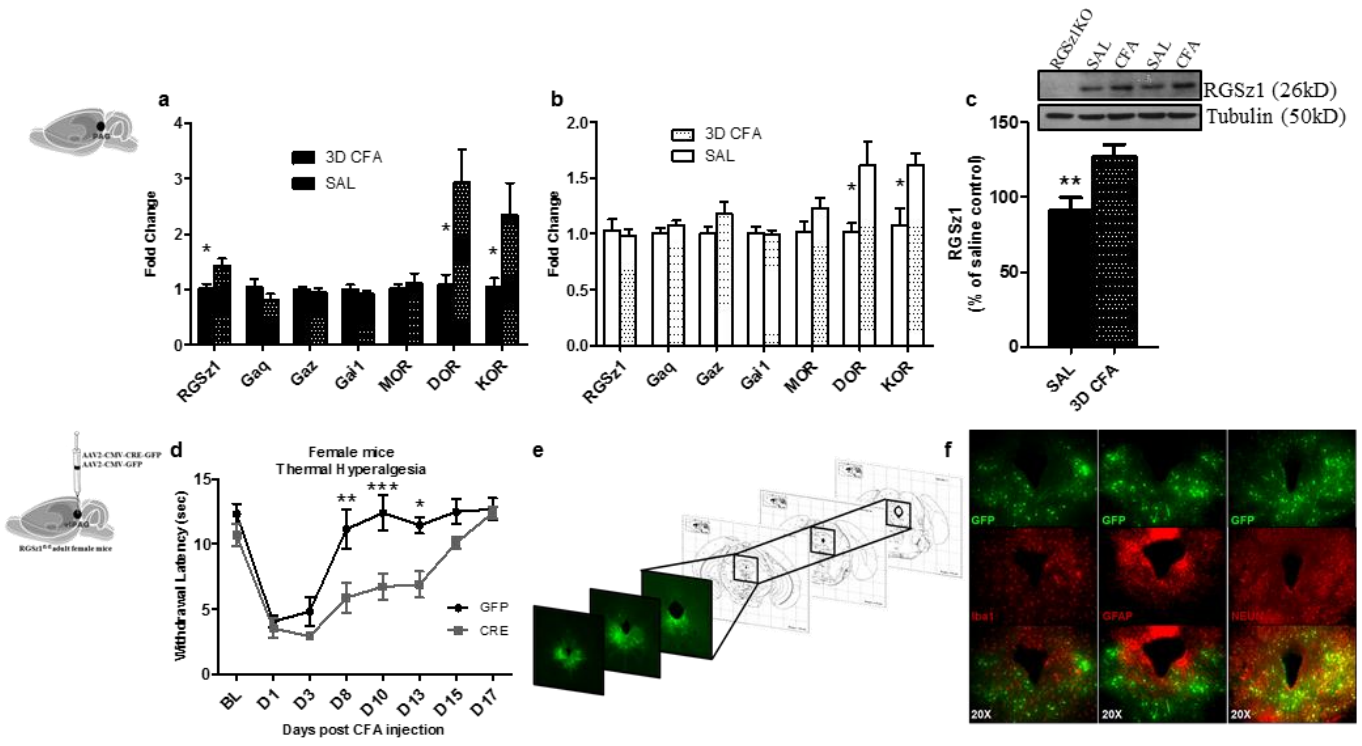


Figure 15. A critical role for RGSz1 in the female PAG under inflammatory pain conditions. (a) RGSz1 mRNA levels are upregulated in the PAG of female mice three days upon inflammatory pain induction [n = 4–6/group; t tests; RGSz1: t(10) = 2.85, Gαq: t(8) = 1.26, Gαz: t(8) = 0.56, Gαi1: t(8) = 0.93, MOR: t(10) = 0.52, DOR: t(10) = 3, KOR: t(10) = 2.16; *p<0.05], **(b)** an adaptation not observed in the PAG of male mice [n = 6-7/group; t tests; RGSz1: t(12) = 0.41, Gαq: t(9) = 1, Gαz: t(9) = 1.53, Gαi1: t(9) = 1.12, MOR: t(12) = 1.56, DOR: t(12) = 2.65, KOR: t(12) = 2.85; *p<0.05]. **(c)** RGSz1 upregulation in the female PAG three days upon inflammatory pain induction can also be detected at the protein level using western blot [n = 9/per group; t test; t(16) = 3.47; **p<0.01]. **(d)** Conditional knockdown of Rgsz1 in neurons in the vlPAG of RGSz1fl/fl female mice by stereotaxic infection of AAV2-CMV-CRE vectors lead to prolonged thermal hyperalgesia [n = 5-7/group; two-way repeated-measures ANOVA followed by Bonferroni's post hoc test, F(28,120) = 11.42; *p<0.05, **p<0.01, ***p<0.001]. **(e)** Verification of AAV2 viral vector distribution in the PAG. **(f)** AAV2 vectors infect neurons (as indicated by NeuN staining) and not astrocytes (as indicated by GFAP staining) or microglia (as indicated by Iba1 staining).

Transcriptomic analysis reveals sex-specific adaptations in serotonin receptor signaling in the PAG of RGSz1KO female mice.

Since the conditional knock-down experimental approach was suggesting a crucial role of RGSz1 in the vlPAG under inflammatory pain-like states, we decided to follow an unbiased whole transcriptome analysis in order to identify gene expression adaptations in the PAG of mice under CFA-induced peripheral inflammation and to determine if these adaptations are influenced by knock-out of RGSz1. As shown in figure 16a, RGSz1KO and RGSz1WT female mice were injected with CFA, thermal hyperalgesia was evaluated using the Hargrave's test on days 1,3,5,7 and 10 post CFA injection, and on day 10 (a timepoint that RGSz1WT mice have recovered from thermal hyperalgesia but RGSz1KO mice still show low thresholds; Fig. 14g) PAG tissue was collected 2 hours after the behavioral assessment. We also included PAG from naïve RGSz1WT and RGSz1KO female mice in order to detect baseline differences in gene expression, as well as PAG from naïve RGSz1WT and RGSz1KO male mice, in order to assess sex-differences in baseline gene expression patterns. Samples were subjected to next generation RNA-seq and results were analyzed as pairwise comparisons leading to four distinct lists of genes: the effect of inflammatory pain in RGSz1WT mice (consisting of genes differentially expressed in RGSz1WT mice 10 days after CFA compared to RGSz1WT naïve mice), the effect of inflammatory pain in RGSz1KO mice (consisting of genes differentially expressed in RGSz1KO mice 10 days after CFA compared to RGSz1KO naïve mice), the effect of *Rgsz1* gene ablation in female mice (consisting of genes differentially expressed in naïve RGSz1KO female mice compared to naïve RGSz1WT female mice) and the effect of *Rgsz1* gene ablation in male mice (consisting of genes differentially expressed in naïve RGSz1KO male mice compared to naïve RGSz1WT male mice). A heatmap representation was used to visualize the gene expression patterns of all four comparisons (Fig. 16b) revealing two important observations. First, knocking-out *Rgsz1* does not have the same impact on gene expression in the PAG of male and female mice. Furthermore, the pattern triggered in the female PAG is resembling the gene expression changes occurring in the PAG of RGSz1WT mice 10 days after the induction of peripheral inflammation. In order to get insight into specific genes and pathways affected in each case, we subjected all four gene lists to canonical pathway analysis by IPA (Fig. 16c). The most significantly affected pathway appeared to be serotonin receptor signaling, with transcripts associated with serotonin production and release expressed in lower levels in the PAG of RGSz1KO female mice, as well as RGSz1WT female mice under inflammatory pain, compared to RGSz1WT naïve female samples (Fig. 16d). Using RT-PCR we indeed validated in separate cohorts of animals these adaptations (Fig. 16e and f) and the fact that they are not present in the PAG of RGSz1KO male mice (Fig. 16g). Since

female mice lacking RGSz1 expression only in vlPAG neurons show a similar phenotype to that of the global RGSz1KO female mice, we also wanted to examine if this conditional deletion in adulthood has any impact on the expression of the serotonin related genes affected in the case of global deletion. Adult female RGSz1fl/fl mice were injected in vlPAG with AAV2-CMV-CRE-EGFP virus or the control AAV2-CMV-EGFP virus, left 2 weeks to recover and for the virus to reach its maximal expression levels, and then PAG was collected and analyzed using RT-PCR. As shown in figure 17h, just knocking-out *Rgsz1* in neurons in the vlPAG of female mice for a period of 2 weeks is enough to trigger a downregulation of enzymes crucial for serotonin production (Tph2, Gch1, Ddc) as well as the serotonin transporter Slc6a4. Given that RGSz1 is a molecule known to act in high proximity to the plasma membrane regulating GPCR signaling, and therefore it is not expected to directly mediate transcriptional changes, we conducted an upstream regulator analysis using IPA in order to identify candidates for the transcriptional control of the affected serotonin related genes (Fig. 16i). Among the most promising hits was β-estradiol, with all the target genes included in the analysis containing ER-binding motifs in their promoters as assessed through in silico transcription factor binding motif analysis (Fig. 16j). Interestingly, the *Rgs20* genomic locus (giving rise to RGSz1 protein) also appears to carry ER-binding motifs.

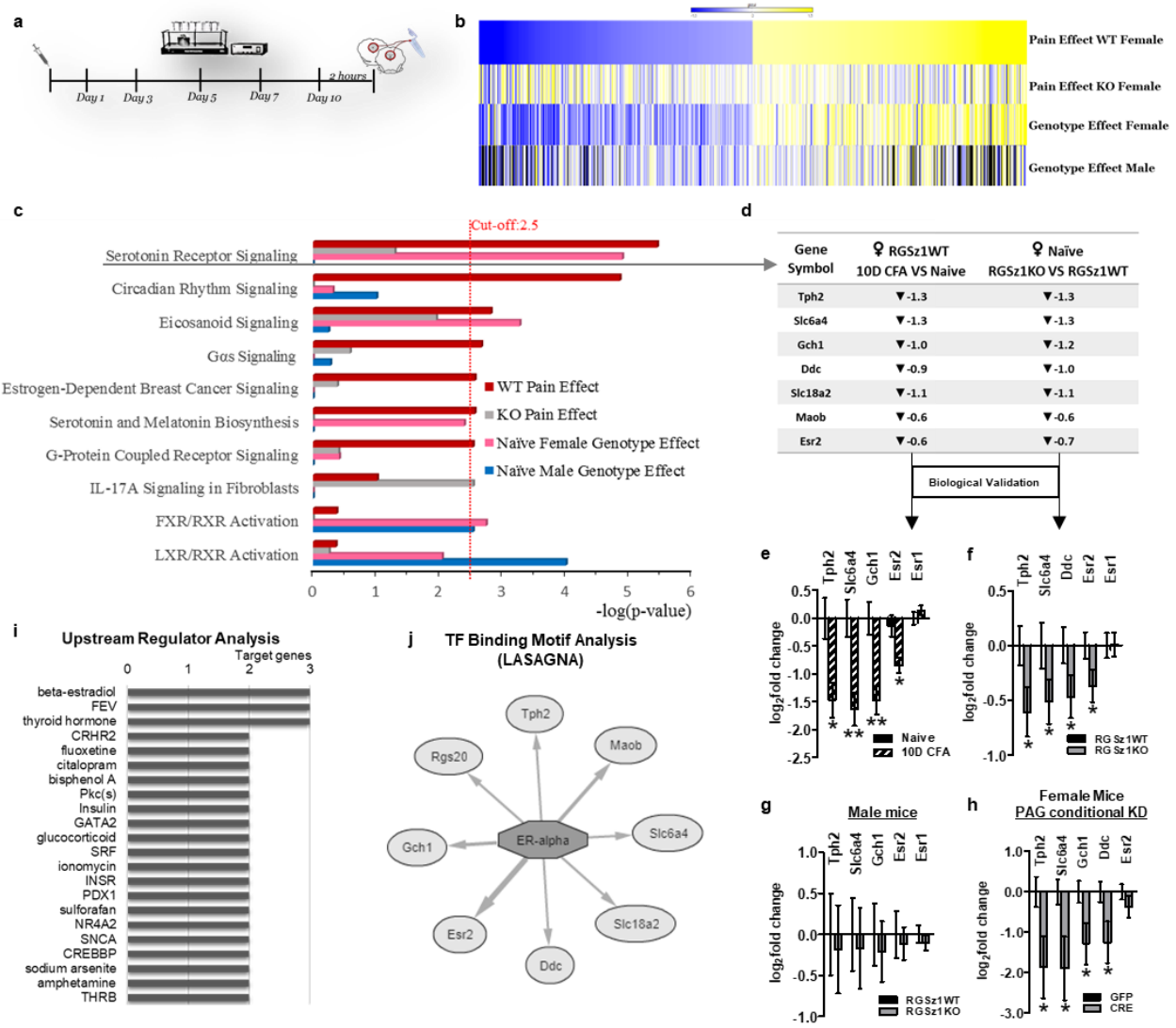
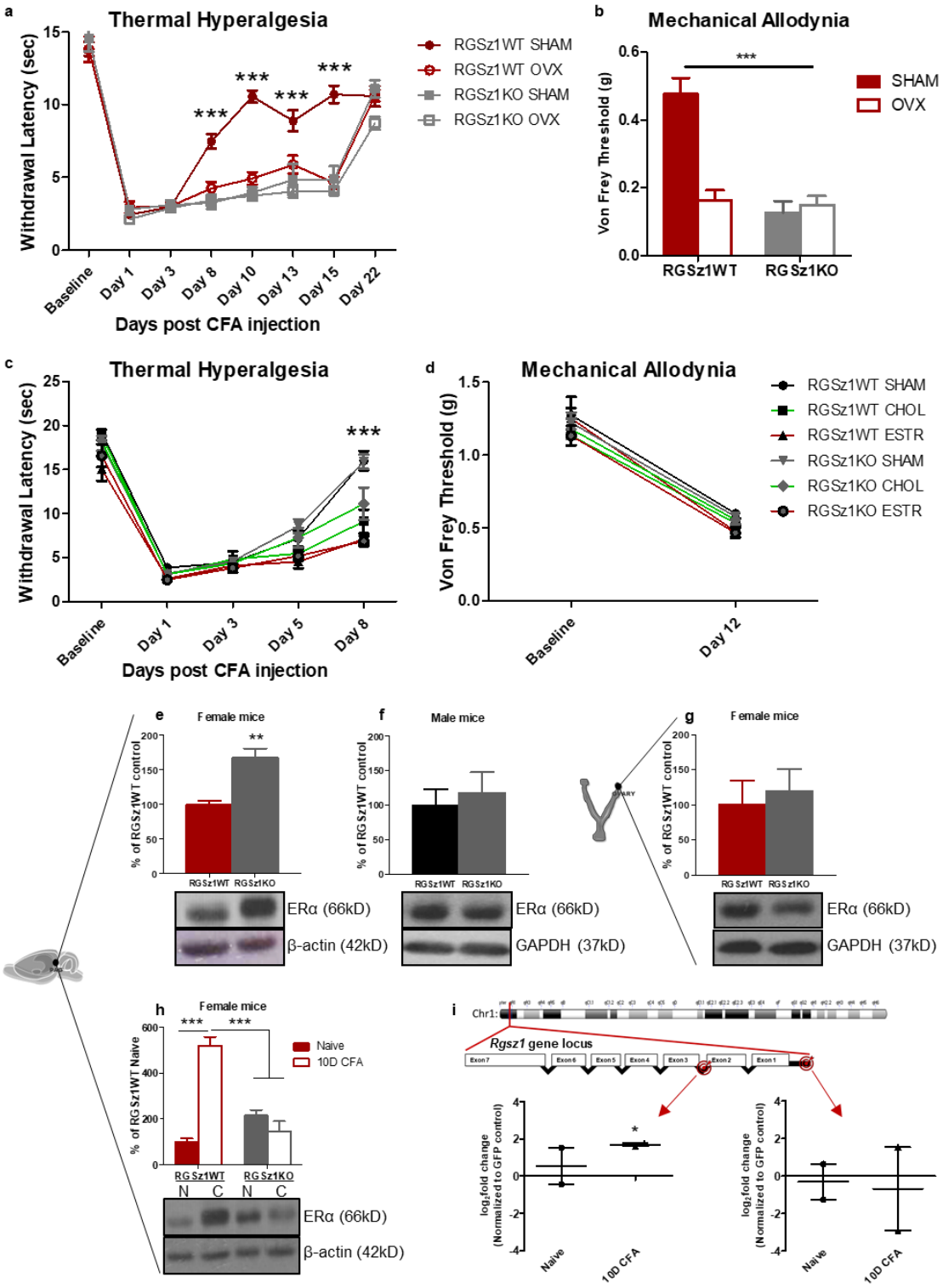


Figure 16. RNA-Seq of PAG tissue from RGSz1WT and RGSz1KO female mice undergoing inflammatory pain. (a) Experimental timeline showing that mice were injected with CFA and monitored for thermal hyperalgesia over a period of 10 days. On day 10 (when RGSz1WT mice have recovered from thermal hyperalgesia) PAG tissue was collected 2 h after behavioral assessment. **(b)** Heatmap analysis showing the overall pattern of gene expression among the four different comparisons. **(c)** IPA canonical pathway analysis revealed the serotonin receptor signaling pathway as the most affected in the case of RGSz1WT mice 10 days after CFA injection and in naïve RGSz1KO female mice. **(d)** Genes belonging to serotonin receptor signaling and the direction of their regulation. **(e-f)** qPCR validation of the above mentioned genes in separate cohorts of animals [RGSz1WT mice Naïve vs 10days CFA: n = 6-8/per group; t tests; Tph2: t(11) = 2.59, Slc6a4: t(13) = 3.6, Gch1: t(13) = 3.83, Esr2: t(13) = 3.12, Esr1: t(14) = 0.95; *p<0.05, **p<0.01. Naïve female mice RGSz1WT vs RGSz1KO: n = 8-14/per group; t tests; Tph2: t(24) = 2, Slc6a4: t(25) = 1.77, Ddc: t(25) = 1.82, Esr2: t(24) = 1.88, Esr1: t(14) = 0.05; *p<0.05]. **(g)** The same genes appear unaffected in the PAG of RGSz1KO male mice [n = 8/per group; t tests; Tph2: t(14) = 0.25, Slc6a4: t(14) = 0.25, Gch1: t(14) = 0.39, Esr2: t(14) = 0.32, Esr1: t(14) = 0.72; p>0.05 for all genes tested]. **(h)** Conditional knock-down of RGSz1 in neurons in the vlPAG of female mice also lead to downregulation of genes associated with serotonin production and release [n = 6-8/per group; t tests; Tph2: t(12) = 2.38, Slc6a4: t(12) = 2.46, Gch1: t(12) = 2.37, Ddc: t(12) = 2.37, Esr2: t(12) = 1.15; *p<0.05]. **(i)** Upstream regulator analysis for the genes depicted in table d. β-estradiol appears among the most prominent upstream regulators and indeed **(j)** transcription factor binding motif analysis suggests potential binding of ERα in the promoter regions of the above stated genes as well as on the promoter region of RGS20/RGSz1.

Evidence for implication of ERα mediated signaling in the phenotype observed in RGSz1KO female mice.

To further test the hypothesis of estrogen as the upstream regulator, we decided to manipulate its levels in RGSz1KO mice and their RGSz1WT controls and test if it has any effect on sensitized behaviors under CFA-induced peripheral inflammation. Adult RGSz1KO and RGSz1WT female mice were ovariectomized, given a 2-week recovery period and then subjected to the CFA model of inflammatory pain. Without circulating estrogen RGSz1WT mice exhibited prolonged thermal hyperalgesia (Fig. 17a) and more intense mechanical allodynia (Fig. 17b), similar to RGSz1KO female mice. Ovariectomy didn't affect sensitized responses of RGSz1KO female mice (Fig. 17a and b). In the same context, we also tested if estrogen supplementation of orchiectomized RGSz1WT and RGSz1KO mice can lead to sensory hypersensitivity. Interestingly, although the lack of testosterone led to a lower threshold of thermal hyperalgesia 8 days after CFA injection, no difference was detected between mice supplemented with β-estradiol implants compared to the cholesterol supplemented controls (Fig. 17c). In terms of mechanical allodynia, all six groups exhibited indistinguishable behavior (Fig. 17d).

Since, estrogen was confirmed behaviorally to affect sensitized responses of female mice under CFA-induced inflammatory pain, we also examined levels and regulation of its main target: estrogen receptor alpha (ERα). Protein levels of ERα appear higher in the PAG of RGSz1KO female mice, as assessed using western blot (Fig. 17e). Importantly, this is not the case in the PAG of RGSz1KO male mice (Fig. 17f) or the ovaries of RGSz1KO female mice (Fig. 17g). Furthermore, there is an upregulation of ERα in the PAG of female mice 10 days after CFA injection, an adaptation prevented in RGSz1KO mice (Fig. 17h). Finally, using ChIP assays we detected binding of ERα at the same timepoint on RGSz1 promoter (Fig. 17i). The *Rgs20* gene locus, predicted as a possible target of ERα (Fig. 16j), contains two distinct promoter regions: one located before exon 1 that gives rise to Ret-RGS and a second one before exon 3 that triggers the expression of RGSz1 (Barker et al., 2001). By designing specific RT-PCR primers for each of the two regions we detected ERα binding only at the second promoter 10 days after CFA injection.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Thermal Hyperalgesia
Withdrawal Latency (sec)
Days post CFA injection
Baseline
Day 1
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RGSz1WT SHAM
RGSz1WT OVX
RGSz1KO SHAM
RGSz1KO OVX
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Mechanical Allodynia
Von Frey Threshold (g)
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RGSz1WT
RGSz1KO
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Thermal Hyperalgesia
Withdrawal Latency (sec)
Days post CFA injection
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Day 3
Day 5
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Mechanical Allodynia
Von Frey Threshold (g)
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β-actin (42kD)
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Male mice
GAPDH (37kD)
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Exon2
Exon1
log2fold change (Normalized to GFP control)

Figure 17. Evidence for implication of ERα mediated signaling in the phenotype observed in RGSz1KO female mice. (a) Ovariectomized (OVX) RGSz1WT mice show prolonged hyperalgesic responses under CFA induced inflammatory pain, a phenotype similar to that of RGSz1KO mice [n = 10-12/per group; two-way repeated-measures ANOVA followed by Bonferroni's post hoc test, $F(21,266) = 33$; ***p<0.001], **(b)** as well as lower mechanical allodynia thresholds [n = 10-12/per group; two-way repeated-measures ANOVA followed by Bonferroni's post hoc test, $F(1,38) = 27.62$; ***p<0.001]. **(c)** Orchiectomized mice supplemented with β-estradiol (ESTR) or cholesterol control (CHOL) subcutaneous implants show overall lower thermal hyperalgesia thresholds 8 days after CFA injection compared to their SHAM operated controls with no difference between RGSz1WT and RGSz1KO mice [n = 8-9/per group; two-way repeated-measures ANOVA followed by Bonferroni's post hoc test, $F(20,184) = 13.34$; ***p<0.001]. **(d)** Mechanical allodynia appears equal between groups [n = 8-9/per group; two-way repeated-measures ANOVA followed by Bonferroni's post hoc test, $F(5,46) = 1.25$]. Protein levels of ERα are higher in **(e)** the PAG of RGSz1KO female mice [n = 5-6/per group; t test; $t(9) = 4.42$; **p<0.01], but not **(f)** in the PAG of RGSz1KO male mice [n = 6/per group; t test; $t(10) = 0.47$] or **(g)** in the ovaries of RGSz1KO female mice [n = 6/per group; t test; $t(10) = 0.43$]. **(h)** Ten days after CFA injection ERα is upregulated in the PAG of female mice and this adaptation is prevented in the case of RGSz1KO female mice [n = 3/per group; two-way ANOVA followed by Bonferroni's post hoc test, $F(1,8) = 30.32$; ***p<0.001]. **(i)** ChIP studies reveal binding of ERα on the *Rgsz1* genomic locus ten days after CFA injection [n = 2 samples/per group (each sample consists of pooled PAG tissue from 5 mice); one-sample t test; gene body: naïve: $t(1) = 0.55$, 10D CFA: $t(1) = 17.48$; promoter region: naïve: $t(1) = 0.32$, 10D CFA: $t(1) = 0.31$; *p<0.05]

Discussion

I. RGS9-2 Modulates Responses to Oxycodone in Pain-Free and Chronic Pain States

Our findings from the first aim of this thesis provide new information on the intracellular pathways involved in behavioral responses to oxycodone, in pain-free as well as in neuropathic pain states. RGS9-2 is a key modulator of drug addiction, and has been shown to have a dynamic role in the actions of several drug of abuse, including cocaine, amphetamine, and morphine (Traynor et al., 2009). Our data reveal that although oxycodone has rewarding, locomotor activating, and analgesic effects that are similar to morphine, RGS9-2 has distinct modulatory roles in the actions of these two MOR-targeting opioids. RGS9-2 promotes the rewarding and locomotor sensitizing effects of oxycodone, and opposes the development of analgesic tolerance. This is the opposite of what we observed in previous studies using morphine, where we found that RGS9-2 opposes the development of morphine reward, and promotes the development of analgesic tolerance (Gaspari et al., 2014; Zachariou et al., 2003). Thus, RGS9-2 appears to uniquely modulate morphine, as analgesic responses to oxycodone, fentanyl, and methadone are positively modulated by RGS9-2 (Psifogeorgou et al., 2011). The distinct phenotypes observed with morphine vs oxycodone may lie in different intracellular mechanisms by which these compounds exert their effects; for example, recruitment of different Gα subunits (Psifogeorgou et al., 2011), or different effectors activated by each drug, although differences owing to receptor specificity or receptor dimer formation should also be considered. We speculate that similar to fentanyl and methadone, oxycodone in the striatum promotes short-term complexes between RGS9-2, MOR, and Gαq subunits, whereas morphine promotes stable complexes between RGS9-2-Gβ5, MOR, and Gαi subunits (Psifogeorgou et al., 2011). Therefore, genetic inactivation of Rgs9 will differentially affect cellular and behavioral responses to oxycodone and morphine. RGS9-2 is very abundant in the striatum, but it may also affect MOR function in the spinal cord (Terzi et al., 2009) and other supraspinal sites expressing MOR, including the periaqueductal gray (PAG) (Garzón et al., 2005). Moreover, the actions of MOR may be mediated in a cell type- or region-specific level by other members of the RGS family, including RGS4 and RGSz proteins (Garzón et al., 2005; Gold et al., 2003). A large number of in vitro and in vivo studies have provided information on the mechanisms underlying the acute and chronic actions of MOR agonists (Koch et al., 2005; Muller and Unterwald, 2004; Pradhan et al., 2006; Quillinan et al., 2011; Raehal and Bohn, 2005; Walwyn et al., 2010). The majority of these studies have focused on morphine and heroin, and

have provided insight into signal-transduction events associated with addiction or the development of analgesic tolerance. Although oxycodone is highly prescribed for the treatment of acute and chronic pain conditions, there is very limited information on the cellular events triggered by repeated oxycodone administration, and the G-protein complexes that modulate oxycodone actions in specific brain regions or cellular populations modulating addiction and analgesia. Several recent reports have used models of reward and drug self-administration to study the actions of oxycodone in the adolescent and adult life (Emery et al., 2015; Niikura et al., 2013; Sanchez et al., 2016; Zhang et al., 2015) and in male vs female groups of animals (Collins et al., 2016). These studies show that exposure to oxycodone in early life affects the sensitivity of opiate analgesia and addiction-related actions of MOR agonists in the adult life. Our studies show that prevention of RGS9-2 activity reduces the rewarding effects of oxycodone, pointing to a potent role of this molecule in addiction-related mechanisms. Recent studies by Emery et al (2015) show that oxycodone and morphine differentially affect responses of dopamine D2/D3 receptors and Akt signaling. Further understanding of the long-term adaptation oxycodone treatment promotes in the reward pathway, and the exact signal-transduction events triggered by oxycodone, will help design better treatment strategies for pain management and identify factors that contribute to oxycodone abuse vulnerability. Chronic pain-induced plasticity in addiction- and analgesia-related brain networks may affect sensitivity to opiate analgesics (Mitsi and Zachariou, 2016; Porreca and Navratilova, 2017; Taylor et al., 2015). Our studies examined the role of the striatal-enriched RGS9-2 in oxycodone actions under neuropathic pain states. Our findings from mice tested at 2 weeks after SNI reveal that neuropathic pain states reduce oxycodone reward sensitivity in both RGS9WT and RGS9KO animals, consistent with the hypothesis that chronic pain states impact the brain reward network (Mitsi and Zachariou, 2016). Decreased reward sensitivity under chronic pain states has been reported by several other groups (Ewan and Martin, 2011; Navratilova et al., 2012; S. Ozaki et al., 2002; Ozaki et al., 2003; Wu et al., 2014), but this is the first study to examine oxycodone reward in the context of chronic pain. Notably, reports also show that long-term inflammatory pain may promote heroin self-administration (Hipólito et al., 2015) and therefore it is important to assess the impact of inflammatory and neuropathic pain in oxycodone self-administration and other addiction-related paradigms. Future studies should monitor reward sensitivity to several pain killers at later time points after nerve injury, when depression symptoms are also present, to understand how long-term pain states affect the function of the reward pathway. In addition to G-protein complexes, neuroimmune and other adaptations affect the function of MOR and other receptors in the brain reward center under chronic pain states, and contribute to the decreased reward sensitivity observed in the CPP paradigm. For example, recent

studies showed that in the rat PAG, MOR may function differently in pain-free vs chronic pain states (Eidson and Murphy, 2013; Mehalick et al., 2013) and have demonstrated the potent role of Toll-like receptors in modulating morphine tolerance in models of acute and long-term inflammatory pain. Our data from SNI groups of mice also support a protective modulatory role of RGS9-2 in oxycodone tolerance: in accord with our findings from experiments using the hot-plate assay, KO of the Rgs9 gene does not affect the acute action of oxycodone on mechanical allodynia following SNI. However, when the drug is administered for consecutive days, the antiallodynic response is abolished in RGS9KO mice after 12 days of treatment, reflecting the development of tolerance. Therefore, RGS9-2 complexes protect against the development of analgesic tolerance to oxycodone in models of acute and chronic pain. RGS9-2 has a prominent role in the modulation of GPCR responses in the striatum, where it is expressed in very high levels, but it is absent or expressed in low amounts in other brain regions expressing MORs (Traynor et al., 2009). We suggest that the phenotypes observed are due to loss of RGS9-2 function primarily in the striatum, and that other members of the RGS family, including RGS7, RGS4, and RGS20 may control MOR function in other brain regions associated with addiction or analgesia, including the locus coeruleus and the PAG (Garzón et al., 2005; Gold et al., 2003; Han et al., 2010). Future work should investigate the signal-transduction and gene expression changes induced by oxycodone exposure in the brain reward center, and the cellular and molecular determinants of oxycodone addiction. While the present study focuses on groups of male mice, future work should further determine the role of RGS9-2 in behavioral responses to oxycodone and other pain killers in male and female animals. Our findings that RGS9-2 modulates the actions of oxycodone in a manner distinct from its modulation of morphine support the notion that although synthetic opioid analgesics produce similar physiological responses, they act via distinct intracellular mechanisms. This knowledge may lead to the development of more efficacious and less addictive compounds and will help developing better strategies for the management of chronic pain.

II. Suppression of RGSz1 function optimizes the actions of opioid analgesics by mechanisms that involve the Wnt/β-catenin pathway

Aim II provides new evidence for a brain region-specific mechanism that modulates functional responses of MOPRs. We show that constitutive deletion of Rgsz1 increases analgesic responses to morphine, methadone, and fentanyl and delays the development of analgesic tolerance in pain-free and pain-like states. Importantly, the rewarding effects of opioids were suppressed after the deletion of Rgsz1. These features indicate that RGSz1 is a promising target for the development of adjunct medications to enhance the efficacy of typical opioid analgesics and lower their abuse potential. Our data also indicate that the modulatory roles of RGSz1 in morphine analgesia and tolerance are the same in both male and female mice, further supporting the value of this molecule as a potent new target for pain management.

By controlling GPCR activity, RGS proteins may dynamically affect the function of ion channels, signal transduction cascades, epigenetic modifiers, and transcription factors (Gerber et al., 2016; Kimple et al., 2011; Sjögren et al., 2010; Terzi et al., 2009). Several members of the RGS family have been shown to modulate the actions of MOPR agonists in vitro and in vivo (Gaspari et al., 2014; Han et al., 2010; Psifogeorgou et al., 2011; Sutton et al., 2016; Zachariou et al., 2003). In our previous studies, we found that RGS9-2 negatively modulates both morphine analgesia and reward-related behaviors (Gaspari et al., 2014; Zachariou et al., 2003). Therefore, improving analgesic effects by inhibition of RGS9-2 would also likely increase the rewarding and locomotor effects of the drug and promote physical dependence. In addition, RGS9-2 has a ligand-dependent role on MOPR function, as it positively modulates the function of MOPRs by fentanyl and oxycodone (Psifogeorgou et al., 2011). Another RGS protein, RGS7, similarly modulates both reward and analgesia-related behaviors (Sutton et al., 2016). The actions of RGS4 in the NAc have a less potent but significant effect on morphine reward, and in the locus coeruleus, they modulate physical dependence but this molecule does not affect analgesia or tolerance to morphine (Han et al., 2010). Although RGS4 and RGS7, are also expressed at moderate to high levels in the PAG (Gold et al., 1997), RGSz1 is unique in its differential modulation of opioid reward and analgesia.

Our study focused on understanding the mechanism by which RGSz1 modulates opioid analgesia and tolerance. Using conditional knockdown approaches, we demonstrate that the development of analgesic tolerance is fostered by RGSz1 in the PAG, a key area involved in the descending control of pain and morphine-induced analgesia (Reichling et al., 1988). Importantly, the actions

of RGSz1 in the PAG do not affect the analgesic efficacy of morphine, or the development of physical dependence. A variety of studies document adaptations in this brain area in models of analgesic tolerance (Bagley et al., 2005; Bobeck et al., 2014; Lane et al., 2005; Macey et al., 2009). However, the detailed mechanisms that contribute to MOPR signal transduction and desensitization in the PAG are not fully understood. Our results indicate that RGSz1 is involved by forming complexes with Gαz and MOPR with chronic opioid use. These findings are in accordance with previous work by Hendry et al. suggesting that compromised Gαz action is associated with morphine tolerance (Hendry et al., 2000).

Furthermore, we applied next-generation RNA sequencing to determine the mechanism by which chronic morphine induces analgesic tolerance and the impact of RGSz1 in such adaptations. In agreement with previously reported data, our findings indicate that analgesic tolerance to morphine is linked to robust adaptations in genes associated with inflammatory cascades (Hutchinson et al., 2011; Johnston et al., 2004; Mélik Parsadaniantz et al., 2015; Watkins et al., 2007), consisting eight of the fifteen most significantly affected pathways identified by IPA. Notably, most of these adaptations were observed only in RGSz1WT mice, and therefore are likely to be linked to morphine tolerance. Furthermore, there was a robust up-regulation of genes associated with serotonin production and release in the PAG of RGSz1KO mice, highlighting the importance of the supraspinal serotonergic system in maintaining analgesic responses (Tao et al., 1998). We observed that several components of the Wnt/β-catenin signaling pathway were down-regulated in the RGSz1WT group at the time the mice become tolerant to morphine. Although there is no known direct link between Wnt/β-catenin signaling and opioid actions, earlier studies have shown that the inhibition of GSK3β, shown to promote the degradation of β-catenin (Clevers, 2006), delays the development of morphine tolerance (Liao et al., 2014; Parkitna et al., 2006). Furthermore, there is evidence for Wnt/β-catenin signaling in the morphological effects of long-term exposure to MOPR agonists, such as reduced dendritic arborization, neurite outgrowth, and neurogenesis in several brain regions (Eisch et al., 2000; Jin et al., 2010; Li et al., 2007; Reyes et al., 2012). Here, we confirm the involvement of this pathway by showing morphine tolerance can develop in RGSz1KO mice by directly antagonizing β-catenin actions in the vlPAG. However, β-catenin overexpression is not sufficient to prevent the development of tolerance in RGSz1WT mice, suggesting that additional factors control transcriptional activation under states of tolerance. We also demonstrated that this effect likely involves the dissociation of Gαz from Axin2 and the formation of complexes with RGSz1 under conditions of morphine tolerance. Our data suggest that Axin2 then translocates to the nucleus, where it may repress β-catenin-mediated transcription (Rennoll et al., 2014).

Wnt/β-catenin signaling is among the most conserved intracellular pathways, playing pivotal roles in cell proliferation, migration, and homeostasis (Clevers, 2006). Therefore, the pharmacological inhibition of Wnt components would broadly affect many tissues and cellular processes in the CNS and periphery. The identification of molecules such as RGSz1 that modulate Wnt/β-catenin signal transduction in a brain region-specific manner may provide a novel avenue for developing adjunct medications to MOPR agonists for the management of chronic pain.

Future work should continue to detail the actions of RGSz1 in the brain reward network and determine the key protein interactions and intercellular pathways by which RGSz1 promotes the rewarding effects of opioids. Overall, our findings provide novel information on the signal transduction networks mediating the effects of MOPR agonists in male and female mice and identify RGSz1 as a novel target for optimizing the analgesic actions of MOPR agonists.

III. A Role of RGSz1 Modulating Sensitized Responses in murine Chronic Pain models

Aim III provides new evidence on sex-dependent molecular mechanisms underlying chronic pain states. Using well-established murine chronic pain models combined with transgenic mouse lines we show that RGSz1 actions in the PAG of female mice are crucial for the intensity and duration of nociceptive responses. Although the contribution of GPCRs in pain processing and analgesia has been extensively studied over the years (Geppetti et al., 2015; Stone and Molliver, 2009), research on the role of RGS proteins has mainly focused on pain pharmacology (Mitsi et al., 2015; Papachatzaki et al., 2011; Psifogeorgou et al., 2011; Stratinaki et al., 2013; Terzi et al., 2012; Traynor, 2012; Zachariou et al., 2003), with only few cases examining their effect on pain pathophysiology per se (Bosier et al., 2015; Taccola et al., 2016; Terzi et al., 2014). Indeed, this is the first report of such a profound effect of an RGS protein on sensitized responses, ranging across several sensory modalities and pain paradigms. Interestingly, this effect appears to be female specific pointing towards the existence of sex-dependent molecular mechanisms involved in pain responses.

Sex differences in pain have been observed both in clinical studies as well as in animal models (Fillingim et al., 2009; Greenspan et al., 2007; Mogil, 2012), but the underlying biological mechanisms are poorly understood. Evidence suggests the involvement of descending modulation pathways, with rodent models of inflammatory pain revealing differences in the PAG-RVM monoaminergic circuit (Loyd et al., 2007; Loyd and Murphy, 2009) and human imaging studies identifying distinct PAG functional connectivity patterns (Atalla et al., 2015; Linnman et al., 2012). In fact, our conditional knock-down approach together with our next generation sequencing results point towards the same direction, highlighting the importance of PAG mediated signaling in female nociceptive responses and revealing that the exact same manipulation (in this case knocking-out RGSz1) can lead to distinct gene expression adaptations in the PAG of male and female mice.

Furthermore, pathway analysis of our sequencing results indicates an important role of serotonin signaling in the PAG of female mice under inflammatory pain conditions. Chronic CFA-induced inflammatory pain leads to a downregulation of genes implicated in serotonin production and release. Intriguingly, suppressing the actions of RGSz1 possess a comparable downregulation of serotonin-related genes in the PAG of female by not male mice. The effect of serotonin in peripheral as well as central pain mechanisms is widely studied (Bardin, 2011; Messing and Lytle, 1977; Sommer, 2004). Polymorphisms of genes encoding serotonin receptors, transporters and

enzymes have been associated with several chronic pain syndromes (Heddini et al., 2014; Treister et al., 2011) and serotonin reuptake inhibitors are among the medications prescribed for chronic pain conditions (Jung et al., 1997; Watson et al., 2011). Importantly, serotonin appears to be a sex-dimorphic system (Carlsson and Carlsson, 1988; Jovanovic et al., 2008; Yang et al., 2015), with evidence suggesting an underlying mechanism involving gonadal hormones (Amin et al., 2005; Bethea et al., 2002; Bethea and Reddy, 2012; Borrow and Cameron, 2014). Indeed, our upstream regulator analysis suggested a similar mechanism by proposing β-estradiol as the number one candidate responsible for the serotonin-related gene expression adaptations observed in the PAG of female mice.

In an attempt to further evaluate this hypothesis, we manipulated the levels of gonadal hormones by ovariectomizing adult mice and then monitoring their behavioral responses in the CFA model of inflammatory pain. Consistent with previous reports (Li et al., 2014; Sanoja and Cervero, 2005, 2008), we observed that ovariectomized mice experience higher levels of hyperalgesia and allodynia, a phenotype resembling the one observed in RGSz1KO female mice. A vast variety of literature supports pain modulation by gonadal hormones (Craft et al., 2004; Craft, 2007; Hassan et al., 2014; Maurer et al., 2016). Several polymorphism of the estrogen receptor genomic locus have been associated with chronic pain syndromes (Colson et al., 2004; Kang et al., 2007; Kim et al., 2010; Ribeiro-Dasilva et al., 2009), knock-out models for estrogen receptors exhibit pain-related phenotypes (Li et al., 2009; Spooner et al., 2007) and estrogen receptor agonists have been shown to possess antinociceptive properties (Cao et al., 2012; Gardell et al., 2008; Leventhal et al., 2006; Lu et al., 2013; Ma et al., 2016).

Despite all the evidence mentioned above and the fact that estrogen receptors exist in several key areas of the pain pathway, such as DRG, superficial laminae of the dorsal horn, midbrain and brainstem nuclei (Pfaff and Keiner, 1973; Takanami et al., 2010; Vanderhorst et al., 2005), to our knowledge there is no report so far examining local adaptations of their expression levels under chronic pain conditions. Our western blot analysis revealed upregulation of ERα in the PAG of female mice 10 days after CFA-induced peripheral inflammation, overlapping with the suppression of serotonin-related gene transcription identified by our RNA sequencing results. Interestingly, this correlation between higher ERα levels and lower transcription of serotonin-related genes was detected, as a baseline adaptation, in the PAG of RGSz1KO female mice too. These results together with the existence of ERE binding elements on the promoters of the downregulated genes suggest a suppression of serotonin production by ERα. A possible explanation could be the recruitment of distinct repressive ERα co-factors under chronic pain

conditions, since ER mediated transcription is highly dependent on co-factors and cellular environment (Hu and Lazar, 2000; McDonnell and Norris, 2002; Shang et al., 2000).

Overall, our results highlight the importance of including female mice in preclinical pain research, suggesting that malfunction of a single protein, in this case RGSz1, can trigger distinct molecular adaptations in female and male mice leading to different behavioral outcomes.

The interplay between estrogen and serotonin under chronic pain conditions could be part of the molecular mechanism underlying female vulnerability to chronic pain syndromes, and could also account for the comorbidity between chronic pain and depression. Given the importance of both estrogen and serotonin signaling in numerous organ systems throughout the body, identifying molecules that affect the signaling fate in restricted areas of the CNS, such as RGSz1 in PAG, would lead to more efficient and specialized therapies.

Future Directions

Overall, the results deriving from the present thesis highlight the importance of targeting novel intracellular molecules for improving opioid analgesics. In particular, RGS proteins represent a promising group of targets, modulating MOPR signaling in a delicate manner that appears to be site-, agonist- or even sex-dependent. This fine tuning upon MOPR activation does not only depend on their enzymatic activity and expression pattern, but also involves their ability to interact and exert influence on key signaling pathways, such as Wnt/β-catenin signaling and estrogen receptor 1-mediated signaling.

Future studies should focus on further characterizing the RGS interactome across several brain areas and the impact of disrupting this form of "molecular communication" on opiate actions. For example, our data suggest that after repeated morphine administration RGSz1 in the PAG indirectly, through its interaction with Gαz, can lead to inactivation of β-catenin mediated transcription promoting the development of analgesic tolerance. Is this mechanism conserved among other brain areas as well? And if so, could it also underlie the effect of RGSz1 in morphine reward? Answering questions like those could lead to the development of adjunct medications that target region-specific interactions in order to improve the analgesic actions of opioids while at the same time eliminate their undesirable side effects.

Furthermore, as research efforts move towards personalized medicine, future studies should focus on identifying polymorphisms that disrupt the actions of RGS proteins and therefore could influence responsiveness and abuse potential of opioid drugs. In fact, a recent study identified a significant correlation between two SNPs in the promoter of the *Rgs9* gene and heroin dependence (Zhu and Zhang, 2015). Our observations using knock-out mouse models suggest that a malfunction in RGS9 would possibly affect responses to oxycodone as well. Whereas, a malfunction of RGSz1 could lead to higher analgesic efficacy and delayed tolerance development upon repeated treatment with opioids. On the other hand, based on the sex-specific phenotypes observed with RGSz1 mutants, a polymorphism leading to malfunction of RGSz1 would possibly be associated with chronic pain syndromes in female subjects.

Combining these two approaches would provide valuable evidence on both the intracellular mechanisms underlying the role of RGS proteins in opiate actions as well as the clinical outcome associated with the disruption of such mechanisms, leading to new personalized medications that would further improve the treatment of severe chronic pain conditions.

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Abbreviations

AAV2: adeno-associated virus 2

AMPA receptor: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

APC: adenomatous polyposis coli

ATP: adenosine triphosphate

BDNF: brain-derived neurotrophic factor

CCI: chronic constriction injury

Ccr1: CC chemokine receptor 1

CFA: complete Freund's adjuvant

ChIP: chromatin immunoprecipitation

CHOL: cholesterol

CMV: cytomegalovirus

CNS: central nervous system

Co-IP: co-immunoprecipitation

CPP: conditioned place preference

CRE: causes recombination

CREB: cAMP response element binding protein

CREBBP: CREB-binding protein

CRHR2: corticotropin releasing hormone receptor 2

CRPS: complex regional pain syndrome

Cys: cysteine string

Ddc: dopa decarboxylase

DEP domain: dishevelled, EGL-10 and pleckstrin domain

DH domain: dbl homology domain

DIX domain: dishevelled and Axin domain

Dkk2: dickkopf WNT signaling pathway inhibitor 2

DNA: deoxyribonucleic acid

DRG: dorsal root ganglia

DS: dorsal striatum

DVL: dishevelled

ECL: enhanced chemiluminescence

EGFP: enhanced green fluorescent protein

ERE: estrogen response element

ERα: estrogen receptor alpha

ERβ: estrogen receptor beta

ESCI: excitotoxic spinal cord injury

Esr1: estrogen receptor 1

Esr2: estrogen receptor 2

ESTR: estrogen

FEV: fifth Ewing variant protein

fl/fl: floxed/floxed

FXR/RXR: farnesoid X receptor/retinoid X receptor

Fzd10: frizzled class receptor 10

Gabre: gamma-aminobutyric acid receptor subunit epsilon

GAP: GTPase-activating protein

GAPDH: glyceraldehyde-3-phosphate dehydrogenase

GATA2: GATA binding protein 2

Gch1: GTP cyclohydrolase 1

GDP: guanosine diphosphate

GEF: guanine nucleotide exchange factor

GFAP: glial fibrillary acidic protein

GFP: green fluorescent protein

GGL domain: G-protein gamma-like domain

GO: gene ontology

GPCR: G-protein coupled receptor

GPER1: G-protein coupled estrogen receptor 1

GRK: G-protein coupled receptor kinase

GSK3β: glycogen synthase kinase 3 beta

GTP: guanosine triphosphate

HCLB: hypotonic cellular lysis buffer

HIV: human immunodeficiency virus

HSV: herpes simplex virus

Htr1A: 5-hydroxytryptamine receptor 1A

Htr2B: 5-hydroxytryptamine receptor 2B

i.p.: intraperitoneal

IASP: international association for the study of pain

Iba1: ionized calcium binding adaptor molecule 1

IL-10: interleukin 10

IL-17A: interleukin 17A

Il1r1: interleukin 1 receptor 1

IP: immunoprecipitation

IPA: ingenuity pathway analysis

KD: knock-down

KO: knock-out

LEF/TCF-1: lymphoid enhancer-binding factor 1

LXR/RXR: liver X receptor/retinoid X receptor

Maob: monoamine oxidase b

mGlu receptor: metabotropic glutamate receptor

MIF: macrophage migration inhibitory factor

MOPR: mu opioid receptor

MPE: maximum possible effect

NAc: nucleus accumbens

NeuN: neuronal specific nuclear protein

NF-Kb: nuclear factor-Kb

Ngfr: nerve growth factor receptor

NMDA receptor: N-methyl-D-aspartate receptor

Nos2: nitric oxide synthase 2

Npy: neuropeptide y

NR4A2: nuclear receptor subfamily 4 group A member 2

NSAID: nonsteroidal anti-inflammatory drugs

OVX: ovariectomy

OXY: oxycodone

PAG: periaqueductal gray

PBS: phosphate-buffered saline

PCP: planar cell polarity protein

PCR: polymerase chain reaction

PDX domain: pancreatic and duodenal homeobox domain

Pdyn: prodynorphin

PFA: paraformaldehyde

PH domain: pleckstrin homology domain

PKA: protein kinase A

PKC: protein kinase C

PLC: phospholipase C

PNL: partial sciatic nerve ligation

PP2A: protein phosphatase 2

Psd95: postsynaptic density protein 95

PTB: phosphotyrosine-binding domain

PX: phosphoinositide-bindind domain

qPCR: quantitative polymerase chain reaction

RBD: Ras-binding domain

RGS: regulator of G-protein signaling

RIPA buffer: radioimmunoprecipitation assay buffer

RNA: ribonucleic acid

RNA-seq: ribonucleic acid sequencing

RT-PCR: real time polymerase chain reaction

RVM: rostral ventral medulla

s.c.: subcutaneous

SAL: saline

SDS: sodium dodecyl sulfate

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

Slc18a2: solute carrier family 18 member A2

Slc6a4: solute carrier family 6 member A4

SNCA: alpha-synuclein

SNI: spared nerve injury

SNL: segmental spinal nerve ligation

Sox2: sex determining region Y box 2

SPA: stimulation-produced analgesia

SRF: serum response factor

SSRI: selective serotonin reuptake inhibitor

TCA: tricyclic antidepressant

THRB: thyroid hormone receptor B

TL: total lysate

TM domain: transmembrane domain

Tph2: tryptophan hydroxylase 2

TRP channel: transient receptor potential channel

TWEAK: TNF related weak inducer of apoptosis

vlPAG: ventrolateral periaqueductal gray

WB: Western blot

WDS: wet dog shake

WHO: world health organization

WT: wild type

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RGS9-2 Modulates Responses to Oxycodone in Pain-Free and Chronic Pain States

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Regulator of G-protein signaling 9-2 (RGS9-2) is a striatal-enriched signal-transduction modulator known to have a critical role in the development of addiction-related behaviors following exposure to psychostimulants or opioids. RGS9-2 controls the function of several G-protein-coupled receptors, including dopamine receptor and mu opioid receptor (MOR). We previously showed that RGS9-2 complexes negatively control morphine analgesia, and promote the development of morphine tolerance. In contrast, RGS9-2 positively modulates the actions of other opioid analgesics, such as fentanyl and methadone. Here we investigate the role of RGS9-2 in regulating responses to oxycodone, an MOR agonist prescribed for the treatment of severe pain conditions that has addictive properties. Using mice lacking the *Rgs9* gene (RGS9KO), we demonstrate that RGS9-2 positively regulates the rewarding effects of oxycodone in pain-free states, and in a model of neuropathic pain. Furthermore, although RGS9-2 does not affect the analgesic efficacy of oxycodone or the expression of physical withdrawal, it opposes the development of oxycodone tolerance, in both acute pain and chronic neuropathic pain models. Taken together, these data provide new information on the signal-transduction mechanisms that modulate the rewarding and analgesic actions of oxycodone.

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INTRODUCTION

Synthetic and semisynthetic opioids, including fentanyl, hydromorphine, and oxycodone are prescribed for the treatment of severe acute or chronic pain conditions (Carise *et al*, 2007; Ling *et al*, 2011; Hermanns *et al*, 2012). Oxycodone in particular (available in several formulations, alone or in combination with non-narcotic analgesics) has been used as an alternative to morphine for the management of severe pain. The increasing numbers of oxycodone-dependent patients, and the rising incidents of deaths from oxycodone overdose (Carise *et al*, 2007; Comer *et al*, 2007; Spiller *et al*, 2009; Ling *et al*, 2011; Butler *et al*, 2011; Dart *et al*, 2015; Ray *et al*, 2016) highlight the need for a better understanding of the mechanism of action of oxycodone analgesia and the factors that determine vulnerability to oxycodone dependence and addiction.

Whereas there is a lot of information from *in vitro* work on the signaling properties of a range of mu opioid receptor (MOR)-targeting compounds, *in vivo* research on MOR agonist signal-transduction mechanisms has focused largely on the study of morphine or heroin (Bailey and Connor, 2005; Muller and Unterwald, 2004; Koch *et al*, 2005; Raehal and Bohn, 2005; Pradhan *et al*, 2006; Walwyn *et al*, 2010; Quillinan *et al*, 2011). Recently, several preclinical studies demonstrated the potent effect of oxycodone exposure in the development of addiction-related behaviors and adaptations in synaptic plasticity, in adolescent and adult animals (Niikura *et al*, 2013; Zhang *et al*, 2013, 2015). These studies have demonstrated that oxycodone has prominent effects on the expression of genes modulating synaptic plasticity in several brain regions, including the dorsal striatum and the hippocampus. Importantly, oxycodone self-administration in early life affects the sensitivity to the rewarding and analgesic actions of the drug in the adult life (Zhang *et al*, 2016). The preclinical and epidemiological findings on oxycodone abuse highlight the need to study the neurochemical and molecular actions of this drug in pain-free as well as in chronic pain states.

RGS9-2 is enriched in the striatum, and has a potent role in the modulation of functional responses of several G-protein-coupled receptors (GPCRs), including MOR and dopamine receptors (Zachariou *et al*, 2003; Rahman *et al*, 2003; Kovoor *et al*, 2005; Terzi *et al*, 2009; Kimple *et al*, 2011). RGS9-2 binds to activated Gα subunits and controls the duration of signal transduction, by regulating how long Gα and $\beta\gamma$ subunits are available to their effectors. In addition, the binding of RGS9-2 to Gα subunits may prevent activation of their effectors, without affecting $\beta\gamma$ complex's

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actions (Hollinger and Hepler, 2002; Traynor *et al*, 2009; Kimple *et al*, 2011). We previously showed that striatal RGS9-2 controls MOR signal transduction and desensitization (Zachariou *et al*, 2003; Psifogeorgou *et al*, 2007; Psifogeorgou *et al*, 2011; Gaspari *et al*, 2014). Several biochemical and *in vivo* studies suggest that RGS9-2 forms complexes with Gα subunits, the Gβ5 protein and other adaptor or scaffolding molecules to dynamically modulate the acute and chronic actions of the opioid analgesic morphine (Psifogeorgou *et al*, 2007; Anderson *et al*, 2007; Jayaraman *et al*, 2009; Psifogeorgou *et al*, 2011; Terzi *et al*, 2011; Gaspari *et al*, 2014). These RGS9-2 complexes affect several aspects of morphine addiction-like behavior in rodents, by negatively modulating reward and physical dependence, whereas they also affect analgesia and the development of analgesic tolerance (Zachariou *et al*, 2003; Terzi *et al*, 2009; Gaspari *et al*, 2014). Interestingly, RGS9-2 has a complex role in the regulation of MOR function, as suggested by more recent findings showing that RGS9-2 acts as a positive modulator of the analgesic effects of methadone and fentanyl (Psifogeorgou *et al*, 2011). Furthermore, recent studies reveal two SNPs located in the promoter region of *Rgs9* gene significantly associated with heroin dependence, highlighting the translational aspect of the above-stated research (Zhu and Zhang, 2015).

Chronic pain leads to plasticity in several brain networks involved in reward, mood, and motivation (Baliki *et al*, 2012; Schwartz *et al*, 2014; Yalcin *et al*, 2014; Baliki and Apkarian, 2015; Mitsi and Zachariou, 2016). We therefore hypothesized that chronic pain states may influence sensitivity to the behavioral effects of oxycodone. Neuropathic pain is a severe chronic disorder, resulting from nerve damage due to injury or various central nervous system disorders. It is characterized by several symptoms, including dysesthesia, mechanical and cold allodynia, hyperalgesia, and often coexists with anxiety and depression (Gruccu, 2007; Shields *et al*, 2003; Stratinaki *et al*, 2013; Terzi *et al*, 2014; Mitsi and Zachariou, 2016).

Here we investigated the role of RGS9-2 in mediating the behavioral responses to oxycodone. Our findings suggest that RGS9-2 acts a positive modulator of oxycodone reward, in both pain-free and neuropathic pain states. Although oxycodone promotes a similar somatic withdrawal syndrome in mice to that observed with morphine (Zachariou *et al*, 2003), RGS9-2 does not affect the expression of somatic withdrawal syndrome to oxycodone. Finally, our study shows that RGS9-2 delays the development of oxycodone tolerance both under pain-free and neuropathic pain states. Overall, these findings provide new information on the cellular mechanisms modulating oxycodone actions and support the hypothesis that distinct modulatory mechanisms control behavioral responses to opiate analgesics.

MATERIALS AND METHODS

Animals

Two- to three-month-old male C57BL/6 mice (Jackson Labs) were used for biochemical studies. Behavioral studies were performed using adult (2–3-month-old) male RGS9-wild-type (RGS9WT) and RGS9-knockout (RGS9KO) mice derived from homozygote breeding from mice backcrossed 20 generations to C57BL/6 background (Zachariou *et al*, 2003). Animals were housed in a 12 h dark–light cycle room according to the IACUC committee of Icahn School of Medicine. For all manually scored behavioral assays (hot-plate test, oxycodone withdrawal, Von Frey testing) experimenters were blinded to the genotype.

Locomotor Activity Assay

Mice were habituated to the locomotor activity apparatus (Med Associates, VT) for 30 min each day for 3 consecutive days after receiving a subcutaneous saline injection. Following that, oxycodone was administered for 3 or 4 consecutive days, once per day, and ambulatory activity was monitored for 30 min immediately after drug injection as described earlier (Charlton *et al*, 2008).

Conditioned Place Preference Test

An unbiased place conditioning procedure was performed as described in earlier studies (Zachariou *et al*, 2003; Gaspari *et al*, 2014). Briefly, baseline preference was monitored for 20 min, animals were conditioned to the drug paired side or to the saline paired side for 45 min on alternate days. After 6 conditioning days animals were tested again for 20 min, having free access to both chambers. Preference was determined as time spent in the drug-paired compartment after conditioning minus the time spent in this compartment at baseline. Animals showing strong bias (over 250 s) for one side at baseline were excluded from the study. For the oxycodone reinstatement studies (Figure 2b), mice were tested for baseline preference (day 1, for 20 min) and then they were conditioned with saline in the morning (45 min) and with a high dose of oxycodone (5 mg/kg, subcutaneously) in the afternoon (45 min) for 2 consecutive days. Place preference was tested the day after the last conditioning session, for 20 min. The extinction section involved saline injections paired to the saline side (morning, 45 min) and the drug side (afternoon, 45 min) for 5 days a week, for 4 weeks, starting 24 h after conditioned place preference (CPP) testing. Mice were evaluated for place preference once a week (20 min testing), until extinction was observed (week 4). For the reinstatement session, mice were injected with a low dose of oxycodone (1.5 mg/kg, subcutaneously) and place preference was monitored for 20 min.

Hot-Plate Assay

Analgesia was measured using a 52°C hot-plate apparatus (IITC Life Sciences, CA), as described previously (Gaspari *et al*, 2014). Animals were habituated in the room for 1 h and then tested for baseline latency to jump or paw lick. Oxycodone was then injected subcutaneously and 30 min later mice were placed on the hot-plate apparatus and latencies to lick the hind paw or jump were monitored. For tolerance studies this procedure was repeated for 6 consecutive days using a high oxycodone dose (20 mg/kg). All hot-plate data are expressed as % of maximal possible effect (MPE = (latency – baseline)/(cutoff – baseline)). A cut-off time of 40 s was used in all hot-plate experiments to avoid tissue damage and inflammation.

Opiate Withdrawal Paradigm

For opiate withdrawal assays, mice were injected with increasing oxycodone doses every 12 h for 4 consecutive days (day 1: 20 mg/kg; day 2: 40 mg/kg; day 3: 60 mg/kg; day 4: 80 mg/kg). On day 5 mice were injected with 80 mg/kg oxycodone in the morning and withdrawal was precipitated 3 h later, using naloxone (NLX) hydrochloride (1 mg/kg, subcutaneously; Sigma, MO). Withdrawal signs (jumps, wet dog shakes, tremor, diarrhea, weight loss) were monitored for 30 min, starting immediately after NLX administration. We monitored the number of jumps, wet dog shakes, and diarhhea events observed in the 30 min period, and % change in weight before and 30 min post withdrawal for weight loss. For tremor and ptosis, we monitored the presence of a sign at the beginning of each 5 min interval during the monitoring period.

Spared Nerve Injury Model

The spared nerve injury (SNI) operation was performed under Avertine (2,2,2-tribromoethanol; Sigma-Aldrich) general anesthesia (Mitsi *et al*, 2015). With the help of a stereomicroscope, skin incision of the left hindlimb at mid-thigh level followed by muscle layers separation revealed the sciatic nerve and its three branches. The common peroneal and the sural nerves were carefully ligated with 6.0 silk suture (Ethicon; Johnson & Johnson Intl.) transected and 1–2 mm sections of these nerves were removed, while the tibial nerve was left intact. Skin was then closed with silk 4.0 sutures (Ethicon; Johnson & Johnson Intl).

Von Frey Test for Mechanical Allodynia

For the assessment of mechanical allodynia, we used Von Frey testing (Shields *et al*, 2003; Mitsi *et al*, 2015) with ascending forces expressed in grams (0.1–3.6 g; Electronic von Frey Anesthesiometer; IITC). Each filament was applied five times in a row against the lateral area of the paw. Hindpaw withdrawal or licking induced by the filament was defined as a positive allodynia response. A positive response in three out of five repetitive stimuli was defined as the allodynia threshold. Mice were habituated to the Von Frey apparatus for 30 min every day for 10 days, before the SNI surgeries. For the tolerance studies, mice were injected subcutaneously for 17 consecutive days with 3 mg/kg oxycodone and allodynia was assessed 1 h after the injection.

Western Blot Analysis

For western blot analysis, nucleus accumbens (NAc) and dorsal striatum (DS) punches were dissected with a 14 gauge syringe needle from 1-mm-thick coronal sections of mouse brain, as described before (Psifogeorgou *et al*, 2011; Gaspari *et al*, 2014). Samples were run in a 10% polyacrylamide gel, and then transferred to a nitrocellulose membrane (Bio-Rad 4.45 μm) for 1 h. Membranes were incubated in blocking buffer (3% filtered non-fat dry milk, in PBS) and incubated overnight at 4°C in PBS with primary antibody. The following antibodies were used: rabbit anti-RGS9-2 (Psifogeorgou *et al*, 2011), a rabbit anti-GAPDH (Cell Signaling; no. 5174), a rabbit anti-β-actin (Cell Signaling; no. 4967). β-Actin or GAPDH were used as loading controls. The next day membranes were incubated with goat anti-rabbit peroxidase-labeled secondary antibody (Jackson Laboratories; no. 111-035-003; 1 : 10.000) in the blocking buffer. The bands were visualized using Super Signal West Pico Extended Duration Substrate (Pierce). Bands were quantified using the Image J Software. For all western blot analysis, sample optical density was normalized to loading control and then expressed as the percentage of the saline-treated group.

Statistical Analysis

For experiments monitoring behavior of the same group of mice over time (locomotor sensitization, CPP reinstatement, and tolerance experiments), we used two-way ANOVA repeated-measures followed by Bonferroni *post hoc* tests. For SNI-CPP experiments and acute oxycodone analgesia that involved plotting several doses (each dose was tested in a separate group of animals), we used two-way ANOVA followed by Bonferroni *post hoc* tests. For withdrawal experiments we used multiple *t*-test analysis. Finally, for western blot analysis studies, the CPP experiment testing 1 mg/kg of oxycodone (Figure 2a), and the morphine analgesia assays, we used *t*-test. Effects were considered significant at $p<0.05$, all data were expressed as mean ± SEM. F values and *p*-values for each experiment are described in the figure legends.

RESULTS

When administered repeatedly, drugs of abuse produce a progressive enhancement of locomotor response (locomotor sensitization). We used the RGS9KO line to investigate the behavioral consequences of *Rgs9* deletion in an oxycodone locomotor sensitization paradigm. As shown in Figure 1a, KO of the *Rgs9* gene does not affect basal locomotor activity nor the acute response to a high oxycodone dose (3 mg/kg subcutaneously). However, RGS9KO mice are less sensitive than RGS9WT controls to the locomotor-sensitizing actions of oxycodone. We next used western blot analysis to determine if repeated oxycodone administration affects the expression of RGS9-2 in the NAc and DS. C57BL/6 mice were habituated to the locomotor chamber for 3 consecutive days, followed by 3 additional days of oxycodone treatment (2 mg/kg, subcutaneously). On day 3, NAc and DS tissues were collected 2 h after drug administration (Figure 1b). Western blot analysis of these samples revealed that this treatment leads to the downregulation of RGS9-2 protein levels both in NAc and DS (Figures 1c and d).

We next investigated the role of RGS9-2 in the rewarding actions of oxycodone. Our earlier work revealed a negative modulatory role of RGS9-2 in the rewarding actions of morphine (Zachariou *et al*, 2003; Gaspari *et al*, 2014). Here our data (Figure 2a) suggest that RGS9-2 acts as a positive modulator of oxycodone actions in the CPP paradigm, as RGS9KO mice are less sensitive to the rewarding actions of this drug (1 mg/kg, subcutaneously). At higher doses both genotypes develop place preference to oxycodone (Figure 2b). We also assessed the role of RGS9-2 in the extinction and reinstatement of oxycodone place

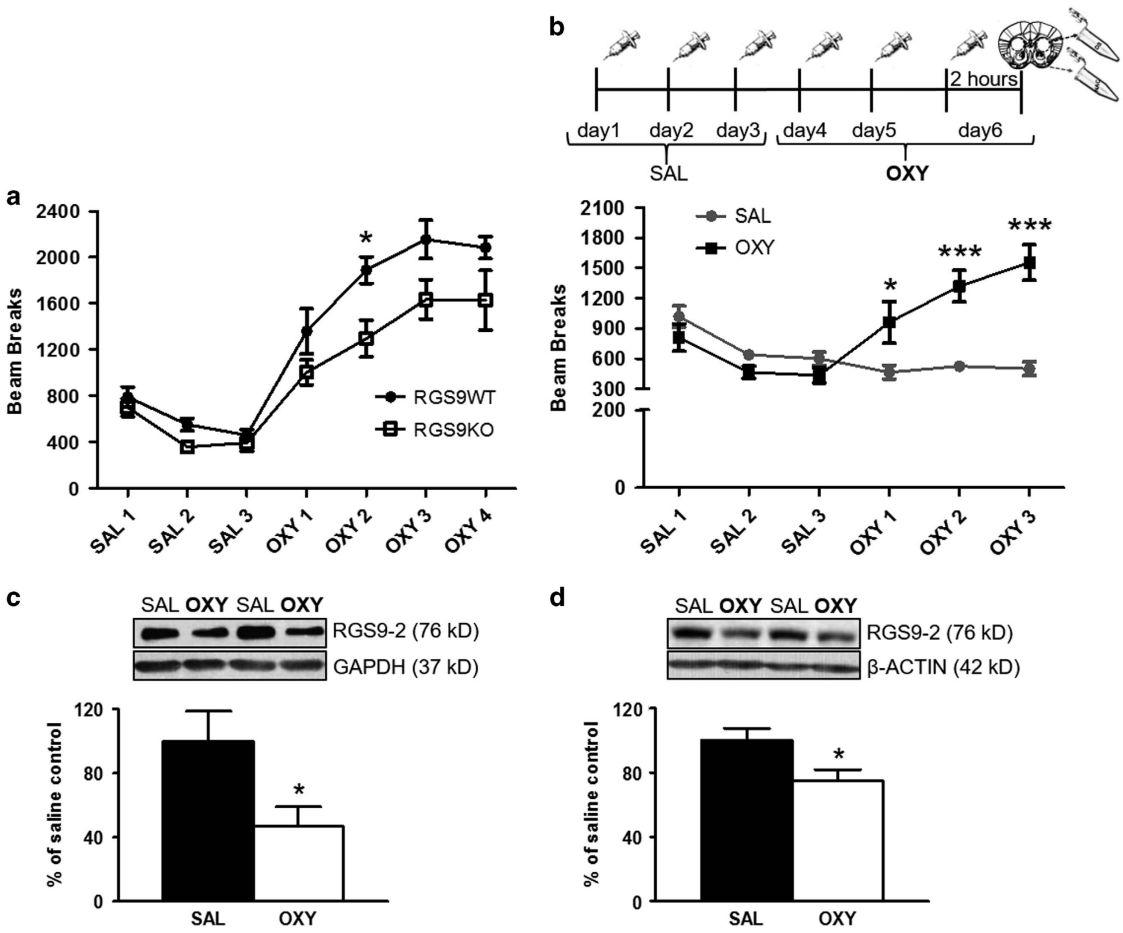

Figure 1 Regulator of G-protein signaling 9-2 (RGS9-2) modulates the locomotor-sensitizing actions of oxycodone. (a) RGS9-knockout (KO) mice are less sensitive to the locomotor-sensitizing actions of oxycodone compared with their RGS9-wild-type (WT) controls (dose: 3 mg/kg, two-way analysis of variance (ANOVA) repeated-measures followed by Bonferroni *posthoc* test, $F(1,48) = 8.22$, $^{*}p < 0.05$). (b) The schematic on top shows the experimental design for tissue collection for our western blot analysis studies. The graph shows the locomotor responses of the cohort used for western blot analysis. Mice were habituated to the locomotor chamber for 3 consecutive days followed by 3 additional days of oxycodone administration (dose: 2 mg/kg, two-way ANOVA repeated-measures followed by Bonferroni *posthoc* test, $F(1,40) = 4.93$, $^{*}p < 0.05$, $^{***}p < 0.001$). On day 3, nucleus accumbens (NAc) and dorsal striatum (DS) tissues were collected 2 h after drug administration. Western blot analysis reveals that RGS9-2 is downregulated in the NAc (c) and DS (d) of mice treated for 3 consecutive days with 2 mg/kg oxycodone (OXY) compared with saline (SAL)-treated controls (*t*-test, $t(19) = 2.429$, $^{*}p < 0.05$ for NAc and $t(9) = 2.433$, $^{*}p < 0.05$ for DS). GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

preference. In this case we used a high dose of oxycodone (5 mg/kg), which induces CPP in both WT and mutant mice. Both genotypes extinguished oxycodone CPP after a month of saline training; however, only RGS9WT mice reinstated CPP following treatment with a low (1.5 mg/kg) oxycodone dose (Figure 2b). Thus, RGS9-2 has a positive modulatory role in the acquisition and reinstatement of oxycodone CPP.

We also investigated if RGS9-2 contributes to the rewarding actions of oxycodone under chronic pain conditions. We used the SNI model of neuropathic pain, and 2 weeks after the induction of nerve injury (when the animals have developed pain-like behaviors, but not depression-like behaviors; Terzi *et al*, 2014), we conditioned RGS9WT and RGS9KO mice to oxycodone. The neuropathic pain-like state decreased sensitivity to oxycodone place preference in RGS9WT mice, as they did not respond to the 1 mg/kg dose that conditions CPP in the pain-free state. RGS9KO mice did not respond to this dose either. When a higher dose was used, we found a similar phenotype to that observed under pain-free states: RGS9WT mice develop a significant place preference, while the RGS9KO show no significant preference to this dose of the drug (Figure 2c; 2 mg/kg: RGS9WT—initial preference of the drug side $= 427 \pm 28$, conditioned preference $= 647 \pm 30$, $t(10) = 5.95$, $p < 0.0001$; RGS9KO—initial preference $= 490 \pm 35$, conditioned preference $= 560 \pm 22$, $t(8) = 1.96$, $p = 0.085$). At a higher dose (3 mg/kg) SNI groups of both RGS9WT and RGS9KO mice develop CPP to oxycodone (Figure 2c).

Our earlier work had also shown that KO of *Rgs9* exacerbated several symptoms of somatic morphine withdrawal, suggesting that RGS9-2 opposes the development of physical dependence (Zachariou *et al*, 2003; Gaspari *et al*, 2014). To determine if RGS9-2 has a similar role in the development of oxycodone physical dependence, we monitored somatic symptoms for 30 min following NLX-precipitated withdrawal. Oxycodone was administered

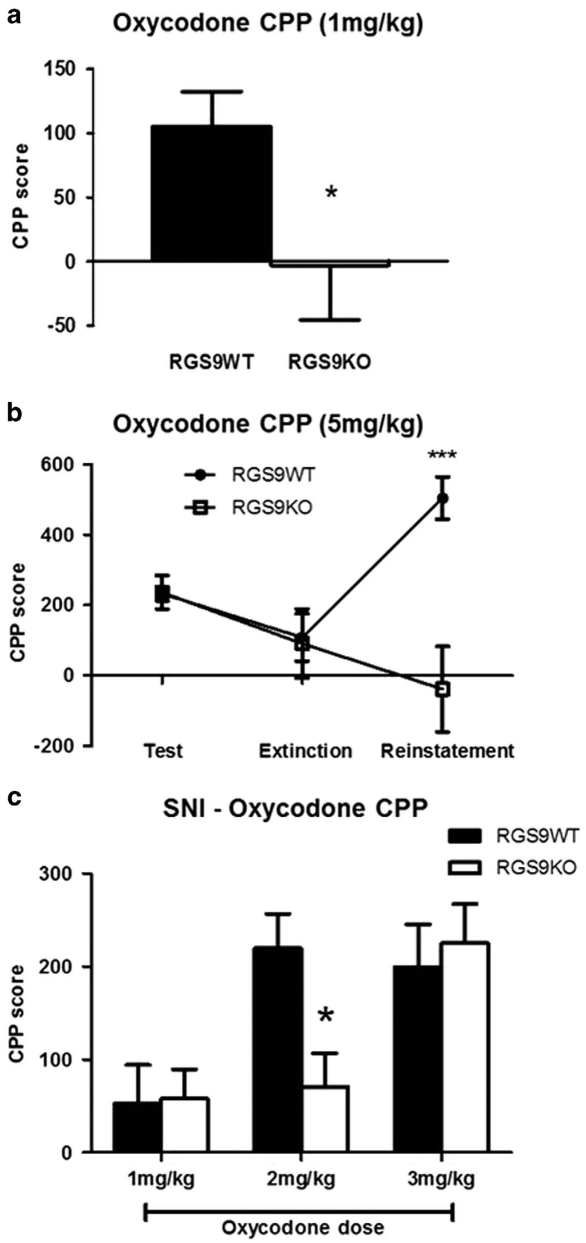

Figure 2 Regulator of G-protein signaling 9-2 (RGS9-2) modulates the rewarding actions of oxycodone. RGS9-2 acts as a positive modulator of oxycodone in the conditioned place preference (CPP) paradigm. (a) RGS9-knockout (KO) mice are less sensitive to the rewarding actions of oxycodone (dose:1 mg/kg, *t*-test, $t(17) = 2.249$, $^{*}p < 0.05$). (b) Furthermore, RGS9-2 has a positive modulatory role in the reinstatement of oxycodone CPP. Both genotypes developed CPP using a high dose of oxycodone (5 mg/kg) and extinguished preference after a month of saline training; however, only RGS9-wild-type (WT) mice reinstated CPP following a low (1.5 mg/kg) dose of oxycodone (two-way analysis of variance (ANOVA) repeated-measures followed by Bonferroni *post hoc* tests, $F(1,16) = 5.51$, $^{***}p < 0.001$). (c) Using the spared nerve injury (SNI) model of neuropathic pain, we demonstrate that RGS9WT and RGS9KO do not develop place preference to a low dose of oxycodone (1 mg/kg). At a higher dose (2 mg/kg), only RGS9WT mice develop preference, whereas both genotypes condition place preference at 3 mg/kg (two-way ANOVA followed by Bonferroni *post hoc* test, $F(1,16) = 5.51$, $^{*}p < 0.05$).

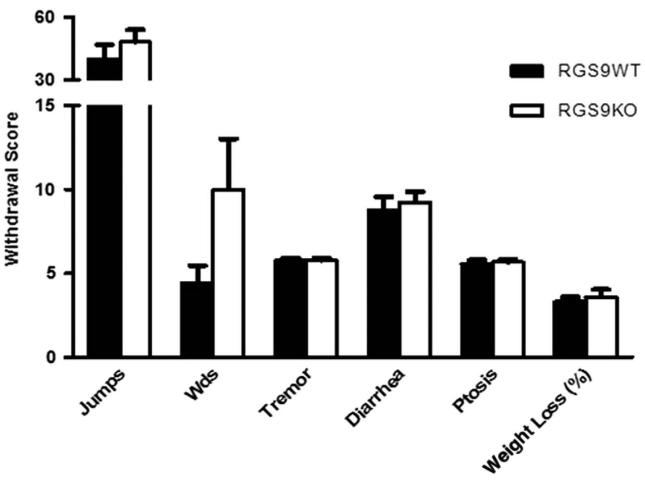

Figure 3 Knockout (KO) of the *Rgs9* gene does not affect the intensity of oxycodone withdrawal symptoms. We used a paradigm of naloxone (NLX)-precipitated withdrawal to determine if regulator of G-protein signaling 9-2 (RGS9-2) has a role in oxycodone physical dependence. Oxycodone was administered for 5 days, twice a day, at increasing doses (20, 30, 40, 60, and 80 mg/kg). We monitored several somatic withdrawal symptoms (jumps, wet dog shakes, tremor, diarrhea, ptosis, and weight loss) for a 30 min period after NLX (1 mg/kg) administration. Oxycodone produced a similar withdrawal behavior to the one observed with morphine, and onset and intensity of symptoms were comparable between RGS9KO mice and their RGS9-wild-type (WT) controls. RGS9KO mice showed a nonsignificant trend for a greater number of wet dog shakes (WDS) (multiple *t*-tests, Jumps: $t(16) = 0.8833$, $p > 0.05$, WDS: $t(16) = 1.742$, $p > 0.05$, Tremor: $t(16) = 0$, $p > 0.05$, Diarrhea: $t(16) = 0.4411$, $p > 0.05$, Ptosis: $t(16) = 0.3780$, $p > 0.05$, weight loss (%): $t(16) = 0.5066$, $p > 0.05$).

for 5 days, twice a day, at increasing doses (20, 30, 40, 60, and 80 mg/kg). Although oxycodone produces similar withdrawal behavior to that seen for morphine, ablation of the *Rgs9* gene did not affect the onset or intensity of withdrawal symptoms (Figure 3).

Finally, we investigated the role of RGS9-2 in the analgesic effects of oxycodone. We used the hot-plate assay to assess the consequences of global *Rgs9* KO on the acute analgesic actions of oxycodone. Our previous work demonstrated that RGS9KO mice show increased responses to morphine in the hot-plate test (Zachariou *et al*, 2003). Here we found that KO of *Rgs9* does not affect the acute analgesic effects of oxycodone in the 52°C hot-plate assay (Figure 4a). The top panel in Figure 4a shows that consistent with our earlier findings (Zachariou *et al*, 2003; Psifogeorgou *et al*, 2011), RGS9KO mice are more sensitive to the analgesic effect of morphine (15 mg/kg, subcutaneously). Western blot analysis showed that acute oxycodone treatment (15 mg/kg) has no effect on RGS9-2 expression in the NAc of C57Bl/6 mice (Figure 4b, NAc tissue dissected 2 h post oxycodone treatment). However, we found that RGS9-2 has a protective role towards the development of oxycodone tolerance, as RGS9KO mice become tolerant to the analgesic effects of the drug earlier than RGS9WT animals (Figure 5a). As shown on the top panel and consistent with our earlier findings, RGS9-2 has the opposite role in the modulation of morphine tolerance, as RGS9KO mice respond to repeated morphine treatment at 4 days, while their WT controls show analgesic tolerance. Furthermore, protein levels of RGS9-2 are

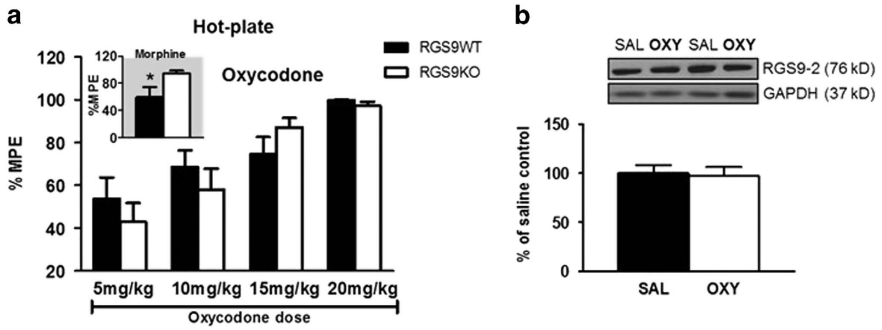

Figure 4 Regulator of G-protein signaling 9-2 (RGS9-2) does not affect the acute analgesic actions of oxycodone. (a) Ablation of the *Rgs9* gene does not affect the acute analgesic effects of oxycodone in the hot-plate assay (each dose corresponds to a separate group of animals, two-way analysis of variance (ANOVA) followed by Bonferroni *post hoc* tests, $F(1,74) = 0.16$, $p > 0.05$). Upper panel: RGS9-knockout (KO) mice are more sensitive to the analgesic actions of morphine (dose: 15 mg/kg, *t*-test, $t(7) = 2$, $^{*}p < 0.05$). (b) Acute administration of a high dose of oxycodone (15 mg/kg) did not have any effect on RGS9-2 protein levels in the nucleus accumbens (NAc) (*t*-test, $t(19) = 0.23$, $p > 0.05$). OXY, oxycodone; SAL, saline.

upregulated in the NAc of mice tolerant to oxycodone (Figure 5b).

The hot-plate test is a measure of acute analgesia, whereas oxycodone is often prescribed for chronic pain conditions. To determine if RGS9-2 affects the development of oxycodone tolerance under chronic pain states, we used the SNI paradigm and monitored responses to the antiallodynic effects of oxycodone in the Von Frey assay for mechanical allodynia. Our earlier work has shown that RGS9-2 affects Von Frey responses the first week after nerve injury, but by day 10, mechanical allodynia levels are not different from those observed in RGS9WT mice (Terzi *et al*, 2014). Here we started the treatment with oxycodone on day 15, when mechanical allodynia levels were identical between genotypes. The drug was administered once a day for 17 consecutive days, and Von Frey responses were monitored 1 h after drug injection. Consistent with our findings using the hot-plate assay, while the antiallodynic effects of oxycodone are maintained in RGS9WT mice throughout the monitoring period, RGS9KO mice show decreased analgesic response to oxycodone by day 12 (Figure 5c).

DISCUSSION

Our findings provide new information on the intracellular pathways involved in behavioral responses to oxycodone, in pain-free as well as in neuropathic pain states. RGS9-2 is a key modulator of drug addiction, and has been shown to have a dynamic role in the actions of several drug of abuse, including cocaine, amphetamine, and morphine (Traynor *et al*, 2009). Our data reveal that although oxycodone has rewarding, locomotor activating, and analgesic effects that are similar to morphine, RGS9-2 has distinct modulatory roles in the actions of these two MOR-targeting opioids. RGS9-2 promotes the rewarding and locomotor sensitizing effects of oxycodone, and opposes the development of analgesic tolerance. This is the opposite of what we observed in previous studies using morphine, where we found that RGS9-2 opposes the development of morphine reward, and promotes the development of analgesic tolerance (Zachariou *et al*, 2003; Gaspari *et al*, 2014). In addition, although deletion of the *Rgs9* gene exacerbates many morphine withdrawal symptoms (Zachariou *et al*, 2003), it does not affect symptoms of oxycodone withdrawal. Thus, RGS9-2 appears to uniquely modulate morphine, as analgesic responses to oxycodone, fentanyl, and methadone are positively modulated by RGS9-2 (Psifogeorgou *et al*, 2011). The distinct phenotypes observed with morphine *vs* oxycodone may lie in different intracellular mechanisms by which these compounds exert their effects; for example, recruitment of different Gα subunits (Psifogeorgou *et al*, 2011), or different effectors activated by each drug, although differences owing to receptor specificity or receptor dimer formation should also be considered. We speculate that similar to fentanyl and methadone, oxycodone in the striatum promotes short-term complexes between RGS9-2, MOR, and $G\alpha_q$ subunits, whereas morphine promotes stable complexes between RGS9-2-Gβ5, MOR, and $G\alpha_i$ subunits (Psifogeorgou *et al*, 2011). Therefore, genetic inactivation of *Rgs9* will differentially affect cellular and behavioral responses to oxycodone and morphine.

RGS9-2 is very abundant in the striatum, but it may also affect MOR function in the spinal cord (Terzi *et al*, 2009) and other supraspinal sites expressing MOR, including the periaqueductal gray (PAG) (Garzón *et al*, 2005), Moreover, the actions of MOR may be mediated in a cell type- or region-specific level by other members of the RGS family, including RGS4 and RGSz proteins (Gold *et al*, 2003; Garzón *et al*, 2005).

A large number of *in vitro* and *in vivo* studies have provided information on the mechanisms underlying the acute and chronic actions of MOR agonists (Muller and Unterwald 2004; Koch *et al*, 2005; Raehal and Bohn, 2005; Pradhan *et al*, 2006; Walwyn *et al*, 2010; Quillinan *et al*, 2011). The majority of these studies have focused on morphine and heroin, and have provided insight into signal-transduction events associated with addiction or the development of analgesic tolerance. Although oxycodone is highly prescribed for the treatment of acute and chronic pain conditions, there is very limited information on the cellular events triggered by repeated oxycodone administration, and the G-protein complexes that modulate oxycodone actions in specific brain regions or cellular populations modulating addiction and analgesia. Several recent reports have used models of reward and drug self-administration to study the actions of oxycodone in the adolescent and adult life

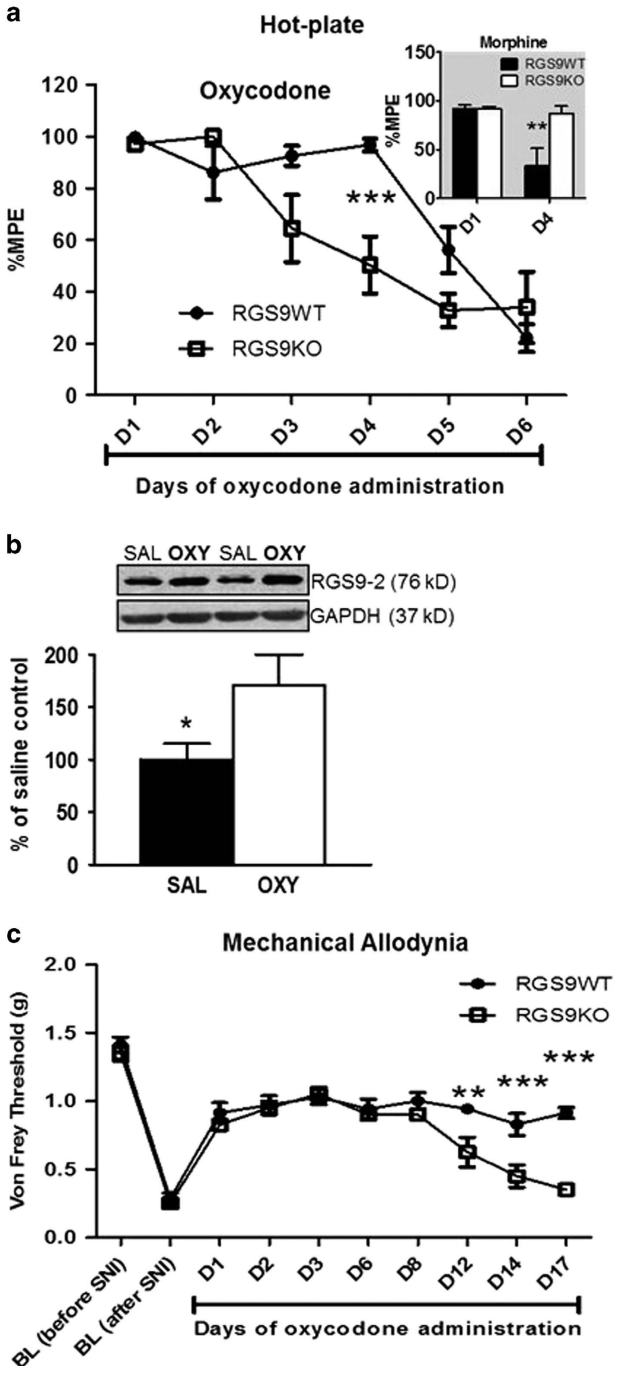

Figure 5 Regulator of G-protein signaling 9-2 (RGS9-2) acts as a negative modulator of oxycodone tolerance. (a) RGS9-knockout (KO) mice become tolerant to the analgesic effects of the drug earlier than their wild-type controls (dose: 20 mg/kg, two-way analysis of variance (ANOVA) repeated-measures followed by Bonferroni *post hoc* test, $F(1,45) = 3.77$, $^{***}p < 0.001$). Upper panel: The opposite phenotype is observed when morphine is used in the hot-plate assay, as RGS9KO mice respond to morphine at a time point their wild-type controls show analgesic tolerance (dose:20 mg/kg, two-way ANOVA repeated-measures followed by Bonferroni *post hoc* test, $F(1,7) = 9.25$, $^{**}p < 0.01$). (b) Furthermore, protein levels of RGS9-2 are upregulated in the nucleus accumbens (NAc) of mice tolerant to oxycodone (*t*-test, $t(28) = 2.14$, $^{*}p < 0.05$). (c) RGS9KO mice experiencing neuropathic pain become tolerant to the antiallodynic effects of the drug earlier than their wild-type controls. Oxycodone treatment was initiated on day 15 after spared nerve injury (SNI) surgery, when mechanical allodynia levels were identical between genotypes. The drug was administered once a day for 17 consecutive days, and Von Frey responses were monitored 1 h after drug injection (dose: 3 mg/kg, two-way ANOVA repeated-measures followed by Bonferroni *post hoc* tests, $F(1,117) = 10.49$, $^{**}p < 0.01$, $^{***}p < 0.001$).

(Niikura *et al*, 2013; Emery *et al*, 2015; Zhang *et al*, 2015; Sanchez *et al*, 2016) and in male *vs* female groups of animals (Collins *et al*, 2016). These studies show that exposure to oxycodone in early life affects the sensitivity of opiate analgesia and addiction-related actions of MOR agonists in the adult life. Our studies show that prevention of RGS9-2 activity reduces the rewarding effects of oxycodone, and also prevents the reinstatement of oxycodone place preference, pointing to a potent role of this molecule in addiction-related mechanisms. Recent studies by Emery *et al* (2015) show that oxycodone and morphine differentially affect responses of dopamine D2/D3 receptors and Akt signaling (Emery *et al*, 2015). Further understanding of the long-term adaptation oxycodone treatment promotes in the reward pathway, and the exact signal-transduction events triggered by oxycodone, will help design better treatment strategies for pain management and identify factors that contribute to oxycodone abuse vulnerability.

Chronic pain-induced plasticity in addiction- and analgesia-related brain networks may affect sensitivity to opiate analgesics (Navratilova and Porreca, 2014; Taylor *et al.*, 2015; Mitsi and Zachariou, 2016). Our studies examined the role of the striatal-enriched RGS9-2 in oxycodone actions under neuropathic pain states. Our findings from mice tested at 2 weeks after SNI reveal that neuropathic pain states reduce oxycodone reward sensitivity in both RGS9WT and RGS9KO animals, consistent with the hypothesis that chronic pain states impact the brain reward network (Mitsi and Zachariou, 2016). Decreased reward sensitivity under chronic pain states has been reported by several other groups (Ozaki *et al*, 2002, 2003; Ewan and Martin, 2011; Navratilova *et al*, 2012; Wu *et al*, 2014), but this is the first study to examine oxycodone reward in the context of chronic pain. Notably, reports also show that long-term inflammatory pain may promote heroin self-administration (Hipolito *et al*, 2015) and therefore it is important to assess the impact of inflammatory and neuropathic pain in oxycodone self-administration and other addiction-related paradigms. Future studies should monitor reward sensitivity to several pain killers at later time points after nerve injury, when depression symptoms are also present, to understand how long-term pain states affect the function of the reward pathway. In addition to G-protein complexes, neuroimmune and other adaptations affect the function of MOR and other receptors in the brain reward center under chronic pain states, and contribute to the decreased reward sensitivity observed in the CPP paradigm. For example, recent studies showed that in the rat PAG, MOR may function differently in pain-free *vs* chronic pain states (Eidson and Murphy, 2013; Mehalick *et al*, 2013) and have demonstrated the potent role of Toll-like receptors in modulating morphine tolerance in models of acute and long-term inflammatory pain.

Our data from SNI groups of mice also support a protective modulatory role of RGS9-2 in oxycodone tolerance: in accord with our findings from experiments using the hot-plate assay, KO of the *Rgs9* gene does not affect the acute action of oxycodone on mechanical allodynia following SNI. However, when the drug is administered for consecutive days, the antiallodynic response is abolished in RGS9KO mice after 12 days of treatment, reflecting the development of tolerance. Therefore, RGS9-2 complexes protect against the development of analgesic tolerance to oxycodone in models of acute and chronic pain.

RGS9-2 has a prominent role in the modulation of GPCR responses in the striatum, where it is expressed in very high levels, but it is absent or expressed in low amounts in other brain regions expressing MORs (Traynor *et al*, 2009). We suggest that the phenotypes observed are due to loss of RGS9-2 function primarily in the striatum, and that other members of the RGS family, including RGS7, RGS4, and RGS20 may control MOR function in other brain regions associated with addiction or analgesia, including the locus coeruleus and the PAG (Han *et al*, 2010; Gold *et al*, 2003; Garzón *et al*, 2005).

Future work will investigate the signal-transduction and gene expression changes induced by oxycodone exposure in the brain reward center, and the cellular and molecular determinants of oxycodone addiction. While the present study focuses on groups of male mice, future work will further determine the role of RGS9-2 in behavioral responses to oxycodone and other pain killers in male and female animals. Our findings that RGS9-2 modulates the actions of oxycodone in a manner distinct from its modulation of morphine support the notion that although synthetic opioid analgesics produce similar physiological responses, they act via distinct intracellular mechanisms. This knowledge may lead to the development of more efficacious and less addictive compounds and will help developing better strategies for the management of chronic pain.

FUNDING AND DISCLOSURE

The authors declare no conflict of interest.

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Biological Sciences

Suppression of RGSz1 function optimizes the actions of opioid analgesics by mechanisms that involve the Wnt/β-catenin pathway

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ABSTRACT

Regulator of G protein signaling z1 (RGSz1), a member of the RGS family of proteins, is present in several networks expressing mu opioid receptors (MOPR). By using genetic mouse models for global or brain region-targeted manipulations of RGSz1 expression, we demonstrate that the suppression of RGSz1 function increases the analgesic efficacy of MOPR agonists in male and female mice and delays the development of morphine tolerance while decreasing the sensitivity to rewarding and locomotor activating effects. Using biochemical assays and next-generation RNA sequencing, we identified a key role of RGSz1 in the periaqueductal gray (PAG) in morphine tolerance. Chronic morphine administration promotes RGSz1 activity in the PAG, which in turn modulates transcription mediated by the Wnt/β-catenin signaling pathway to promote analgesic tolerance to morphine. Conversely, the suppression of RGSz1 function stabilizes Axin2-Gαz complexes near the membrane and promotes β-catenin activation, thereby delaying the development of analgesic tolerance. These data show that the regulation of RGS complexes, particularly those involving RGSz1-Gαz, represents a promising target for optimizing the analgesic actions of opioids without increasing the risk of dependence or addiction.

Significance Statement: Opioids are used to alleviate severe pain, but their long-term use leads to analgesic tolerance, dependence, and addiction. Here, we target specific intracellular pathways to dissociate the analgesic actions of opioids from addiction-related effects. Using genetically modified male and female mice in models of addiction and analgesia, we reveal a key role of an intracellular modulator of the mu opioid receptor, RGSz1, in opioid actions. We applied next-generation sequencing and biochemical assays to delineate the mechanism of RGSz1 action in the mouse periaqueductal gray. Findings from this work point to novel

intracellular pathways that can be targeted to optimize the actions of opioids for the treatment of chronic pain.

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INTRODUCTION

Mu opioid receptor (MOPR) agonists, such as morphine, are prescribed for the treatment of severe acute or chronic pain conditions, but their long-term use is hindered by numerous side effects and by the development of dependence and addiction (1–4). Recent evidence highlights the rise of physical dependence and addiction in patients treated with opioid analgesics (5, 6). As research efforts aim to develop more efficient and safer morphine-like compounds (6, 7) and opioid alternatives, a more detailed understanding of the mechanisms mediating the analgesic effects of MOPR agonists is needed. Although information on the cellular and molecular adaptations associated with repeated exposure to opioids is emerging (8–13), the detailed brain region- and cell type-specific signal transduction events triggered by MOPR activation are not fully understood.

RGSz1, one product of the *RGS20* gene (14), is a member of the Rz subfamily (composed of GAIP [RGS19], RGSz1, RGSz2 [RGS17]) of regulator of G protein signaling (RGS) proteins (15–17) and is expressed in several brain regions (18–20). RGSz1 accelerates the hydrolysis of GTP-bound Gαz more than 600-fold and may modulate the GTP hydrolysis of other Gαi subunits (18, 19). As with other RGS proteins, RGSz1 may serve as an effector antagonist for Gα subunits (21, 22). RGSz1 has been shown to modulate signal transduction from MOPRs and serotonin receptor 1A both *in vitro* (23, 24) and *in vivo* (25, 26), whereas knockout of the Gαz subunit accelerates the development of morphine tolerance (27, 28). Although a number of studies have documented brain region-specific effects of other RGS proteins (RGS9-2, RGS7, and RGS4) (29–33) on the functional responses of MOPRs, the role of RGSz1 in opioid actions is not known. We hypothesized that the expression and activity of RGSz1 are altered by chronic

opioid use and that the manipulation of RGSz1 expression will impact the development of tolerance to the drug and its analgesic and rewarding effects.

Here, we show that chronic morphine administration increases RGSz1 expression in the periaqueductal gray (PAG), a brain region that plays a key role in modulating endogenous analgesia and responses to MOPR agonists (34). Knockout of *Rgsz1* (RGSz1KO) increased the analgesic efficacy of opioids and decreased the sensitivity to the rewarding and locomotor activating effects of morphine without impacting physical dependence. Global deletion or conditional knockdown of *Rgsz1* in the ventrolateral (vl) PAG delayed the development of morphine tolerance in pain-free and chronic pain states. Co-immunoprecipitation (co-IP) assays indicated that under states of morphine tolerance, there is an increased formation of complexes between Gαz and MOPR in the PAG. Next-generation RNA sequencing (RNA-Seq) revealed a robust downregulation of Wnt/β-catenin signaling in the PAG of morphine tolerant mice. Our Co-IP and Western blot analyses also suggested that RGSz1 competes with Axin2 (a member of the RGS protein family and a key component of the Wnt/β-catenin pathway that controls the nuclear translocation of β-catenin) for binding to Gαz. Using a morphine tolerance regimen, we showed that knockout of RGSz1 stabilizes Axin2/Gαz complexes, permitting the nuclear translocation of β-catenin and the transcription of target genes. Therefore, interfering with RGSz1 actions may be useful for promoting the analgesic effects of MOPR agonists while attenuating their addiction-related effects.

RESULTS

Regulation of RGSz1 by chronic morphine

We initially performed Western blot analyses to test the hypothesis that repeated morphine administration regulates RGSz1 levels in brain regions associated with analgesic responses. We applied a 4-day hot plate morphine tolerance paradigm, and monitored analgesic responses for 4 consecutive days, in groups of mice treated with morphine (15mg/kg). RGSz1 protein levels in the PAG were upregulated 2 hours after the last morphine injection compared to that in saline-treated controls (Fig. 1a). No RGSz1 protein was detected in PAG samples from RGSz1KO mice. This morphine treatment regimen had no effect on RGSz1 expression in the nucleus accumbens (NAc), dorsal striatum, or thalamus (Suppl Fig. S1). Notably, morphine treatment upregulated Gαz levels in the PAG of both RGSz1WT and RGSz1KO mice (Fig. 1b), but levels of other Gα subunits expressed in the PAG (Gαi3, Gαo, and Gαq) were not altered (Fig. 1c–e). To further evaluate the association between RGSz1 and Gαz complexes in the PAG, we performed co-IP assays and found that morphine acutely (within 30 min) promoted the dissociation of Gαz from MOPRs (Fig. 1f). However, chronic morphine treatment induced the converse, promoting MOPR-Gαz complex formation (Fig. 1g).

RGSz1 knockout enhances the analgesic efficacy of MOPR agonists and delays morphine tolerance

We next examined the role of RGSz1 in the analgesic actions of morphine and other MOPR agonists. In the hot plate test, male RGSz1KO mice show enhanced analgesia at lower doses of morphine compared with the responses of RGSz1WT controls (Fig. 2a). This phenotype was also observed in female RGSz1KO mice (Fig. 2a, inset). The sensitivity to the analgesic effects of fentanyl and methadone was also increased in RGSz1KO mice compared with that of their RGSz1WT counterparts (Fig. 2b). We repeated the hot plate assay for 8 consecutive days to

assess the impact of RGSz1 on the development of morphine analgesic tolerance. By day 3, RGSz1WT mice show reduced responses to morphine due to the development of tolerance. Analgesic responses were significantly different between genotypes, indicating that the development of tolerance to morphine treatment (15 mg/kg, s.c.) is delayed in male (Fig. 2c) and female (Fig. 2c, inset) RGSz1KO mice. We similarly assessed morphine tolerance under inflammatory pain conditions. Thermal hind paw hyperalgesia after intraplantar injection of Complete Freud's adjuvant (CFA) was assessed by the Hargreaves test (35). Similar to our observations of animals in pain-free states, morphine analgesia was reduced in RGSz1WT mice after 3 days of treatment, whereas it was maintained in RGSz1KO mice throughout the testing period (Fig. 2d).

Interestingly, whereas RGSz1 knockout enhanced the analgesic responses to morphine, it decreased the rewarding effects. Specifically, knockout of RGSz1 decreased the sensitivity to low morphine doses in the place preference paradigm (Fig. 3a–c). When a higher dose of morphine was applied, both genotypes showed conditioned place preference (Fig. 3d). We also assessed locomotor activation, which is increased in rodents by repeated opioid administration (36). Knockout of RGSz1 lowered the locomotor activation in response to repeated morphine administration (Fig. 3e). Importantly, RGSz1KO mice showed normal responses to other reward-related behaviors, such as sucrose preference [sucrose consumption (% of total amount consumed): RGSz1WT, 60.4±6.2; RGSz1KO, 69.7±6.2].

RGSz1 in PAG neurons modulates analgesic tolerance

We hypothesized that RGSz1 modulates the analgesic and rewarding effects of MOPR agonists via actions in distinct brain regions. To test this, we used viral approaches to conditionally knockdown RGSz1 in specific brain regions of adult mice. On the basis of our initial Western blot analysis, we targeted the PAG. AAV2-CMV-CRE-GFP or an AAV2-CMV-GFP control virus was injected into the vlPAG of $RGSz1^{fl/fl}$ mice. Two weeks later, when maximal viral expression

is achieved, mice were tested in the morphine tolerance paradigm. The development of morphine tolerance was significantly delayed by knockdown of RGSz1 in the vlPAG (Fig. 4a), corresponding to a 50% reduction of RGSz1 protein levels (Fig. 4b). As shown in Figure 4b and d and consistent with previous reports (37), the virus infects only neuronal cells and not astrocytes. Interestingly, the knockdown of RGSz1 in the NAc did not affect the development of analgesic tolerance (Fig. 4c). Notably, the knockdown of RGSz1 in the vlPAG of male mice did not affect analgesic responses to morphine in the hot plate assay [hot plate latencies (%MPE) for 12 mg/kg morphine: AAV2-CMV-GFP, 50.3±6.7; AAV2-CMV-CRE-GFP, 44.2±8.4]. Furthermore, the actions of RGSz1 in the vlPAG did not affect the development of physical dependence. After treating mice for 5 days with escalating doses of morphine, withdrawal was precipitated with naloxone hydrochloride (1 mg/kg, s.c.). We immediately began monitoring several somatic withdrawal symptoms in AAV2-CMV-CRE-injected RGSz1$^{fl/fl}$ mice and their AAV2-CMV-GFP-injected controls, including jumps, wet-dog shakes, tremor, ptosis, diarrhea, and weight loss for a period of 30 min (31). As shown in Figure 4e, withdrawal symptoms were similar between the two groups of mice. Thus, RGSz1 actions in the vlPAG promote the development of analgesic tolerance without impacting the development of physical dependence.

Morphine distinctly alters gene expression patterns in the PAG of RGSz1KO and RGSz1WT mice

Repeated exposure to opioids promotes long-term adaptations in gene expression and neuronal plasticity (38, 39). We applied next-generation RNA-Seq to understand the impact of morphine tolerance and the influence of RGSz1 on gene expression in the PAG. PAG tissue samples were collected from mice on day 4 of the morphine hot plate assay, when analgesic tolerance emerges in RGSz1WT mice (Fig. 5a). After pooling tissue from two animals per sample, RNA from naïve and morphine-treated RGSz1WT and RGSz1KO mice was analyzed. A heatmap analysis comparing treated versus naïve mice for each genotype shows that morphine tolerance

altered gene expression patterns in RGSz1WT mice, but that a much larger number of genes was affected in the RGSz1KO group (Fig. 5b). The number of genes regulated by morphine in each genotype is shown in a Venn diagram in Figure 5c. Our findings reveal that only ¼ of the genes regulated by morphine are common between genotypes, whereas ¾ of the genes with altered expression are unique to RGSz1WT or RGSz1KO mice (Fig. 5c). A gene ontology analysis indicated that the majority of genes regulated by chronic morphine in the PAG of RGSz1WT and RGSz1KO mice are associated with transcription and cell proliferation/differentiation, whereas genes associated with ion/transmembrane transport, cell adhesion, and behavior are also altered in RGSz1KO mice. We further investigated this observation using Ingenuity Pathway Analysis (IPA), and indeed, distinct pathways in the PAG were affected in each genotype, with Wnt/β-catenin signaling as the top pathway affected in RGSz1WT mice ($P<0.001$) and serotonin receptor signaling targeted in RGSz1KO mice ($P<0.001$) (Fig. 6a). Importantly, the pathway analysis of the preexisting gene expression adaptations in the PAG of RGSz1KO naïve mice does not reveal alterations in any of the above-stated pathways (Suppl Fig. S3). Figure 6b summarizes the changes in several components for each of these pathways after morphine treatment. We further validated the results by qPCR in separate cohorts of animals. Twenty selected genes per genotype were tested, and around 70% of the tested genes were validated. Differential gene expression patterns for a subset of the validated genes are presented in figure 6c.

Wnt/β-catenin signaling is essential for analgesic responses to morphine

Several key molecules in the Wnt/β-catenin signaling pathway were downregulated by chronic morphine treatment in the PAG of RGSz1WT but not RGSz1KO mice (shown in blue in Fig. 7a). Furthermore, narrowing our lists of differentially regulated genes to known β-catenin targets (40) revealed that those genes in RGSz1WT mice were primarily downregulated (Fig. 7b). We

hypothesized that analgesic responses to morphine are maintained in RGSz1KO mice because β-catenin remains active, whereas signaling in this pathway is suppressed in RGSz1WT mice. Western blotting confirmed this hypothesis, as higher levels of active phospho-β-catenin (ser675) and inactive phospho-GSK3β (ser9) were observed in the PAG of RGSz1KO mice after 4 days of morphine treatment (Fig. 7c and d). Furthermore, antagonism of β-catenin activity in the vlPAG of RGSz1KO mice by expressing a dominant-negative form of the protein (40) induced tolerance similar to that seen in RGSz1WT controls, whereas overexpressing β-catenin itself in the vlPAG of RGSz1WT mice during morphine treatment was not sufficient to delay the development of tolerance (Fig. 7e; for viral validation, see Suppl Fig. S4). We therefore hypothesized that in addition to β-catenin, other components of this pathway are necessary to obtain the prolonged analgesic response phenotype observed in RGSz1KO mice. It is also possible that the lack of an observed phenotype when β-catenin was overexpressed in the vlPAG may be related to compensatory changes in the expression of destruction complex components resulting from the high levels of transgene expression. In the absence of stimulation, β-catenin is bound to Axin2 in the cytoplasm, which targets it for proteasomal degradation (41). Wnt signaling releases β-catenin for translocation to the nucleus. Because Axin2 contains an RGS domain that has been shown to interact with Gα subunits (42–44), we hypothesized that an interaction between Axin2 and Gαz indirectly affects Wnt/β-catenin signaling. This hypothesis was confirmed by our next set of biochemical assays. Although the total levels of Axin2 in the PAG were not affected by repeated morphine administrations (Fig. 8a), the interaction with Gαz in the synaptosomal fraction was significantly decreased in RGSz1WT mice, as indicated by co-precipitation of Gαz with Axin2 (Fig. 8b). However, this interaction was maintained in RGSz1KO mice that did not exhibit morphine tolerance (Fig. 8b). The dissociation of Axin2 from Gαz was accompanied by higher levels of Axin2 in the nuclear fractions of the samples, as shown in Figure 8c. Furthermore, a correlation analysis reveals a

significant negative association between the amount of Axin2 bound to Gαz in the synaptosomal fraction and the amount of Axin2 present in the nuclear fraction (Fig. 8d). The translocation of Axin2 to the nucleus was previously shown to block β-catenin-mediated transcription (45).

DISCUSSION

Our findings provide new evidence for a brain region-specific mechanism that modulates the functional responses of MOPRs. We show that constitutive deletion of *Rgsz1* increases analgesic responses to morphine, methadone, and fentanyl and delays the development of analgesic tolerance in pain-free and chronic pain states. Importantly, the deletion of *Rgsz1* decreases the rewarding effect of morphine. These features indicate that RGSz1 is a promising target for the development of adjunct medications to enhance the efficacy of typical opioid analgesics and lower their abuse potential. Our data also indicate that the modulatory roles of RGSz1 in morphine analgesia and tolerance are the same in both male and female mice, further supporting the value of this molecule as a potential new target for pain management.

By controlling GPCR activity, RGS proteins may dynamically affect the functions of ion channels, signal transduction cascades, epigenetic modifiers, and transcription factors (15–17, 46). Several members of the RGS family have been shown to modulate the actions of MOPR agonists *in vitro* and *in vivo* (29–33). In our previous studies, we found that RGS9-2 negatively modulates both morphine analgesia and reward-related behaviors (29, 31). Therefore, the inhibition of RGS9-2 not only improves the analgesic effects but would also likely increase the rewarding and locomotor effects of the drug and promote physical dependence. In addition, RGS9-2 has a ligand-dependent role on MOPR function, as it positively modulates the effects of fentanyl and oxycodone (30, 47). Another RGS protein, RGS7, similarly modulates both reward- and analgesia-related behaviors (32). RGS4 in the NAc has a less potent but significant effect on morphine reward, and in the locus coeruleus, it modulates physical dependence; however, this molecule does not affect analgesia or tolerance to morphine (33). Although RGS4 and RGS7 are also expressed at moderate-to-high levels in the PAG (48), RGSz1 is unique in its differential modulation of opioid reward and analgesia.

Our study focused on understanding the mechanism by which RGSz1 modulates opioid analgesia and tolerance. Using conditional knockdown approaches, we demonstrate that the

development of analgesic tolerance is fostered by RGSz1 in the vlPAG, a key area involved in the descending control of pain and morphine-induced analgesia (34). Importantly, the actions of RGSz1 in the vlPAG do not affect the development of physical dependence. A variety of studies document adaptations in this brain area in models of analgesic tolerance (49–52). However, the detailed mechanisms that contribute to MOPR signal transduction and desensitization in the PAG are not fully understood. Our results indicate that RGSz1 forms complexes with Gαz and MOPRs after chronic opioid use. These findings are in accordance with previous work by Hendry et al. suggesting that compromised Gαz actions are associated with morphine tolerance (27). As mentioned above, several components of the MOPR desensitization machinery may affect the development of analgesic tolerance. This study highlighted the role of RGSz1, Gαz and components of the β-catenin pathway in morphine tolerance.

Our group has used next-generation sequencing in knockout mouse models and with pharmacological treatment to monitor adaptations in gene expression under pain states and to identify the pathways involved (53, 54). We applied this methodology to determine the mechanism by which chronic morphine induces analgesic tolerance and the impact of RGSz1 in such adaptations. In agreement with previously reported data, our findings indicate that analgesic tolerance to morphine is linked to robust adaptations in genes associated with inflammatory cascades (55–58), involving eight of the fifteen most significantly affected pathways identified by IPA. Notably, most of these adaptations were observed only in RGSz1WT mice and therefore are likely to be linked to morphine tolerance. Furthermore, there was a robust upregulation of genes associated with serotonin production and release in the PAG of RGSz1KO mice, highlighting the importance of the supraspinal serotonergic system in maintaining analgesic responses (59). We observed that several components of the Wnt/β-catenin signaling pathway were downregulated in the RGSz1WT group at the time the mice become tolerant to morphine. Although there is no known direct link between Wnt/β-catenin

signaling and opioid actions, earlier studies have shown that the inhibition of GSK3β, which promotes the degradation of β-catenin (41), delays the development of morphine tolerance (60, 61). Furthermore, there is evidence for Wnt/β-catenin signaling in the morphological effects of long-term exposure to MOPR agonists, such as reduced dendritic arborization, neurite outgrowth, and neurogenesis in several brain regions (62–65). Here, we confirm the involvement of this pathway by showing morphine tolerance can develop in RGSz1KO mice when β-catenin actions in the vlPAG are directly antagonized. However, β-catenin overexpression is not sufficient to delay the development of tolerance in RGSz1WT mice, suggesting additional factors control transcriptional activation as tolerance develops, or that the robust overexpression of β-catenin affected the function of components of the destruction pathway. Our data suggest that states of morphine tolerance are associated with increased abundance of Axin2 in the nucleus, where it may repress β-catenin-mediated transcription (45) Wnt/β-catenin signaling is among the most conserved intracellular pathways, playing pivotal roles in cell proliferation, migration, and homeostasis (41). Therefore, the pharmacological inhibition of Wnt components would broadly affect many tissues and cellular processes in the CNS and the periphery. The identification of molecules such as RGSz1 that modulate Wnt/β-catenin signal transduction in a brain region-specific manner may provide a novel avenue for developing adjunct medications to MOPR agonists for the management of chronic pain.

Future work will continue to detail the actions of RGSz1 in the brain reward network and determine the key protein interactions and intercellular pathways by which RGSz1 promotes the rewarding effects of opioids. Finally, we plan to investigate if RGSz1 modulates the analgesic effects of MOPR agonists in other brain regions and the intracellular pathways involved. Overall, our findings provide novel information on the signal transduction networks mediating the effects of MOPR agonists in male and female mice and identify RGSz1 as a novel target for optimizing the analgesic actions of MOPR agonists.

MATERIALS AND METHODS

Animals

For these studies, we used 2–3-month-old male and female RGSz1 knockout (RGSz1KO) and wild-type (RGSz1WT) or RGSz1-floxed ($RGSz1^{fl/fl}$) mice derived from homozygote breedings. The RGSz1KO and $RGSz1^{fl/fl}$ lines are on a DBA background, backcrossed to C57Bl/6 mice for three and two generations, respectively. For the RGSz1 locus targeting strategy, see Supplemental Figure S2. The primers used for genotyping are listed in Supplemental Table S7. Mice were housed with a 12-h dark/light cycle according to the Animal Care and Use Committee of the Icahn School of Medicine at Mount Sinai.

CFA model

Chronic inflammation was induced by an intraplantar injection of 25 μl complete Freund's adjuvant (diluted 1:1 in saline; Sigma-Aldrich) in the left hind paw (66). Morphine treatment (3 mg/kg) was initiated 24 h later and repeated for four consecutive days (day 1–4).

Hot plate analgesia assays

Analgesia was measured using a 52°C hot plate apparatus (IITC Life Sciences, CA) as previously described (29). Animals were habituated in the room for 1 h, and the baseline latencies to jump or lick the hind paw were measured. Morphine was administered subcutaneously (s.c.) and 30 min later, mice were placed on the hot plate and the latencies to jump or lick the hind paw were monitored. A cutoff time of 40 s was used to avoid tissue damage and inflammation (31). For tolerance studies, this procedure was repeated for 4 to 8 consecutive days using a high dose of morphine (15 mg/kg, s.c.). Data are expressed as percentages of maximal possible effect [MPE = (latency – baseline)/(cutoff – baseline)].

Hargreaves assay

Morphine tolerance under inflammatory pain conditions was assessed by measuring thermal hyperalgesia in the Hargreaves test. For this assay, mice were placed in a Plexiglas box with a glass bottom and the latency to withdraw the injured (CFA-injected) hind paw was measured after a high-intensity heat beam (30%) was applied to the mid-plantar area (IITC Life Sciences). Two measurements were obtained with a 10-min interval, and the average was defined as the thermal nociceptive threshold. A cutoff time of 20 s was used to avoid tissue damage (67).

Stereotaxic virus injection surgery

Conditional deletion of *Rgsz1* was achieved by bilateral stereotaxic injections of the AAV2-CMV-CRE-EGFP (University of South Carolina Viral Core Facility) into the NAc or vlPAG of RGSz1$^{fl/fl}$ mice. Control animals received injections of AAV2-CMV-EGFP vectors. Stereotaxic coordinates for viral vector injections were as follows: NAc (with respect to bregma): AP, +1.6 mm; AL, +1.5 mm; and DV, -4.4 mm at 10° from the midline; vlPAG (with respect to lambda): AP, +0.6 mm; ML, +0.8 mm; and DV, -2.8 mm at 22° from the midline. HSV-β-catenin (HSV-β-cat), HSV-β-catenin dominant negative (HSV-β-catDN), and HSV-GFP vectors were generated by R.L.N. (40).

Conditioned place preference test

An unbiased place-conditioning procedure was performed using a two-chamber place-conditioning system (Med Associates, Inc., VT) (29, 31). Briefly, after monitoring baseline preferences for 20 min on day 1, animals were conditioned to the drug-paired or saline-paired side for 45 min on alternate days. After six conditioning sessions, animals were tested for 20 min, and the results are presented as the time spent in the drug-paired compartment at baseline compared to that on test day.

Locomotor activation paradigm

Mice received a saline injection (s.c.) and were habituated to the locomotor apparatus (Med Associates, Inc.) for 30 min each day for 3 consecutive days. Following that, mice received injections of morphine (10 mg/kg, s.c.), and ambulatory activity was monitored for 30 min on 5 consecutive days (47).

Opiate withdrawal paradigm

Mice were injected intraperitoneally (i.p.) with escalating morphine doses every 12 h for 4 consecutive days (day 1, 20 mg/kg; day 2, 40 mg/kg; day 3, 60 mg/kg; day 4, 80 mg/kg). On the fifth day, they were injected with 80 mg/kg morphine in the morning and withdrawal was precipitated 3 h later using naloxone hydrochloride (1 mg/kg, s.c.; Sigma, MO) (31). Withdrawal signs (jumps, wet-dog shakes, tremor, diarrhea, and weight loss) were monitored for 30 min after naloxone administration. Data are expressed as the percentages of the AAV2-GFP injected control. For tremor and ptosis, we monitored the presence of signs at the beginning of each 5-min interval during the monitoring period (29).

Western blotting, subcellular fractionations, and co-IP assays

Two 14-gauge PAG punches (derived from a single animal) were used per sample for all assays. Western blotting was performed as previously described (53). For subcellular fractionation, samples were homogenized using a pestle in 50 μl of hypotonic cell lysis buffer (HCLB) plus protease and proteasome inhibitors, incubated on ice for 45 min, and centrifuged at 2,000 rpm at 4°C for 10 min, and supernatants were collected as the crude cytoplasmic fractions. The remaining nuclear pellets were washed twice with 100 μl HCLB plus inhibitors, resuspended in 25 μl nuclear lysis buffer with inhibitors, incubated for 2 h on ice, and

centrifuged at 13,200 rpm at 4°C for 5 min, and the supernatants were collected as the crude nuclear fractions. To obtain the synaptosomal fractions, the crude cytoplasmic fractions were centrifuged twice to remove the remaining nuclei (2,000 rpm at 4°C for 10 min). The supernatants were collected and centrifuged at 10,000 rpm at 4°C for 10 min. Supernatants were collected as the cytoplasmic fractions and the synaptosomal pellets were resuspended in 20 μl HCLB.

Co-IPs for MOPR-Gαz were performed using whole lysate samples as previously reported (30). For co-IPs with Axin2, 1 μl of antibody was added to each synaptosomal fraction (co-IP with anti-GFP was used as the negative control), and samples were incubated overnight at 4°C on a circular rotator. Each sample was then incubated for an additional 3 h with 10 μl anti-rabbit Dynabeads (Thermo Fisher Scientific). Co-IP proteins were washed three times with 700 μl blocking solution using a magnetic stand and then resuspended in 10 μl RIPA buffer. The following rabbit antibodies were used: anti-RGSz1 (provided by Elliott Ross), anti-Gαz (#3904; Cell Signaling), anti-Gαi3 (Lilly Jiang, UTSW Medical Center) (68), anti-Gαo (#3975; Cell Signaling), anti-Gαq (Paul Sternweis, UTSW Medical Center) (69), anti-MOPR (#24216; Immunostar), anti-phospho-β-catenin (ser675, #4176; Cell Signaling), anti-β-catenin (#9562; Cell Signaling), anti-phospho-GSK3β (ser9 #9336; Cell Signaling), anti-GSK3β (#9315; Cell Signaling), anti-Axin2 (#ab32197; Abcam), anti-GADPH (#5174; Cell Signaling), anti-β-actin (#4967; Cell Signaling), and anti-GFP (#ab290; Abcam). Mouse anti-β-tubulin III (#T8578; Sigma), GADPH, or β-actin antibodies were used as the loading control. Data are calculated as the optical density ratio of sample/loading control or IP/total lysate in the case of co-IPs and are expressed as the percentages of the control group. Bands were quantified using Image J software (53).

RNA-Seq and qPCR

Four biological replicates per group were used for the RNA-Seq studies. PAG punches from two animals were pooled per biological replicate (four 14-gauge punches per sample). Total RNA was isolated with TRIzol and the integrity was confirmed with an Agilent 2100 Bioanalyzer (53). mRNA-Seq libraries were prepared using a TruSeq RNA sample preparation kit v2 (Illumina). Sequencing was performed using the Illumina HiSeq 2000 apparatus. Read alignment, read counting, and differential analysis were performed using TopHat2 (70), HTSeq (71), and voom-limma R package (72), respectively. For all four gene lists, a cut-off of p-value <0.05 and $\log_2$(fold change) <-0.5 or >0.5 was used to generate gene lists for further bioinformatics analysis. qPCR was performed using SYBR green and analyzed using the $\Delta\Delta C_T$ method. Information on primers used for biological validations is provided in Supplemental Table S7.

Bioinformatics analysis

Heatmaps were generated using GENE-E, Venn diagrams were generated using VennPlex Version 1.0.0.2 (NIH), pathway analysis was conducted using IPA, and Gene Ontology (GO) analysis was conducted using DAVID. For pathway analysis, a cut-off of p-value <0.003 was applied to the output pathways.

Statistical analysis

For the experiments monitoring behavior of the same group of mice over time (Fig. 2c and d, 3a–e, 4a and c, 7e, and Suppl Fig. S2b), we used two-way repeated-measures ANOVAs followed by Bonferroni's *post hoc* tests. For two-factor designs (Fig. 1b–g, 2a, 7c and d, and Suppl Fig. S2c), we used two-way ANOVAs followed by Bonferroni's *post hoc* tests. For data containing a single independent variable (Fig. 1a, 2a upper panel, 4b, and Suppl Fig. S2d and e), we used unpaired two-tailed *t* tests. For the data in graphs depicting multiple individual single-factor comparisons (Fig. 2b, 4e, and 6c), we used multiple *t* tests. Correlation analysis

(Fig. 8d) was contacted using Spearman's rho. Error bars are depicting ±SEM. F and *t* values for each data set are provided in the Figure legends.

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FIGURE LEGENDS

Figure 1. Morphine regulates RGSz1 and Gαz in the PAG. (a) RGSz1 protein levels were elevated in the PAG of RGSz1WT mice treated for 4 consecutive days with morphine [15 mg/kg; *n* = 16/group; *t* test, *t*(30) = 2.144; **P*<0.05]. Chronic morphine also increased the levels of Gαz in the PAG (b), which occurred in both RGSz1WT and RGSz1KO mice [15 mg/kg; *n* = 11–12 per group; two-way ANOVA followed by Bonferroni's *post hoc* test; F(1,42) = 11.94; **P*<0.05)], but not the levels of Gαi3 [F(1,42) = 0.247] (c), Gαo [F(1,42) = 0.124] (d), or Gαq [F(1,43) = 0.366] (e)(dose: 15 mg/kg; *n* = 11–12 per group; two-way ANOVA followed by Bonferroni's *post hoc* test; *P*>0.05 for all three subunits). (f) Co-IP analysis revealed that Gαz dissociated from MOPRs 30 min after an acute morphine injection [15 mg/kg; *n* = 3–4/group; two-way ANOVA followed by Bonferroni's *post hoc* test, F(1,11) = 17.84; **P*<0.05]. (g) However, chronic treatment with morphine for 4 consecutive days promoted MOPR-Gαz complexes measured 30 min after the last injection [15 mg/kg; *n* = 4 per group; two-way ANOVA followed by Bonferroni's *post hoc* test, F(1,12) = 32.94; **P*<0.05, ****P*<0.001]. S: saline, M: morphine, TL: total lysate.

Figure 2. Role of RGSz1 in morphine-induced analgesia. (a) Analgesic responses to morphine are enhanced in RGSz1KO mice compared with those in RGSz1WT controls [each dose was tested in a separate group of mice; *n* = 8–10/group; two-way ANOVA followed by Bonferroni's *post hoc* test, F(1,64) = 9.28; **P*<0.05, ***P*<0.01]. A similar phenotype was observed in female mice [inset: dose, 7.5 mg/kg; *n* = 10–13/group; *t* test, *t*(21) = 2.885; ***P*<0.001]. (b) RGSz1KO mice show increased analgesic responses compared with those in the RGSz1WT mice to fentanyl [0.125 mg/kg; *n* = 9–11 per genotype; *t* test; *t*(18) = 4.677; ****P*<0.001] and methadone [5 mg/kg; *n* = 11‑12/group; *t* test, *t*(21) = 2.519; **P*<0.05]. (c) The development of tolerance to 15 mg/kg morphine was delayed in male [*n* = 7/group; two-way ANOVA repeated-measures followed by Bonferroni's *post hoc* test, F(1,12) = 20.99; ****P*<0.001]

and female [inset: *n* = 6–7/group; two-way ANOVA repeated-measures followed by Bonferroni's *post hoc* test, F(1,11) = 22.89; ***P<0.001] RGSz1KO mice. (d) Delayed development of morphine tolerance was also observed under inflammatory pain states. Withdrawal latencies of hind paws with CFA-induced inflammation were significantly higher in RGSz1KO mice after 3 and 4 days, demonstrating that morphine (3 mg/kg) analgesia was maintained [*n* = 4–5/group; two-way ANOVA repeated-measures followed by Bonferroni's *post hoc* test, F(1,7) = 18.59; **P<0.01, ***P<0.001]. MPE: maximum possible effect.

Figure 3. RGSz1 modulates reward-like behaviors. (a–d) Knockout of RGSz1 affects the rewarding effect of low morphine doses. RGSz1KO mice did not show conditioned place preference with 1 mg/kg and spent less time in the drug-paired side when 3 mg/kg of morphine was used. At higher doses (8 mg/kg), both genotypes showed conditioned place preferences to morphine [two-way repeated-measures ANOVA followed by Bonferroni's *post hoc* test; 0.5 mg: *n* = 8/group, F(1,14) = 1.12; 1 mg: *n* = 13/group, F(1,24) = 0.33, **P<0.01; 3 mg: *n* = 11–12/group, F(1,21) = 2,94, **P<0.01, ***P<0.001; 8 mg: *n* = 16–19/group, F(1,33) = 0.65, **P<0.01, ***P<0.001]. (e) Furthermore, RGSz1KO male mice showed lower locomotor activity in response to repeated morphine administrations than RGSz1WT control mice [10 mg/kg; *n* = 9–10/group; two-way ANOVA repeated-measures followed by Bonferroni's *post hoc* test, F(1,17) = 11.16; *P<0.05, **P<0.01, ***P<0.001].

Figure 4. *Rgsz1* knockdown in vlPAG neurons suppresses morphine tolerance. (a) Conditional knockdown of *Rgsz1* in neurons in the vlPAG of RGSz1$^{fl/fl}$ mice by stereotaxic infection of AAV2-CMV-CRE vectors maintained the analgesic effects of morphine in the hot plate assay over several days [15 mg/kg; *n* = 7/group; two-way repeated-measures ANOVA followed by Bonferroni's *post hoc* test, F(1,12) = 11.34; *P<0.05, **P<0.01, ***P<0.001]. (b) Western blot analysis verifying RGSz1 downregulation in the PAG of CRE virus-infected mice [*n*

= 4–5/group; *t* test, *t*(7) = 2.663; **P*<0.05] and verification of AAV2 viral vector distribution in the vlPAG. AAV2 vectors infect neurons (as indicated by NeuN staining) and not astrocytes (as indicated by GFAP staining). (c) The effect of *Rgsz1* knockdown on the development of morphine tolerance was region specific, and no effect was observed when neurons of the NAc were targeted [15 mg/kg; *n* = 6–7/group; two-way repeated-measures ANOVA followed by Bonferroni's *post hoc* test, F(1,11) = 0.08]. (d) Western blot analysis verifying RGSz1 downregulation in the NAc of CRE virus-infected mice [*n* = 9-10/group; *t* test, *t*(17) = 2.123; **P*<0.05] and verification of AAV2 viral vector distribution in the NAc. (e) Knockdown of RGSz1 in the vlPAG does not affect the intensity of naloxone-precipitated morphine withdrawal symptoms (*n* = 9–10/group; *t* tests).

Figure 5. RNA-Seq of PAG tissue from morphine-treated RGSz1WT and RGSz1KO mice. (a) Experimental timeline showing that mice were treated for 4 consecutive days with 15 mg/kg morphine and tested in the hot-plate assay to evaluate analgesic responses. On day 4 (when analgesic tolerance emerges in RGSz1WT mice) PAG tissue was collected 2 h after morphine administration. (b) Heatmap analysis showing the effect of drug treatment on gene expression in RGSz1WT and RGSz1KO mice. The overall patterns of gene expression regulation were similar between the two genotypes, but a much larger group of genes was affected in the PAG of RGSz1KO mice. (c) Venn diagram depicting the actual numbers of genes affected in each group and their direction of regulation and associated Gene ontology (GO) categories. Pie chart depicts results of a GO analysis of the 225 genes regulated by morphine in the PAG of both genotypes. Bars graphs show GO of genes uniquely regulated by morphine in the PAG of RGSz1WT (red) and RGSz1KO (light gray) mice.

Figure 6. Chronic morphine affects different intracellular pathways in the PAG of RGSz1KO and RGSz1WT mice. (a) IPA analysis of the genes regulated by chronic morphine

revealed distinct canonical pathways were affected in RGSz1WT and RGSz1KO mice (cut-off: *p-value*<0.003). (b) Selected genes in the canonical Wnt/β-catenin signaling or inflammatory response-related cascades and G-protein coupled/serotonin receptor signaling pathways and the direction of their regulation. (c) qPCR validation of a subset of genes in a separate cohort of animals by genotype [*n* = 7–8/group; *t* tests, RGSz1WT: *Ddc*, *t*(13) = 2.135; *Gch1*, *t*(13) = 2.2; *Pdyn*, *t*(13) = 2.27; *Bdkrb1*, t(14) = 2.154; *Wnt9a*, *t*(14) = 3.4; *Cxcl12*, *t*(14) = 3.69; *Ccr1*, *t*(12) = 2.99; *Il1r1*, *t*(14) = 2.44; *Cxcr4*, *t*(14) = 6.08; RGSz1KO: *Ddc*, *t*(13) = 2.29; *Tph2*, *t*(13) = 2.4, *Gch1*, *t*(13) = 2.36; *Pdyn*, *t*(13) = 2.9; *Wnt9a*, *t*(14) = 0.87; *Il1r1*, *t*(12) = 5.49; *NPY*, t(14) = 2.86; *Gabre*, *t*(14) = 2.98; **P*<0.05, ***P*<0.01, ****P*<0.001]. ns: not significant.

Figure 7. RGSz1 modulates the activity of the Wnt/β-catenin signaling pathway. (a) Schematic depiction of Wnt/β-catenin signaling pathway components (in blue) that are affected by chronic morphine in the PAG of RGSz1WT mice only. (b) Bar graph showing the subset of genes that are known β-catenin targets and the direction of their regulation after chronic morphine administration. Notably, the majority of genes in the RGSz1WT group were downregulated. (c) Western blot analysis revealed regulation of active phospho-β-catenin (ser675) only in the PAG of RGSz1KO mice after 4 consecutive days of morphine administration [15 mg/kg; *n* = 14–15/group; two-way ANOVA followed by Bonferroni's *post hoc* test, F(1,53) = 7.37; **P*<0.05], with total β-catenin levels remaining unaffected [*n* = 11–12/group; two-way ANOVA followed by Bonferroni's *post hoc* test, F(1,40) = 0.18]. (d) Levels of inactive phospho-GSK3β (ser9) were also increased in the PAG of morphine-treated RGSz1KO mice [15 mg/kg; two-way ANOVA followed by Bonferroni's *post hoc* test; for phospho-GSK3β: *n* = 14–15/group; F(1,53) = 11.65, ***P*<0.01; for total GSK3β: *n* = 11–12/group; F(1,40) = 1.004]. (e) Antagonizing the activity of β-catenin in the vlPAG by overexpressing a dominant-negative form in RGSz1KO mice led to the development of analgesic tolerance to morphine [15 mg/kg; *n* = 7 for RGSz1KO-

HSV-GFP and 12 for RGSz1KO-HSV-β-catDN; two-way repeated-measures ANOVA followed by Bonferroni's *post hoc* test, F(1,51) = 9.9; **P*<0.05, ****P*<0.001). However, overexpressing β-catenin in the vlPAG of RGSz1WT mice did not delay the development of analgesic tolerance [15 mg/kg; *n* = 9 for RGSz1WT-HSV-GFP and 10 for RGSz1WT-HSV-β-cat; two-way repeated-measures ANOVA followed by Bonferroni's *post hoc* test, F(1,51) = 0.14]. S: saline, M: morphine.

Figure 8. RGSz1 knockout prevents dissociation of Axin2-Gαz complexes after chronic morphine. (a) Protein levels of Axin2 in the PAG are not affected by morphine treatment in RGSz1WT or RGSz1KO mice [15 mg/kg; n = 12/group; two-way ANOVA followed by Bonferroni's *post hoc* test, F(1,44) = 0.19]. (b) Co-IP assays on synaptosomal fractions revealed a reduced association of Axin2 with Gαz in RGSz1WT but not in RGSz1KO mice after repeated morphine administration [15 mg/kg; *n* = 3/group; two-way ANOVA followed by Bonferroni's *post hoc* test; F(1,8) = 7.6; ***P*<0.01]. (c) Morphine tolerance in RGSz1WT also led to higher levels of Axin2 in the nucleus [15 mg/kg; *n* = 3/group; two-way ANOVA followed by Bonferroni's *post hoc* test; F(1,8) = 7; **P*<0.05]. (d) Correlation analysis reveals a negative association between the amount of Axin2 bound to Gαz in the synaptosomal fractions and the amount of Axin2 in the nuclear fractions of the same samples from RGSz1WT but not RGSz1KO mice. (e) Schematic depicting working hypothesis. In the PAG of tolerant mice, higher levels of RGSz1 and stabilized complexes between MOPR and Gαz lead to a reduced Axin2-Gαz interaction, thereby enabling the function of Axin2-containing destruction complexes, as well as the translocation of Axin2 to the nucleus and silencing β-catenin-mediated transcription. In the absence of RGSz1, Axin2 is maintained in the cytoplasm and forms complexes with Gαz, whereas β-catenin may translocate to the nucleus to promote transcriptional activity. S: saline, M: morphine, TL: total lysate

Figure 1

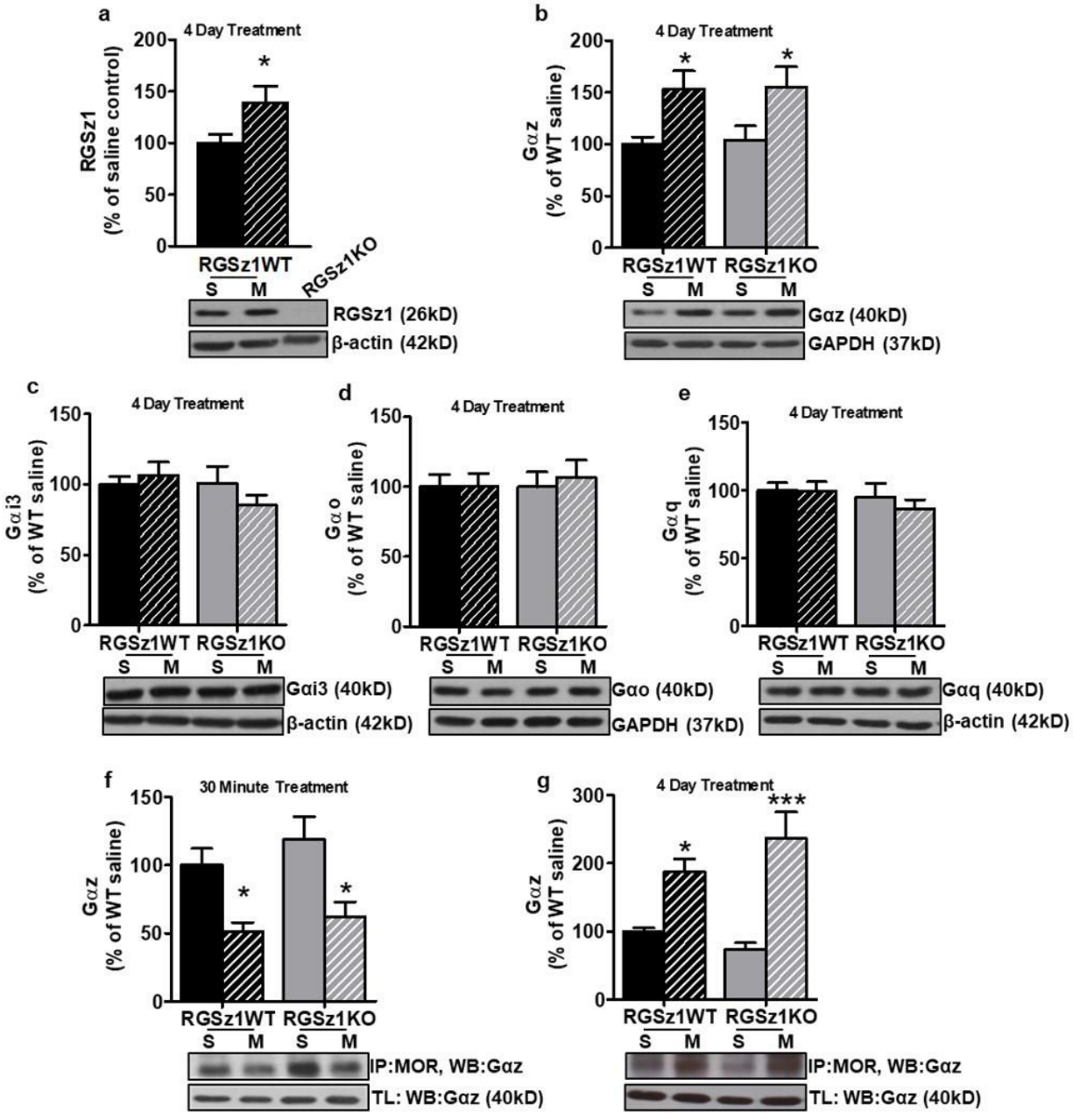

a
4 Day Treatment
RGSz1 (% of saline control)
200
150
100
50
0
*
RGSz1WT
RGSz1KO
S M
RGSz1 (26kD)
β-actin (42kD)
b
4 Day Treatment
Gαz (% of WT saline)
200
150
100
50
0
*
*
RGSz1WT RGSz1KO
S M S M
Gαz (40kD)
GAPDH (37kD)
c
4 Day Treatment
Gαi3 (% of WT saline)
150
100
50
0
RGSz1WT RGSz1KO
S M S M
Gαi3 (40kD)
β-actin (42kD)
d
4 Day Treatment
Gαo (% of WT saline)
150
100
50
0
RGSz1WT RGSz1KO
S M S M
Gαo (40kD)
GAPDH (37kD)
e
4 Day Treatment
Gαq (% of WT saline)
150
100
50
0
RGSz1WT RGSz1KO
S M S M
Gαq (40kD)
β-actin (42kD)
f
30 Minute Treatment
Gαz (% of WT saline)
150
100
50
0
*
*
RGSz1WT RGSz1KO
S M S M
IP:MOR, WB:Gαz
TL: WB:Gαz (40kD)
g
4 Day Treatment
Gαz (% of WT saline)
300
200
100
0
*
***
RGSz1WT RGSz1KO
S M S M
IP:MOR, WB:Gαz
TL: WB:Gαz (40kD)

Figure 2

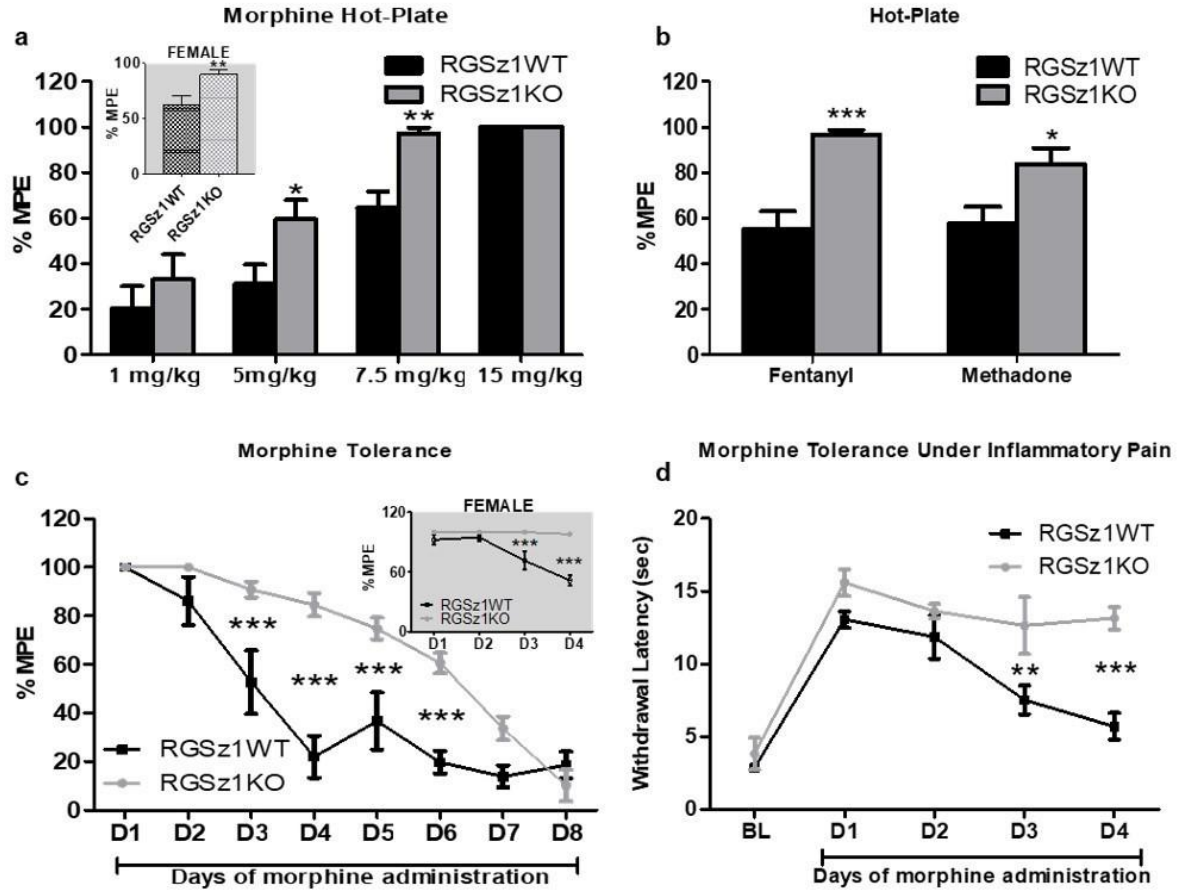

a
Morphine Hot-Plate
FEMALE
% MPE
RGSz1WT
RGSz1KO
1 mg/kg
5mg/kg
7.5 mg/kg
15 mg/kg
b
Hot-Plate
Fentanyl
Methadone
c
Morphine Tolerance
Days of morphine administration
d
Morphine Tolerance Under Inflammatory Pain
Withdrawal Latency (sec)
BL
D1
D2
D3
D4
D5
D6
D7
D8

Figure 3

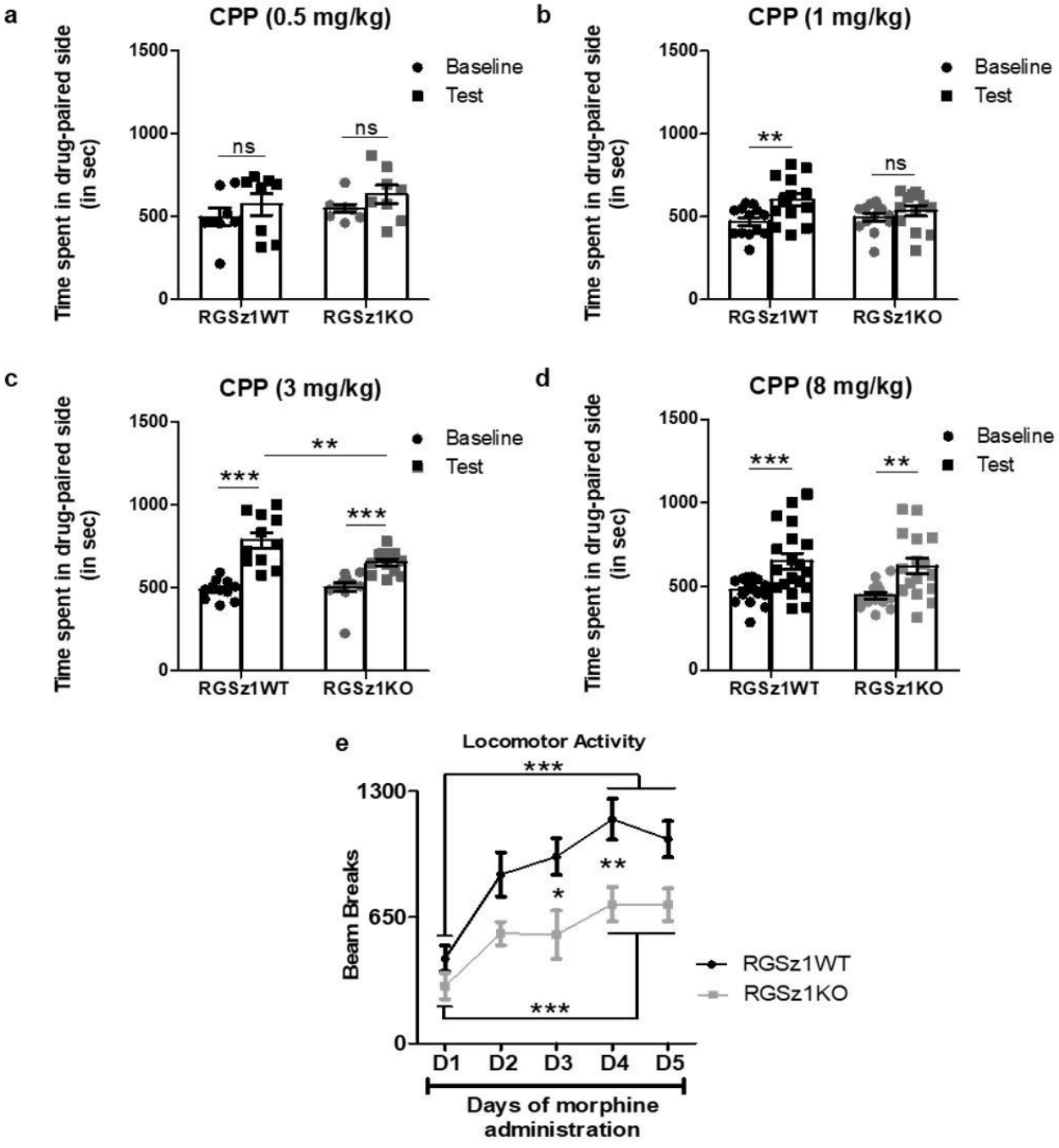

a
CPP (0.5 mg/kg)
Time spent in drug-paired side (in sec)
1500
1000
500
0
Baseline
Test
ns
ns
RGSz1WT
RGSz1KO
b
CPP (1 mg/kg)
Time spent in drug-paired side (in sec)
1500
1000
500
0
Baseline
Test
**
ns
RGSz1WT
RGSz1KO
c
CPP (3 mg/kg)
Time spent in drug-paired side (in sec)
1500
1000
500
0
Baseline
Test
**
***
***
RGSz1WT
RGSz1KO
d
CPP (8 mg/kg)
Time spent in drug-paired side (in sec)
1500
1000
500
0
Baseline
Test
***
**
RGSz1WT
RGSz1KO
e
Locomotor Activity
***
1300
650
0
Beam Breaks
**
*
***
RGSz1WT
RGSz1KO
D1
D2
D3
D4
D5
Days of morphine administration

Figure 4

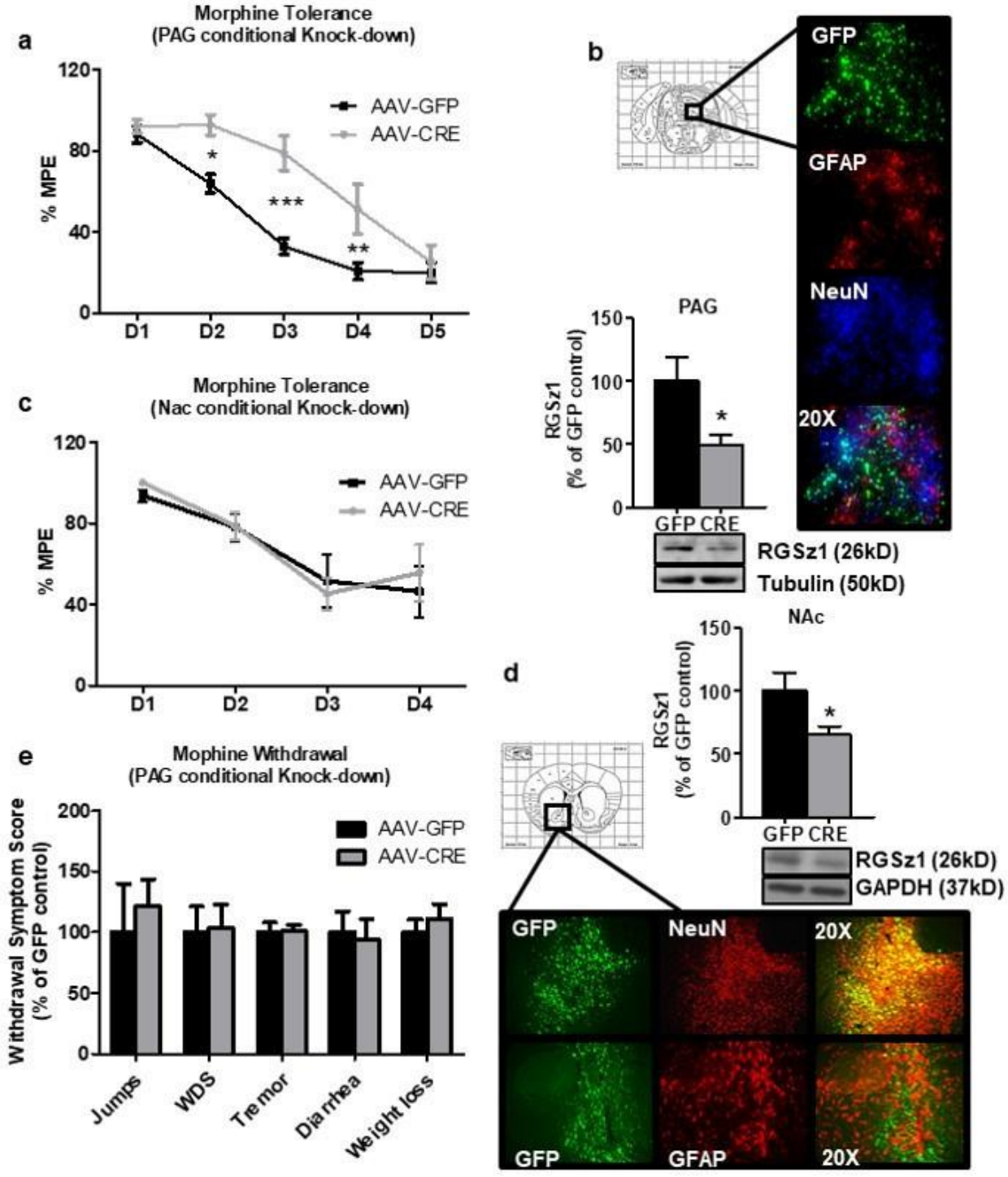

a
Morphine Tolerance
(PAG conditional Knock-down)
% MPE
AAV-GFP
AAV-CRE
D1
D2
D3
D4
D5
b
GFP
GFAP
NeuN
20X
PAG
RGSz1 (% of GFP control)
GFP CRE
RGSz1 (26kD)
Tubulin (50kD)
c
Morphine Tolerance
(Nac conditional Knock-down)
d
NAc
GAPDH (37kD)
e
Mophine Withdrawal
(PAG conditional Knock-down)
Withdrawal Symptom Score
(% of GFP control)
Jumps
WDS
Tremor
Diarrhea
Weight loss

Figure 5

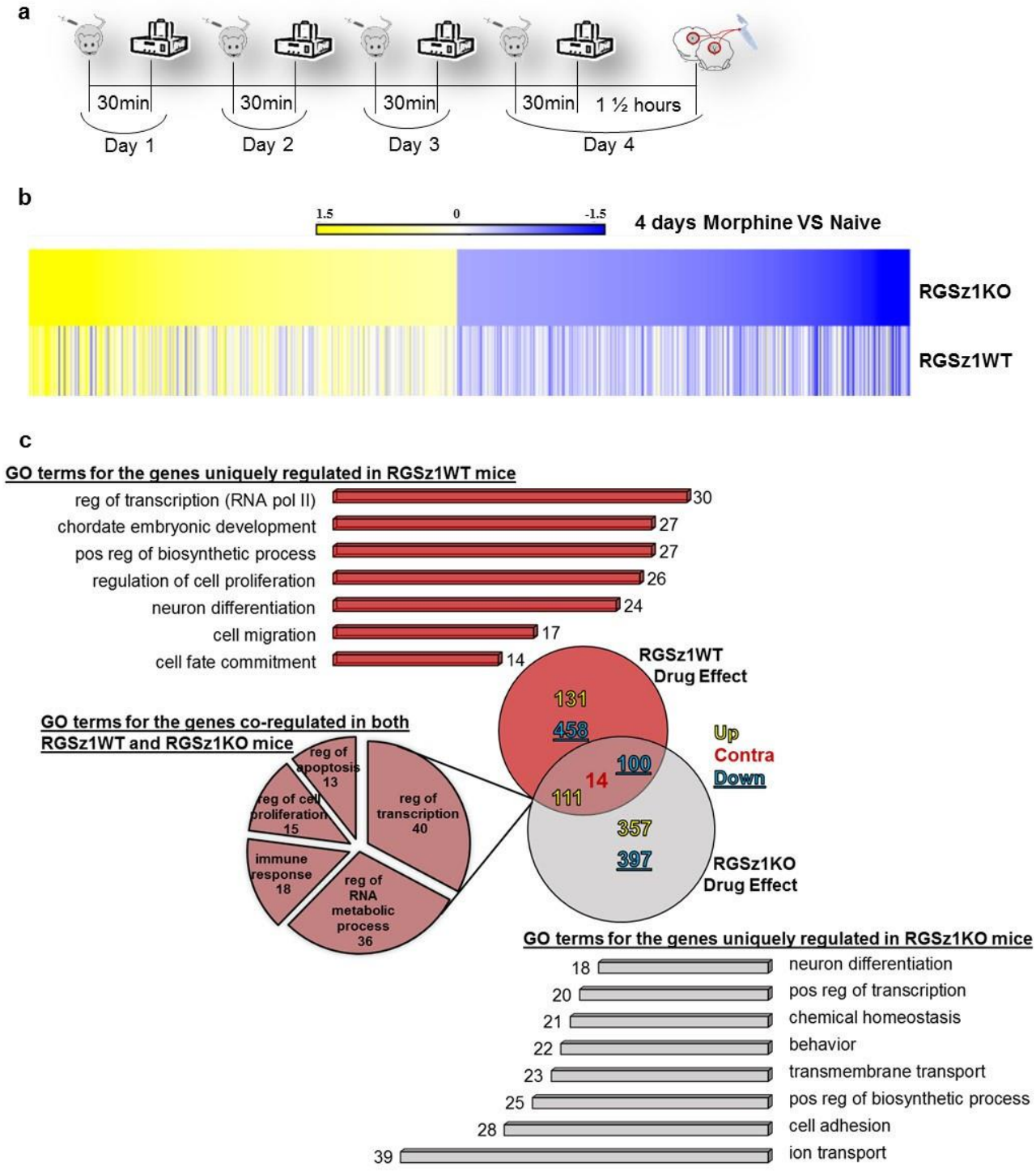
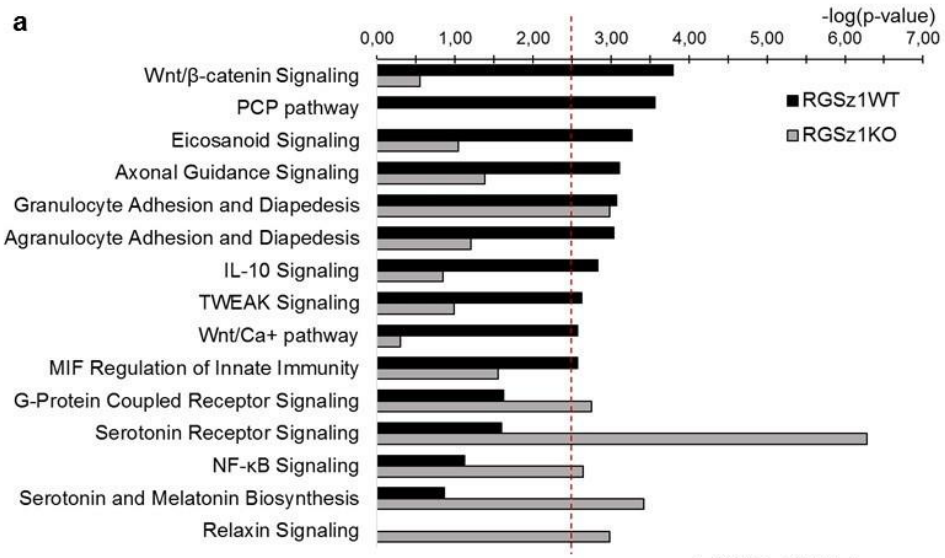

a
30min
30min
30min
30min
1 ½ hours
Day 1
Day 2
Day 3
Day 4
b
1.5
0
-1.5
4 days Morphine VS Naive
RGSz1KO
RGSz1WT
c
GO terms for the genes uniquely regulated in RGSz1WT mice
reg of transcription (RNA pol II) 30
chordate embryonic development 27
pos reg of biosynthetic process 27
regulation of cell proliferation 26
neuron differentiation 24
cell migration 17
cell fate commitment 14
RGSz1WT Drug Effect
131
458
Up
Contra
Down
100
14
111
357
397
RGSz1KO Drug Effect
GO terms for the genes co-regulated in both RGSz1WT and RGSz1KO mice
reg of apoptosis 13
reg of cell proliferation 15
reg of transcription 40
immune response 18
reg of RNA metabolic process 36
GO terms for the genes uniquely regulated in RGSz1KO mice
18 neuron differentiation
20 pos reg of transcription
21 chemical homeostasis
22 behavior
23 transmembrane transport
25 pos reg of biosynthetic process
28 cell adhesion
39 ion transport

Figure 6



b

GENE	RGSz1WT	RGSz1KO
Axin2	↓	—
Dkk2	↓	—
Fzd10	↓	—
Sox2	↓	—
Wnt7a	↓	—
Wnt9a	↓	—
Pdyn	↑	↑
Gch1	↑	↑
Ddc	↑	↑
Tph2	—	↑
Npy	—	↑
Junb	↓	—
Ccr1	↓	—
Il1r1	↑	↑
Gabre	—	↑
Htr2b	—	↓
Ngfr	—	↑
Slc6a4	—	↑
Fos	↑	↑
Nos2	—	↓

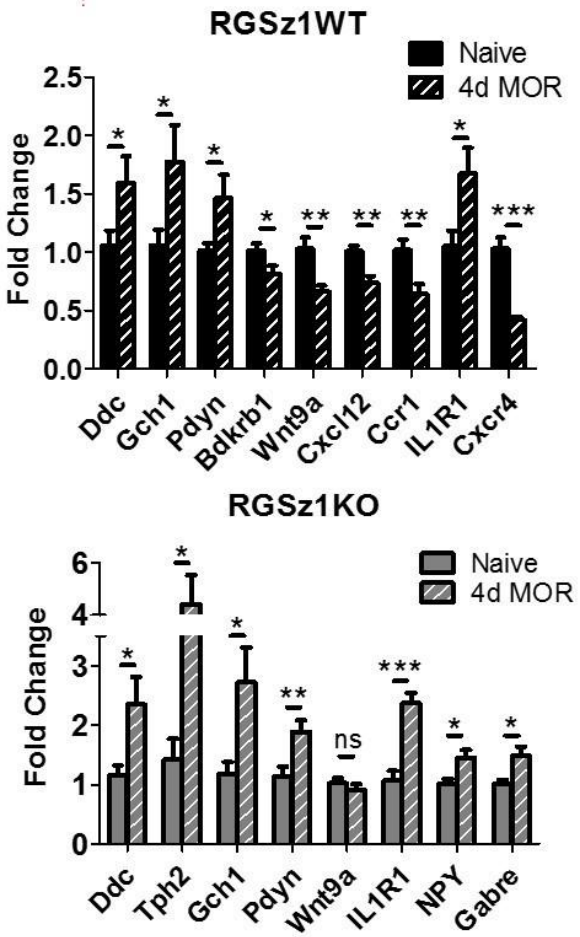


Figure 7

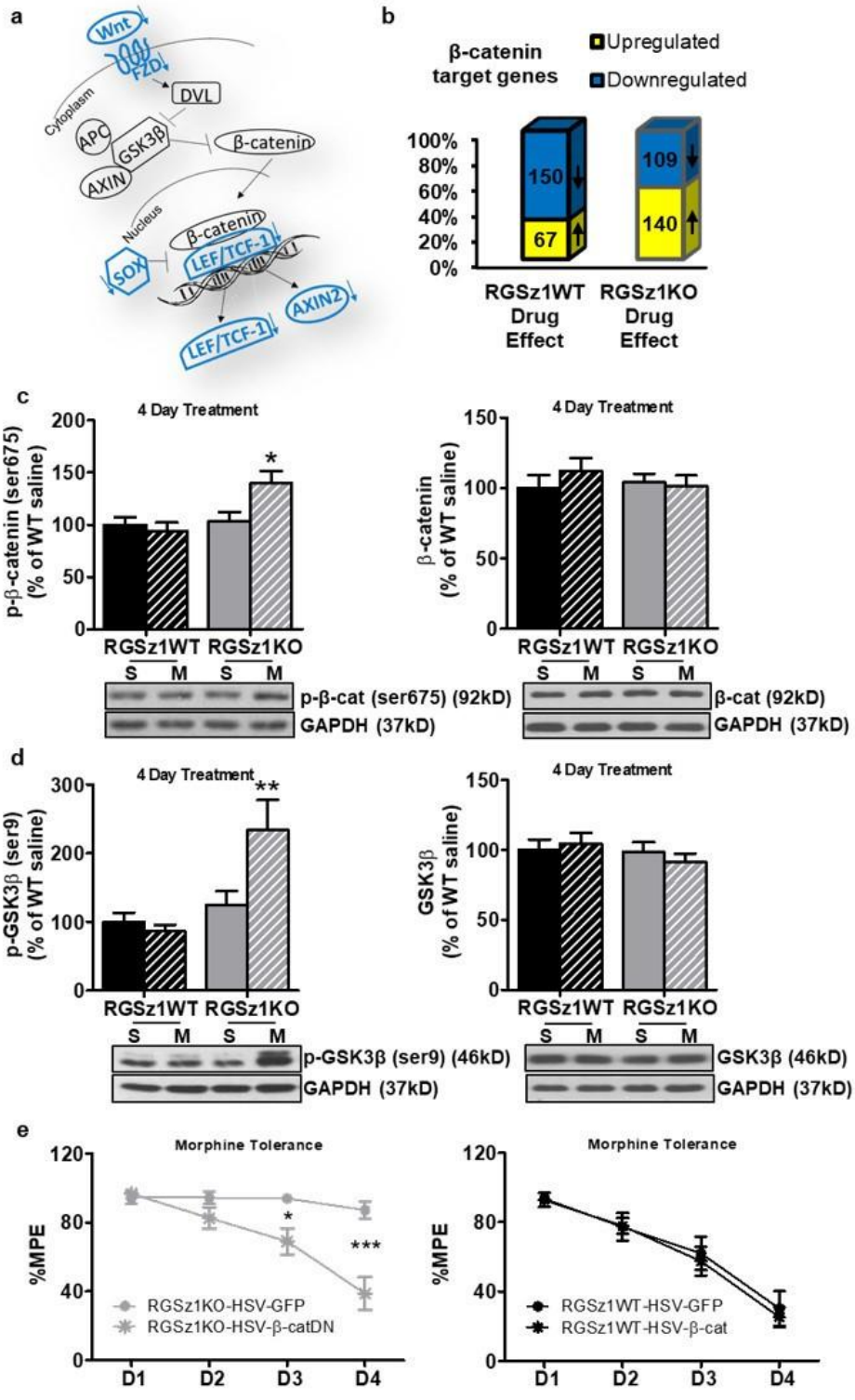
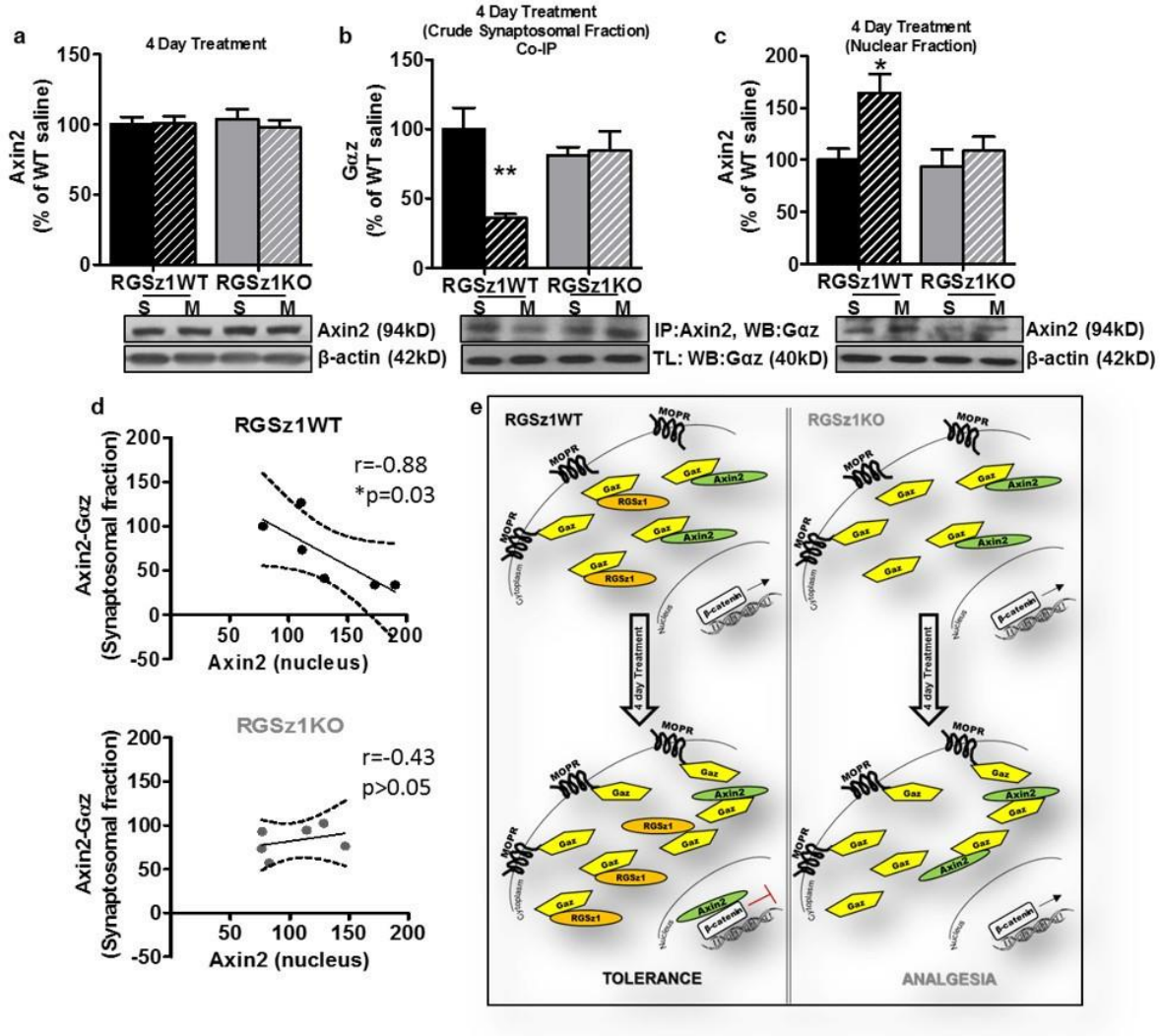

a
Wnt
FZD
DVL
Cytoplasm
APC
GSK3β
AXIN
β-catenin
Nucleus
β-catenin
SOX
LEF/TCF-1
AXIN2
LEF/TCF-1
b
β-catenin target genes
Upregulated
Downregulated
100%
80%
60%
40%
20%
0%
150
67
109
140
RGSz1WT Drug Effect
RGSz1KO Drug Effect
c
4 Day Treatment
p-β-catenin (ser675) (% of WT saline)
RGSz1WT RGSz1KO
S M S M
p-β-cat (ser675) (92kD)
GAPDH (37kD)
4 Day Treatment
β-catenin (% of WT saline)
RGSz1WT RGSz1KO
S M S M
β-cat (92kD)
GAPDH (37kD)
d
4 Day Treatment
p-GSK3β (ser9) (% of WT saline)
RGSz1WT RGSz1KO
S M S M
p-GSK3β (ser9) (46kD)
GAPDH (37kD)
4 Day Treatment
GSK3β (% of WT saline)
RGSz1WT RGSz1KO
S M S M
GSK3β (46kD)
GAPDH (37kD)
e
Morphine Tolerance
%MPE
RGSz1KO-HSV-GFP
RGSz1KO-HSV-β-catDN
D1 D2 D3 D4
Morphine Tolerance
%MPE
RGSz1WT-HSV-GFP
RGSz1WT-HSV-β-cat
D1 D2 D3 D4

Figure 8


a
4 Day Treatment
Axin2 (% of WT saline)
RGSz1WT RGSz1KO
S M S M
Axin2 (94kD)
β-actin (42kD)
b
4 Day Treatment
(Crude Synaptosomal Fraction)
Co-IP
Gαz (% of WT saline)
**
IP:Axin2, WB:Gαz
TL: WB:Gαz (40kD)
c
4 Day Treatment
(Nuclear Fraction)
Axin2 (% of WT saline)
*
Axin2 (94kD)
β-actin (42kD)
d
RGSz1WT
r=-0.88
*p=0.03
Axin2-Gαz (Synaptosomal fraction)
Axin2 (nucleus)
RGSz1KO
r=-0.43
p>0.05
e
RGSz1WT
RGSz1KO
MOPR
Gαz
Axin2
RGSz1
β-catenin
4 day Treatment
TOLERANCE
ANALGESIA