ROLE OF THE REGULATOR OF G PROTEIN SIGNALING 2 (RGS2) FOR NEURONAL AND SYSTEM FUNCTION

by

Jing Han

Submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy

Thesis Advisor: Stefan Herlitze, PhD

Department of Neurosciences

CASE WESTERN RESERVE UNIVERSITY

May 2007
CASE WESTERN RESERVE UNIVERSITY
SCHOOL OF GRADUATE STUDIES

We hereby approve the dissertation of

Jing Han

__________________________

candidate for the Ph.D. degree *.

Evan Deneris

(signed)______________________________

(chair of the committee)

Lynn Landmesser

__________________________

Stefan Herlitze

__________________________

George Dubyak

__________________________

__________________________

3/21/2007

(date) _______________________

* We also certify that written approval has been obtained for any proprietary material contained therein.
# Table of Contents

## Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table of Contents</td>
<td>1</td>
</tr>
<tr>
<td>List of Figures</td>
<td>3</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>5</td>
</tr>
<tr>
<td>Abstract</td>
<td>6</td>
</tr>
<tr>
<td><strong>Chapter 1. Introduction</strong></td>
<td>8</td>
</tr>
<tr>
<td>G protein coupled receptor, G-protein subunits and G protein modulation of ion channels</td>
<td>9</td>
</tr>
<tr>
<td>Regulators of G Protein Signaling (RGS) Protein</td>
<td>11</td>
</tr>
<tr>
<td>RGS2</td>
<td>16</td>
</tr>
<tr>
<td>RGS2(-/-) Mouse</td>
<td>17</td>
</tr>
<tr>
<td>The serotonergic (5-HT) system</td>
<td>20</td>
</tr>
<tr>
<td><strong>Chapter 2. RGS2 determines short-term synaptic plasticity in hippocampal neurons by regulating G\textsubscript{i\textsubscript{o}} mediated inhibition of presynaptic Ca\textsuperscript{2+} channels</strong></td>
<td>29</td>
</tr>
<tr>
<td>Abstract</td>
<td>30</td>
</tr>
<tr>
<td>Introduction</td>
<td>31</td>
</tr>
<tr>
<td>Results</td>
<td>34</td>
</tr>
<tr>
<td>Discussion</td>
<td>47</td>
</tr>
<tr>
<td>Experimental Procedures</td>
<td>53</td>
</tr>
<tr>
<td>Figures</td>
<td>58</td>
</tr>
<tr>
<td>Supplemental Methods</td>
<td>81</td>
</tr>
</tbody>
</table>
Chapter 3. RGS2 regulates serotonergic signaling in mouse brain 85

Abstract 86
Introduction 87
Results 91
Discussion 96
Experimental Procedures 102
Figures 107

Chapter 4. Discussion 117

Predictions for the modulation of ion conductances by RGS2 and the consequences for influencing the physiological properties and transmitter release of serotonergic neurons 118

Putative effects of RGS2 on neuronal morphology 122

Expression of RGS2 or RGS2 mutant (RGS2N148A) specifically in the serotonergic system to elucidate the function of RGS2 in this transmitter system 124

Possible Therapeutic Uses of RGS2-Related Drugs 128

References 131
List of Figures

Chapter 2

Figure 1. RGS2 expressed in hippocampal neurons targets to synaptic sites and regulates short-term synaptic plasticity. 58

Figure 2. Cultured hippocampal neurons from RGS2(-/-) and wild-type mice have comparable G protein expression and make comparable amounts of synaptic contacts. 61

Figure 3. RGS2 regulates the probability of synaptic vesicle release and Ca$^{2+}$ dependence of transmitter release. 64

Figure 4. Synaptic vesicle recycling as well as spontaneous release properties are not altered in hippocampal neurons from RGS2(-/-) mice. 66

Figure 5. RGS2 regulates synaptic plasticity through PTX-sensitive pathways. 69

Figure 6. The RGS2 mutant N149A, which is able to affect the G_q pathway but not the G_{i/o} pathway, is unable to rescue the increased PPR in RGS2(-/-) autapses but acts as a dominant negative mutant in wild-type neurons. 71
Figure 7. G protein mediated Ca\(^{2+}\) channel inhibition is increased in hippocampal neurons from RGS2(-/-) mice, while BoNT-A has similar effects on the release probability and PPR of neurons from wild-type and RGS2 knock-out mice.  

Supplemental Figure.

Chapter 3

Figure 1. RGS2 mRNA is detected in YFP positive, serotonergic neurons at early developmental stages.

Figure 2. The morphology of the serotonergic system at postnatal and embryonic stages is not altered in RGS2(-/-) mice.

Figure 3. The G protein modulation of Ca\(^{2+}\) channels is increased in serotonergic neurons of RGS2(-/-) mice in comparison to wild-type mice suggesting that the basal G protein activity is increased.

Figure 4. The membrane properties of YFP positive serotonergic neurons from hindbrain (DR) slices are different between RGS2(-/-) and wild-type mice.

Figure 5. Spontaneous firing of serotonergic neurons in B7 was reduced in RGS2 (-/-) mice.
Acknowledgements

I would like to thank my thesis advisor Dr. Stefan Herlitze for helping me through all the difficult time during my graduate career. I also thank my former and present committee members: Dr.s Lynn Landmesser, Evan Deneris, George Dubyak, Bryan Ross and Iain Robinson for their tremendous encouragement and support. Many people in the Neuroscience Department gave me generous help. Particularly, I want to thank Christi Wylie for helping me with my serotonin study. Lastly, I want to thank all the members in the Herlitze lab for providing such a good environment and their excellent work in finishing my paper.
ROLE OF THE REGULATOR OF G PROTEIN SIGNALING 2 (RGS2) FOR NEURONAL AND SYSTEM FUNCTION

Abstract

by

Jing Han

RGS2, one of the small members of the regulator of G protein signaling (RGS) family, is highly expressed in brain and regulates Gi/o as well as Gq coupled receptor pathways. RGS2 modulates anxiety, aggression and blood pressure in mice suggesting that RGS2 regulates synaptic circuits underlying animal physiology and behavior. How RGS2 in brain influences synaptic activity was unknown. We therefore analyzed the synaptic function of RGS2 in hippocampal neurons and the system function of RGS2 in the serotonergic system by comparing electrophysiological recordings from RGS2 knock-out and wild type mice. Our study from hippocampal neurons provides a general mechanism of the action of the RGS family containing RGS2 by demonstrating that RGS2 increases synaptic vesicle release by down-regulating the Gi/o mediated presynaptic Ca^{2+} channel inhibition. Our results from serotonergic neurons reveal that loss of RGS2 leads to changes in the physiological properties of the neurons, including changes in membrane potential and spontaneous firing which are mediated by increased G protein activity in these neurons. Our results
suggest that RGS2 regulates precisely the firing and output of serotonergic neurons in the brain, which provides for the first time an explanation of how regulation of RGS2 expression can modulate the function of neuronal circuits underlying behavior.
CHAPTER 1

INTRODUCTION
**G protein coupled receptor, G-protein subunits and G protein modulation of ion channels**

G protein signaling interconnects extracellular signals with intracellular effectors and is involved in many physiological and pathological processes. G protein coupled receptor (GPCR) activation via agonist binding initiates the exchange of GDP for GTP on the Gα subunit, allowing the dissociation of its Gβγ counterpart to interact with different effectors such as ion channels. The hydrolysis of GTP to GDP on the Gα subunit leads to the re-association of the Gβγ dimer with the Gα subunit and termination of the signal (Hamm, 1998). There are four major classes of Gα proteins categorized by their structure and functional homologies: Gαs, Gαi/o, Gαq, and Gα12/13. These four classes of Gα proteins recognize different effectors leading to changes of various cellular functions. For example, Gαs stimulates adenylyl cyclases (AC), Gαi/o inhibits adenylyl cyclases (AC), and Gαq activates phosphoinositide-specific phospholipase C (PI-PLC) isozymes.

Although Gα subunits have long been considered as the G protein subunit responsible for activation of downstream effector proteins, more and more studies have shown that G protein βγ-complexes also play a prominent role in regulating different effectors. The G-protein regulated inward-rectifier K+ channels (GIRK) were the first identified target of the βγ-complex (Huang et al., 1995).
Voltage-gated calcium channels are also regulated by Gβγ-complexes. In the 1980s, several studies demonstrated that the role of G proteins in the inhibition of calcium currents by GPCR activation is through a direct, membrane-delimited second messenger pathway suggesting the close proximity of the G protein and the calcium channel during channel inhibition (Forscher et al., 1986). In 1996, two groups independently reported that Gβγ subunits are responsible for modulating calcium channels (Herlitze et al., 1996a; Ikeda, 1996a; Jarvis et al., 2000). Through the transfection of cell lines or primary neurons, it was shown that Gβγ subunits mimicked GPCR induced modulation of the channel leading to tonic inhibition of the N- and P/Q-type calcium current. This inhibition could be transiently reversed by a high depolarizing prepulse, one important criteria of the voltage dependent inhibition of presynaptic calcium channels (Elmslie et al., 1990).

N- and P/Q-type calcium channels are concentrated at synaptic terminals and regulate transmitter release. Modulation of synaptic calcium channels has been shown to negatively regulate synaptic strength. All Gi/o coupled GPCRs including GABA₉ receptors, α2-adrenoceptors, µ and δ opioid receptors can regulate presynaptic calcium channels via the membrane-delimited pathway. For example Takahashi and colleagues showed that in the calyx of Held GABA₉ receptors suppress transmitter release through G protein–coupled inhibition of calcium currents at the presynaptic terminal (Takahashi et al., 1998). In addition to the inhibition of presynaptic calcium channels synaptic strength seems to also be
regulated downstream of the calcium channel by the GPCRs. In 2001 Blackmer et al. reported that in the reticulospinal/motoneuron synapse of the lamprey, serotonin’s inhibitory effect on synaptic transmission occurs downstream of calcium influx and is also mediated by Gβγ subunits (Blackmer et al., 2001). Recently, Gerachshenko et al characterized the molecular pathway of this effect. They demonstrated that the C terminus of the SNARE protein SNAP-25 is the target of Gβγ for presynaptic inhibition (Gerachshenko et al., 2005). These findings suggest that Gβγ subunits can also modulate synaptic transmission via a direct interaction with the transmitter release machinery.

Thus, activation of presynaptic GPCRs coupling to the Gi/o α subunits down-regulates synaptic transmitter release through either modulation of N- or P/Q-type calcium channels or direct interaction with synaptic vesicle release machinery. In both cases, the availability of free Gβγ subunits is the key factor in the presynaptic inhibition effect.

**Regulators of G Protein Signaling (RGS) Protein**

The duration of G-protein signal is determined by the kinetic properties of the Gα subunit. The fast activation and inactivation of GPCR signals are not consistent with the slow intrinsic GTPase rate of the Gα subunit, indicating the existence of additional regulators that speed up the G protein cycle. In fact, the termination of the G protein signal is accelerated by a superfamily of GTPase-activating proteins (GAPs) known as RGS proteins. Since the first RGS gene, SST2, was
recognized in 1980’s from genetic studies in yeast, more than 30 mammalian RGS family members have been identified as key components in the G-protein signaling pathways. All RGS proteins share a 120-residue homologous domain, the RGS domain, which is responsible for the binding of RGS protein to the $G\alpha$ subunit.

How do RGS Proteins Turn off G-protein Signals?

Three different mechanisms can account for the fast termination of the G protein signal by RGS proteins. First, RGS proteins function as GTPase activating proteins (GAPs) and accelerate the intrinsic GTPase activity of $G\alpha$ subunit up to 1000-fold (Berman et al., 1996a; Berman et al., 1996b; Hepler et al., 1997; Hunt et al., 1996). In vitro studies demonstrated that RGS proteins bind directly to the active GTP-bound $G\alpha$ subunit and stabilize the GDP-M$^{2+}$-AlF$_4^-$-bound $G\alpha$ subunit, which mimics the transition state during GTP hydrolysis (Berman et al., 1996a). The crystal structure of RGS4 protein bound to $G_i\alpha_1$-GDP-AlF$_4^-$ showed that the active core of the RGS domain formed a four-helix bundle, which stabilized the transition state for hydrolysis of $G\alpha$-bound GTP by interacting with three flexible switch regions of the $G\alpha$ subunit. Additionally, several studies have demonstrated that the Asn$^{128}$ residue of RGS4 is critical for GAP activity of RGS4. This residue is important for the ability of RGS4 to bind and stabilize the transition state of the $G\alpha$ subunit, and is involved in substrate binding and the hydrolytic event. Asn128 is highly conserved among most RGS proteins. Mutations of this residue can either decrease or abolish the apparent affinity of
RGS for the $G_\alpha$ subunit and the GTP hydrolysis. Thus, RGS proteins reduce the amount of active $G_\alpha$ subunits at the steady-state level and inhibit their signaling.

Second, RGS proteins interfere with the binding between GTP-bound $G_\alpha$ subunits and their effectors by physical interaction (De Vries et al., 2000). For example, RGS2 was found to antagonize the activation of adenylate cyclase by $G_\alpha$s. This blocking principle was first demonstrated in studies showing that RGS4 and RGS19 inhibit Gq-mediated signaling when activated by the non-hydrolysable GTP-analogue, GTP\textsubscript{S} (Hepler et al., 1997). In this case RGS4 inhibits ALF\textsubscript{4}-mediated G-protein activation of PLC. Additionally, it was later demonstrated that some other RGS proteins, including RGS2, RGS3 and RGS10, can inhibit G-protein signaling of a constitutively active Gq subunit which lost its GTPase activity (Scheschenka et al., 2000). Thus, RGS proteins can bind to active $G_\alpha$ subunits and block effector activation independent of GAP activity.

Third, RGS proteins can regulate the availability of G proteins by inhibiting guanine nucleotide dissociation. For example, RGS12 and RGS14 not only have GAP activity that regulates $G_\alpha i$ and $G_\alpha o$-mediated signaling but also act as guanine nucleotide dissociation inhibitors (GDIs) on $G_\alpha i$ via a C-terminal GoLoco motif (Kimple et al., 2001). The GoLoco domain selectively interacts with GDP-bound $G_\alpha$ subunits and prevents the GDP release, leading to inhibition of G protein activation.
The Biological Function of RGS Proteins

Most functional assays of RGS proteins involve in vitro expression studies. RGS proteins can suppress various G protein signaling pathways when exogenously expressed in mammalian cells (De Vries et al., 2000). In HEK293 cells, overexpression of RGS3 and RGS8 releases carbachol-induced N-type calcium channel inhibition, overexpression of GAIP and RGS4 reduces SST-induced inhibition of adenylyl cyclase and inhibits IP3 formation by bradykinin, and overexpression of RGS1, RGS2, RGS3, RGS4, RGS16 reduces IL-8 and carbachol-induced MAPK activation. Expression of RGS proteins in different cell types has been shown to regulate different G protein signaling pathways. For example, RGS3 was reported to suppress IP3 release induced by GnRH in COS-1 cells, to decrease ET-1-induced Ca\(^{2+}\) responses and MAPK activation in HMC cells, and to reverse inhibition of insulin secretion induced by epinephrine in HIT-15 cells. Surprisingly, very little is known about the physiological role of endogenous RGS proteins.

To study the function of RGS proteins in mammalian systems, two strategies have been utilized: disruption of endogenous RGS function and genetic knockout methods. In 2000, Jeong and Ikeda used RGS-insensitive mutants of G\(\alpha\) subunits designed by Lan et al to show that \(\alpha2\)-adrenergic inhibition of N-type calcium currents in rat sympathetic ganglia neurons is inhibited by endogenous RGS proteins (Jeong and Ikeda, 2000; Lan et al., 1998). Expression of RGS-insensitive G\(\alpha\)o subunits slowed calcium channel recovery from norepinephrine-
induced inhibition, which is due to the loss of GAP function of endogenous RGS proteins on the mutant Go subunits. The first RGS knockout mouse line was generated in 2000 (Chen and Lambert, 2000). It was reported that rod outer segments (ROS) membranes from RGS9-1 knockout mice hydrolyze GTP more slowly than ROS membranes from control mice. The functional consequence was that the flash responses of RGS9(-/-) rods rose normally, but recovered much more slowly in comparison to wild-type animals. For the first time, this study demonstrated the negative regulatory effect of endogenous RGS proteins on a specific physiological pathway. At a later time, I will introduce another RGS knockout mouse, the RGS2(-/-) mouse, which was used in my study.

RGS proteins play a role in regulating synaptic transmission

G protein coupled receptors (GPCRs) are found at presynaptic and postsynaptic terminals and are involved in the regulation of neuronal excitability. RGS accelerates both the onset and decay of G protein mediated signals. This implies that RGS proteins are essential for physiological precise signaling events such as synaptic transmission in the CNS that involve G protein-coupled receptor cascades and ion channels. Additionally, several studies have demonstrated the modulation of presynaptic Ca\textsuperscript{2+} channels of the N-, P/Q- and R-type (Jarvis and Zamponi, 2001). These studies revealed that RGS accelerates the onset and offset of transmitter mediated inhibition of presynaptic Ca\textsuperscript{2+} channels and also pointed to a role of RGS in altering the amount of inhibition for the presynaptic Ca\textsuperscript{2+} channels. Despite these findings, in vivo evidence is still lacking.
RGS2

RGS proteins are widely expressed in a tissue-specific manner (Willars, 2006). Among the RGS family RGS2 plays a prominent role in the brain (Kehrl and Sinnarajah, 2002). RGS2 belongs to the small subfamily of RGS proteins, including RGS1, RGS4, RGS5, RGS10, RGS13, RGS16, RGS18, and RGS-GAIP. All these RGS have small N-terminal and C-termini, which flank the RGS domain. RGS2 is a 211 amino acid protein, consisting of the 120 amino acid RGS domain, a short C-terminus and a conserved N-terminal domain, which is critical for plasma membrane targeting and association with other G-protein signaling partners.

RGS2 is widely expressed throughout the brain. Since there is no specific RGS2 antibody available, most expression studies of RGS2 have been done by detecting RGS2 mRNA. Northern blot and in situ hybridization studies showed that RGS2 mRNA is expressed in the cerebral cortex, several thalamic and hypothalamic nuclei and hindbrain regions (Grafstein-Dunn et al., 2001). Excitatory stimuli can dramatically and transiently up-regulate RGS2 mRNA levels in specific brain areas. For example, injection of cocaine or amphetamine increases RGS2 mRNA levels in rat striatum suggesting a role for RGS2 in the dopaminergic system (Ingi et al., 1998). In amygdala, cortex, hippocampus and striatum RGS2 expression can be increased by electroconvulsive seizures. Also, in PC12 cells the Gs pathway activator forskolin greatly increases RGS2 mRNA
expression (Pepperl et al., 1998). These results suggest a regulatory role of RGS2 in synaptic transmission and synaptic plasticity in neuronal systems.

RGS2, Gi/o versus Gq

*In vitro* single turnover assays indicated that RGS2 is 10-fold more potent than RGS4 in blocking Gq-mediated activation of PLCβ (Heximer et al., 1999). This is due to the lower affinity of RGS2 for the Gαi/o subunit and higher affinity for the Gαq subunit. RGS2 also inhibits the activity of adenylyl cyclase isoforms, such as adenylyl cyclase III, V, and VI (Sinnarajah et al., 2001). Although the structural basis for this inhibition is still unclear, there is some evidence suggesting direct interaction between RGS2 and adenylyl cyclases. Unfortunately, these functional studies have been done *in vitro* relying on exogenous expression methods. For this reason, the functions of endogenous RGS2 proteins on signaling pathways are still unknown.

**RGS2(-/-) Mouse**

In 2000, Oliveira-dos-Santos and colleagues generated RGS2 deficient mice (RGS2/-/-) in a C57BL/6J background (Oliveira-Dos-Santos et al., 2000). These mice are fertile and grow normally at the expected Mendelian frequency but reveal several phenotypes. First, impaired immunity was identified in RGS2(-/-) mice. To evaluate the T cell responses, LCMV-induced footpad swelling reactions were tested in RGS2(+/-) and RGS2(-/-) mice. RGS2(-/-) mice had
significant decreased reaction compared to the controls, suggesting that RGS2 proteins are important for normal T cell function.

Although RGS2(-/-) mice did not show apparent defects in motor responses, circadian activity, exploratory behavior, motor coordination, or spatial learning and memory, they showed a higher anxiety and less aggression in males. To evaluate the anxiety phenotype, a light/dark preference test was used. RGS2(-/-) mice showed significant increased preference for the dark environment compared with RGS(+/+) controls, indicating increased anxiety. Aggression was measured by using displacement from a plastic tube. RGS2(-/-) male mice were significantly less aggressive compared with their RGS2(+/+) littermates, whereas RGS2(-/-) female mice exhibited normal levels of aggression. These results suggested that RGS2 is involved in regulating neuronal circuits for anxiety and aggression.

To further understand these behavioral changes in RGS2(-/-) mice, the authors characterized the neuronal developmental and physiological effects caused by RGS2 deficiency in these mice. The number of neurons in the hippocampus, cortex, cerebellum, and brain stem were not changed in RGS2(-/-) mice. Interestingly, dendritic morphology and branching were altered by RGS2 deficiency. Specifically, the CA1 region of RGS2(-/-) mice demonstrated significant lower density of apical and basilar spines when compared with RGS2(+/+) control littermates. Furthermore, a significant reduction of the
electrical input/output relationship was identified in the CA1 neurons. These results indicate that RGS2 is involved in synaptic development, at least in hippocampus, and the physiological properties of neuronal system.

In addition to the anxiety and aggression phenotypes, recent studies demonstrated that both RGS2(+/−) and RGS2(−/−) mice exhibited a strong hypertensive phenotype (Heximer et al., 2003). Considering the wide expression of RGS2 in the brain, Gross et al. hypothesized that central nervous system mechanisms may contribute to hypertension in RGS2(−/−) mice (Gross et al., 2005). They found that urinary norepinephrine concentration and daily urinary norepinephrine excretion were significantly elevated in RGS2(−/−) mice compared with wild-type controls. Also, there was a larger decrease in blood pressure in response to intraperitoneal application of a low dose of the α1-adrenergic receptor antagonist prazosin. Environmental stress sensitivity was increased in RGS2(−/−) mice compared with controls. These studies indicated that elevated sympathetic outflow to the vessel may play a role for the increased blood pressure in RGS2(−/−) mice.

Thus, RGS2 deficient mice provide us with the great opportunity to study the neuronal and system functions of endogenous RGS2 proteins. Both hypertension and anxiety are regulated by the transmitter serotonin and therefore suggest that the serotonergic transmitter system is regulated by RGS2 proteins.
The serotonergic (5-HT) system

The serotonergic (5-HT) system is a diffusively organized projection system consisting of a small number of neurons. The cell bodies of serotonergic neurons are located in the brainstem raphe nuclei and in some regions of the reticular formation (Tork, 1990). Serotonergic neurons project their axons to almost all regions of the central nervous system, including cerebral cortex, limbic structures, basal ganglia, brainstem and the gray matter of spinal cord. The caudal raphe neurons in the B1, B2 and B3 nuclei project descending fibers to the motor and autonomic systems in the spinal cord. The rostral neurons in the B4-B9 nuclei, including the pontine, doral, and median raphe nuclei, project to the midbrain and forebrain area (Adell et al., 2002; Rubenstein, 1998).

Is there a connection between the serotonergic transmitter system in the brain and hypertension?

Hypertension is a physical sign common to a large group of pathophysiologial disorders and involves the heart, brain, and kidney (Laragh and Brenner, 1990). The CNS plays an important role in the regulation of the cardiovascular system. Early studies revealed that changes in serotonin levels in brain caused alterations in blood pressure (Laragh and Brenner, 1990), suggesting that serotonin is involved in central blood pressure regulation. In fact two major serotonin pathways have been suggested to be involved: the areas of the raphe nuclei (B7 and B8 containing the dorsal raphe nuclei) and the B3 group of the medulla (Frishman et al., 1995; Laragh and Brenner, 1990; Ramage, 2001). The
B7-B8 neurons project to the hypothalamus presumably acting as a vasopressor pathway, while the B3 group project to the cell bodies of preganglionic sympathetic nerves in the thoracic spinal cord acting on sympathetic regulation of blood pressure. Thus, investigation of the effects of RGS2 in the serotonergic system is important to reveal the regulatory role of RGS2 in blood pressure.

Is there a connection between the serotonergic transmitter system in the brain and anxiety?
Anxiety disorders have been identified as mental illnesses, which are characterized by pathologic worry and associated psychiatric and physical symptoms. To understand the nature of anxiety related disorders, animal models representing anxiety-like behavior have been studied. Evidence from different resources demonstrated that the anxiety related behavior involves the inter-connection of multiple brain regions including the cerebral cortex, the amygdala, the hypothalamus and the hippocampus (Bear et al., 2001). For example, modulation of the GABAergic and noradrenergic systems plays a significant role in anxiety and its treatment. However, long-term treatment of anxiety involves the manipulation of the serotonergic transmitter system, suggesting that anxiety is in particular regulated by the transmitter serotonin.

Successful treatment of anxiety using serotonin-related drugs points to the serotonergic system as a key modulator of anxiety. Selective serotonin re-uptake inhibitors (SSRIs) have become the first-line treatment for anxiety in the past two
decades (Ballenger, 2001). SSRIs can selectively block the re-uptake of serotonin following the release from serotonergic neurons leading to increased extracellular concentration of serotonin and enhanced synaptic strength in the brain (Smith et al., 2000). The 5-HT1A receptor partial agonists, for example buspirone, are also used as effective treatment of anxiety disorders (Ballenger, 2001). Many studies support that serotonin participates in the modulation of emotional states. Increased serotonin levels in the brain are correlated with reduced anxiety, while decreased serotonin level in the brain or loss of the serotonergic transmitter system in mice induces anxiety behaviors (Gross and Hen, 2004; Hendricks et al., 2003). Genetic components of anxiety have been identified. For example, 5HT-transporter (5HTT) gene variations were found to be linked to anxiety. Populations, including both adults and infants, who carry two short alleles of the 5HTT gene have decreased cellular 5HTT activity and increased anxiety (Auerbach et al., 1999; Lesch, 2001). The relationship between 5HTT activity and anxiety was further supported by the 5HTT deficient mouse which is more anxious (Holmes et al., 2003). Additional mouse models, where a specific component of the serotonergic system was deleted, confirmed the relationship between anxiety and the serotonergic system. A good example is the Pet-1 knockout mouse. Pet-1 is a transcription factor specifically expressed in serotonergic system preceding the expression of serotonin (Hendricks et al., 1999). Pet-1 knock-out mice have dramatically reduced numbers of the serotonergic neurons in the raphe nuclei throughout development, and are more anxious in both elevated plus maze and open field tests (Hendricks et al., 2003).
Another example demonstrating the relationship between serotonin and anxiety is the 5HT-1A receptor knock-out mouse. Several groups independently generated mice lacking 5HT-1A receptors on three different backgrounds (Heisler et al., 1998; Parks et al., 1998; Ramboz et al., 1998). All three mouse lines are more anxious. This behavioral change is consistent with the fact that 5HT-1A receptor agonists have anxiolytic effects (Griebel et al., 1995). Interestingly, 5HT-1A seem to be involved in the development of anxiety circuits, since a conditional knock-out of the receptor 4 weeks after birth does not induce anxiety related phenotypes as seen with the permanent knock-out of the receptor. Additionally, single nucleotide polymorphisms associated with anxiety have been identified in the human 5HT-1A gene (Strobel et al., 2003) and the mono-amino-oxidase A (MAOA) gene (Caspi et al., 2002), the enzyme which is involved in the metabolization of serotonin, dopamine and noradrenaline.

Thus, all these findings suggest that serotonin is involved in modulating anxiety and anxiety related behavior.

Regulation of the activity of the serotonergic system by G protein signaling

Serotonin release from serotonergic neurons act via ligand gated (5HT-3 receptors) and G protein coupled receptors (5HT-1 and -2) either on the serotonergic neuron itself or on the target circuits involved in emotional behaviors such as the amygdala, prefrontal cortex, hypothalamus and hippocampus. Several lines of evidence suggest that an alteration in GPCR function causes
changes in the behavior. GPCRs couple to three main G protein pathways on the intracellular site, i.e. the Gs, Gq and Gi/o pathway. Modulation of anxiety involves at least the 5HT-1A and -1B receptors, which couple to the Gi/o pathway (Chiavegatto et al., 2001; Fish et al., 1999; Lyons et al., 1999; Miczek et al., 1998; Olivier et al., 1995). In general, activation of the Gi/o pathway reduces or dampens the activity of the neurons and the neuronal circuit via regulating ion conductances, such as $K^+$ and $Ca^{2+}$ currents, over the cell membrane. 5HT-1A and -1B receptor agonists cause anti-aggressive effects in rodents (Miczek et al., 1998; Olivier et al., 1995).

**Auto- and hetero-regulation of the serotonergic system**

The activity of the serotonergic transmitter system is regulated via transmitter release from local neurons and/or afferents to the raphe nuclei (hetero-regulation) and in particular via auto-regulatory mechanisms arising from the serotonergic neurons themselves (auto-regulation).

Auto-regulation: The midbrain raphe serotonin neurons elicit spontaneous action potentials (AP), with a regular, slow firing pattern (1-5 AP/s) (Aghajanian and Vandermaelen, 1982; Vandermaelen and Aghajanian, 1983). Application of serotonin onto the neurons leads to a decreased firing rate. This autoregulation is mediated via 5HT-1A receptors, which are located somatodendritically (Aghajanian et al., 1972; Blier and de Montigny, 1987; VanderMaelen et al., 1986). Activation of the 5HT-1A receptor causes hyperpolarization via opening $K^+$ channels.
channels (Aghajanian and Lakoski, 1984; Yoshimura and Higashi, 1985) and inhibition of high voltage activated (HVA) Ca\(^{2+}\) channels (Penington and Fox, 1994; Penington and Kelly, 1990). Interestingly, other 5HT receptors such as 5HT-1B, 5HT-2, 5HT-3, and 5HT-7 do not seem to play a role in the somatodendritic regulation of the serotonergic neuron firing rate (Pineyro and Blier, 1999), pointing to the important functional contribution of the Gi/o pathway in auto-regulation of the serotonergic system. At the presynaptic terminal 5HT-1B and 5HT-1D receptors have been described to be involved in the auto-regulation of 5HT-release. Activation of these receptors leads to a decrease in the transmitter release most likely via inhibition of presynaptic Ca\(^{2+}\) channels. Again these effects have been attributed to the Gi/o pathway (Pineyro and Blier, 1999).

Hetero-regulation: The hetero-regulation of the serotonergic firing rate involves various transmitter systems. As expected the GABAergic input into the serotonergic neurons reduces neuronal firing, while glutamatergic input increases the firing (Becquet et al., 1993; Levine and Jacobs, 1992). Other transmitters acting on GPCRs, rather than ion channels, such as noradrenaline (\(\alpha_2\)-adrenergic receptor (Gi/o) (Baraban and Aghajanian, 1980), dopamine (D\(_2\) receptors (Gi/o) ((Ferre and Artigas, 1993; Ferre et al., 1994), substance P (Gq/11) (Kerwin and Pycock, 1979; Reisine et al., 1982), bombesin (neuromedin B receptor (Gq) (Pinnock et al., 1994) and cholecystokinin (CCKA receptor (Gi/o) (Boden et al., 1991) have also been described in modulating the firing of serotonin neurons. The GPCR activation leads to an increase in the firing rate
and not to a decrease as observed for serotonin. It is important to note that these effects seem to be mediated via activation of Gq as well as Gi/o pathways. This result is surprising in particular for the activation of $\alpha_2$-adrenergic receptor and D$_2$ receptors which both couple to Gi/o pathways. Activation of the Gi/o pathway normally results in inhibition rather than activation of the neuronal firing (see for example (Ehrengruber et al., 1997; Li et al., 2005)). It has been suggested that one possible mechanism of action is via inhibition of a voltage activated $K^+$ current ($I_A$) (Aghajanian, 1985). This $K^+$ current counteracts the depolarizing phase of the AP. However, this has to be analyzed in more detail, since very little information is available about the G protein modulation of this current type via the Gi/o or Gq pathway.

Thus, the serotonergic transmitter system negatively regulates its own firing activity and transmitter release via 5HT autoreceptors coupling to the Gi/o pathway. Input from other neuronal circuits including noradrenergic and dopaminergic transmitter system can modulate the activity of the serotonergic system via activating the Gi/o and/or Gq pathway. The exact mechanism for the hetero-regulation remains to be determined.

The connection between RGS2 and the serotonergic system.

As stated earlier, RGS2(-/-) mice are more anxious and less aggressive with decreased synaptic transmission levels compared with the wild-type controls suggesting that the involvement of RGS2 in synaptic transmission and plasticity
events in the brain is critical for both the physiology of neurons as well as for behavioral output. The behavioral changes in the RGS2(−/−) mouse are supported by the fact that RGS2 has recently been identified as a quantitative trait for anxiety (Yalcin et al., 2004) and variations within the RGS2 gene are expected to play a role for the development of anxiety in humans (Leygraf et al., 2006). Thus, RGS2 is involved in modulating anxiety.

**In situ** hybridization analysis indicated that RGS2 mRNA is expressed in the dorsal raphe (DR), which contain serotonergic neurons (Grafstein-Dunn et al., 2001). As mentioned above, five subfamilies of 5-HT receptors including 5HT-1A, -1B and -1D have been identified in the serotonergic system, and recent studies performed in heterologous expression systems showed that these receptor pathways are modulated by RGS proteins including RGS2 (Ghavami et al., 2004). For example, overexpression of full-length RGS2 protein significantly decreased Gαq-mediated signaling by 5HT-2A receptors.

Thus, RGS2 is expressed in the serotonergic transmitter system and is capable of modulating 5HT receptors. Investigating the function of RGS2 within this important transmitter system provides great opportunities to understand whether RGS2 modulates the cellular G protein signaling to determine the physiology and development of serotonergic neuronal circuits involved in modulation of anxiety like behaviors. This is the goal of the second part of my thesis.
The goal of my thesis is to understand the neuronal as well as the system function of RGS2. To examine the role of RGS2 in regulating short-term synaptic plasticity, I performed electrophysiological analysis in autaptic hippocampal cultures from both RGS2(-/-) mice and wild-type controls. My study shows for the first time that RGS2 modulates synaptic strength through regulating basal activity of Gi/o-mediated calcium channel inhibition. To explore the functions of RGS2 within a neuronal circuit which underlies the behavioral phenotype (anxiety) of RGS2(-/-) mice, I compared the developmental and physiological properties of serotonergic system between RGS2(-/-) mice and the wild-type controls. RGS2 deficiency did not affect the gross morphology of the serotonergic system, but altered the spontaneous firing and membrane properties of serotonergic neurons. This is most likely due to an increased G protein activity in the RGS2(-/-) neurons.
CHAPTER 2

RGS2 DETERMINES SHORT-TERM SYNAPTIC PLASTICITY IN HIPPOCAMPAL NEURONS BY REGULATING GI/O MEDIATED INHIBITION OF PRESYNAPTIC CA\textsuperscript{2+} CHANNELS
Abstract

RGS2, one of the small members of the regulator of G protein signaling (RGS) family, is highly expressed in brain and regulates Gi/o as well as Gq coupled receptor pathways. RGS2 modulates anxiety, aggression and blood pressure in mice suggesting that RGS2 regulates synaptic circuits underlying animal physiology and behavior. How RGS2 in brain influences synaptic activity was unknown. We therefore analyzed the synaptic function of RGS2 in hippocampal neurons by comparing electrophysiological recordings from RGS2 knock-out and wild type mice. Our study provides a general mechanism of the action of the RGS family containing RGS2 by demonstrating that RGS2 increases synaptic vesicle release by down-regulating the Gi/o mediated presynaptic Ca\(^{2+}\) channel inhibition and therefore provides for the first time an explanation of how regulation of RGS2 expression can modulate the function of neuronal circuits underlying behavior.
Introduction

G protein signaling couples extracellular signals with intracellular effectors. G protein coupled receptor (GPCR) activation via neurotransmitters initiates the exchange of GDP for GTP on the Gα subunit, allowing the dissociation of the Gβγ subunit enabling it to interact with different effectors such as ion channels. The hydrolysis of GTP to GDP on the Gα subunit leads to the reassociation of the Gβγ dimer with the Gα subunit and termination of the signal (Hamm, 1998). The termination of the G protein signal is accelerated by a superfamily of GTPase accelerating proteins (GAPs) known as RGS proteins. Besides their function as GAPs for the termination of the G protein signals, RGS proteins can act as effector antagonists by blocking the Gq pathway (De Vries et al., 2000).

GPCRs are found at presynaptic and postsynaptic terminals and are involved in the regulation of neuronal excitability. RGS proteins accelerate both the onset and decay of G protein mediated signals (Herlitze et al., 1999; Zerangue and Jan, 1998). This implies that RGS are essential for precise physiological signaling events such as synaptic transmission in the central nervous system which involves G protein coupled receptor cascades and ion channels. In addition, several studies demonstrate the modulation of presynaptic Ca^{2+} channels of the N-, P/Q- and R-type (Jarvis and Zamponi, 2001) in heterologous expression systems. These studies reveal that RGS accelerates the onset and offset of transmitter mediated inhibition of presynaptic Ca^{2+} channels and also
points to a role of RGS in altering the amount of inhibition for the presynaptic Ca\textsuperscript{2+} channels.

Among the RGS family, RGS2 plays a prominent role in the brain. A regulatory role of RGS2 in synaptic transmission and plasticity was suggested when RGS2 knock-out mice revealed a decrease in synaptic activity in hippocampal CA1 neurons probably correlated with increased anxiety of the mice (Oliveira-Dos-Santos et al., 2000). Genetic dissection of a behavioral quantitative trait locus also revealed that RGS2 is a modulator of anxiety in mice (Yalcin et al., 2004). RGS2 expression has been shown to be rapidly upregulated in various brain regions like the hippocampus by excitatory stimuli (Burchett et al., 1998; Ingi et al., 1998) and it is therefore likely that regulation of RGS2 protein levels in neurons regulates synaptic output and behavior.

Thus, the abundance of GPCRs at presynaptic terminals and their involvement in presynaptic Ca\textsuperscript{2+} channel inhibition, the functional interaction between RGS2 and presynaptic Ca\textsuperscript{2+} channels in heterologous expression systems as well as neurons, and the fact that RGS2(-/-) mice reveal reduced synaptic activity accompanied with behavioral changes suggest that RGS2 acts at the presynaptic terminal. However, direct evidence for this hypothesis is still lacking. We therefore examined the effect of RGS2 on synaptic transmission by exogenously expressing RGS2 in cultured hippocampal neurons and comparing these to recordings from RGS2(-/-) mice. Our data suggest that RGS2 regulates synaptic
output via modulation of basal G protein activity of the Gi/o but not the Gq pathway at the presynaptic terminal. This result is surprising, given the fact that RGS2 has been described for its high affinity for the Gq, but not the Gi/o pathway.
Results

RGS2 is endogenously expressed in cultured hippocampal neurons and colocalizes with the synaptic marker synaptobrevin 2

The goal of this study was to understand if and how RGS2 regulates synaptic transmission. A well established model system for performing such experiments is to compare defined synaptic parameters between knock-out and wild-type autaptic hippocampal neurons and to rescue the effects observed in knock-out cultures by exogenously expressing the wild-type protein (see for example (Bekkers and Stevens, 1991; Calakos et al., 2004; Rhee et al., 2002)). Since no specific antibodies are commercially available to detect RGS2 protein (supplemental Figure 1), we performed in situ hybridization and found that RGS2 mRNA is detected in hippocampal neurons from wild-type but not RGS2(-/-) mice (Figure 1A). Exogenous expression of RGS2 tagged with YFP at its N- and/or C-termini revealed a punctate staining pattern (Figure 1B). These puncta are partially colocalized with the synaptic marker synaptobrevin 2 (Figure 1B), suggesting that RGS2 is transported to synaptic sites.

RGS2 regulates short term synaptic plasticity

To determine if RGS2 was able to modulate synaptic transmission, we characterized short-term synaptic plasticity of autaptic synapses, which
hippocampal neurons make onto themselves when cultured alone on micro-islands. Synaptic transmitter release via two depolarizing pulses separated by 50 ms (20 Hz stimulation) and the ratio between the first and the second elicited excitatory postsynaptic current (EPSC) were compared (paired pulse ratio (PPR)). To investigate the possibility that RGS2 may function during synaptic transmitter release our initial experiments were performed on rat hippocampal neurons, while later studies were performed on mouse cultures. Exogenous expression of RGS2 in wild-type autaptic hippocampal cultures from rat as well as from mice reduced the PPR and induced paired-pulse depression (PPD) (Figure 1C, D and E). In contrast, recordings from autaptic hippocampal neurons from RGS2(-/-) mice revealed paired-pulse facilitation (PPF) and thus an increase in the PPR (Figure 1C and E). Furthermore, the PPF was depressed by exogenous expression of RGS2 to PPRs found in the wild-type cultures (Figure 1E). These results suggest that RGS2 regulates short term synaptic plasticity leading to PPF when absent or at low concentrations (Figure 1E), and PPD when available at high concentrations in hippocampal neurons (Figure 1D). To determine if the decrease in PPR is due to changes in expression of various molecules involved in RGS signaling, we compared expression levels of several G proteins and RGS proteins in wild-type and RGS2(-/-) neurons. No significant differences in protein levels of $\alpha_{i_1}$, $\alpha_{i_3}$, $\alpha_o$, $\alpha_{q/11}$, $\beta_2$ and RGS4 were observed by Western Blot Analysis in wild-type neurons versus RGS2(-/-) neurons (Figure 2A). In addition, we compared the relative mRNA expression levels of RGS5, 7 and 8 between wild-type and RGS2(-/-) cultures and also
observed no significant changes (Figure 2B). Increases in PPR may also reflect changes in neuronal circuit formation due to reduced number of spines and synaptic contacts formed as originally suggested for CA1 hippocampal neurons from RGS2(-/-) mice (Oliveira-Dos-Santos et al., 2000). We did not detect a reduction in the number of synapses formed in RGS2(-/-) neurons when compared with wild-type neurons (Figure 2C-E). This is well supported by the comparison of the mean EPSC amplitudes. While the EPSC amplitudes are significantly different between the RGS2(-/-) and wild-type neurons for the first EPSC during a 20 Hz stimulation, comparison of the EPSC amplitudes elicited by the second pulse during the 20 Hz stimulation revealed no differences, since the RGS2(-/-) neurons have a larger facilitation than wild-type neurons (Figure 2F).

The probability of synaptic vesicle release is reduced in RGS2(-/-) mice due to a shift in the Ca$^{2+}$ dependence of transmitter release

To gain a deeper understanding of the precise action of RGS2 on synaptic transmitter release we analyzed several parameters of synaptic transmission in more detail. A change in the PPR may be caused by an alteration in the vesicle release probability (Thomson, 2000). A low release probability may underlie PPF, while a high release probability may cause PPD. We therefore first compared the probability of synaptic vesicle release between RGS2 knock-out and wild-type neurons, which can be examined by comparing the size of the readily releasable vesicle pool (RRP) to the number of vesicles released by a single action-
potential. The RRP is defined as the number of vesicles released during application of a hypertonic solution (Rosenmund and Stevens, 1996). We found that the vesicle release probability is reduced from the wild-type value of 2.2% to 1.7% in RGS2(-/-) mice when the RRP was compared to the EPSC in each experiment (Figure 3A and B), while no differences in the mean RRP size nor the mean EPSC size could be detected due to the small number of neurons analyzed (wild-type: EPSC (15.4 ± 2.38 (pC) n=21), RRP (763 ± 127 (pC) n=21); RGS2(-/-): EPSC (14.1 ± 2.18 (pC) n=24), RRP (873 ± 108 (pC) n=24).

This reduction in the probability of release could be caused by a decrease in the amount of available vesicles, an alteration in the recruitment and recycling of the vesicles, a change in the coupling of the vesicles to the release machinery, or via a reduction in Ca\(^{2+}\) influx through presynaptic Ca\(^{2+}\) channels. We first analyzed if the vesicle recycling process within the synaptic terminal was altered. It has previously been observed that GABA\(_{B}\) receptors in the calyx of Held reduce the refilling of synaptic vesicles via cAMP dependent signaling (Sakaba and Neher, 2003) and that RGS2 negatively regulates several forms of adenylyl cyclase, which could lead to a change in the presynaptic cAMP levels (Kehrl and Sinnarajah, 2002). We therefore depleted the vesicles from the synapses using 30 depolarizing stimuli at a frequency of 20 Hz and analyzed how long it took for the synapse to recover the EPSC amplitude to the level measured before vesicle depletion (Figure 4A). Neither the time constant of EPSC depletion (Figure 4A) or recovery (Figure 4B) nor the amount of recovery of the EPSC (Figure 4B) or the
size of the RRP (Figure 4C and D) was altered when RGS2 was absent. This suggests that vesicle recycling or priming was not altered in the RGS2(-/-) mice. To exclude postsynaptic and structural/morphological changes of the synapse caused by the absence of RGS2, we also analyzed the miniature EPSCs (mEPSCs) (Figure 4E). mEPSC amplitude (Figure 4F) and mEPSC frequency (Figure 4G and H) were not altered in RGS2(-/-) neurons in comparison to wild-type neurons, suggesting that RGS2 does not alter vesicle size or the postsynaptic response to transmitter release.

Differences in PPF and PPD can be caused by changes in the Ca\(^{2+}\) entry during repetitive stimulations resulting in an alteration of the number of transmitter quanta (vesicles) released (Fisher et al., 1997; Zucker, 1999). Therefore, we analyzed the Ca\(^{2+}\) dependence of the transmitter release by increasing the external Ca\(^{2+}\) concentration between 1 to 10 mM Ca\(^{2+}\) and measured the change in EPSC size during 0.2 Hz depolarizing pulses. In the absence of RGS2 protein, the midpoint of the Ca\(^{2+}\) dependence of transmitter release curve was shifted to higher Ca\(^{2+}\) concentration (Figure 3C and D). This suggests alterations in the Ca\(^{2+}\) influx through voltage dependent Ca\(^{2+}\) channels, an alteration in the coupling between presynaptic Ca\(^{2+}\) channels and the release machinery and/or coupling of Ca\(^{2+}\) to the vesicle release.

**RGS2 acts on synaptic transmission via modulating Gi/o but not Gq pathways**
RGS2 accelerates and/or inhibits Gi/o pathways and inhibits Gq coupled receptor pathways in cells (Kehrl and Sinnarajah, 2002). Gi/o as well as downstream effectors of the Gq pathways modulate synaptic transmission. For example, classical presynaptic inhibition is caused by the inhibition of presynaptic Ca\textsuperscript{2+} channels via Gi/o-pertussis toxin (PTX) sensitive GPCR activation (Stevens, 2004). Downstream components of the Gq pathway such as DAG increase vesicle priming (Brose et al., 2000) and PKC has been suggested to regulate the size and the refilling rate of the vesicle pool (Morgan et al., 2005). Therefore alterations in the Gq as well as Gi pathway in the RGS2(-/-) neurons might underlie the observed changes in PPR. In order to investigate whether the increased PPF in RGS2(-/-) neurons is mediated by Gi/o or Gq coupled pathways we analyzed transmission in the presence of pertussis toxin (PTX), which blocks the Gi/o pathway and YM-254890, a specific blocker of the Gq/11 pathway (Takasaki et al., 2004). We found that incubation of the autaptic neurons with PTX 24 h prior to the experiments abolished PPF and reduced the PPR of RGS2(-/-) neurons from 1.16 to 0.98, i.e. to levels observed in the wild-type neurons (1.03 ± 0.02 (n=25), Figure 5). In wild-type neurons PTX did not alter the PPR (Figure 5B). In contrast, blocking the Gq pathway by application of 10 ng/ml YM-254890 did not change PPF in neurons from knock-out or wild-type mice (Figure 5A and 5B). To demonstrate that YM-254890 can effectively inhibit Gq pathways at 10 ng/ml we monitored the Gq induced PIP\textsubscript{2} hydrolysis with the described PIP\textsubscript{2} sensor PH-EGFP in HEK293 cells (Stauffer et al., 1998). HEK293
cells were transfected with the mAChR-M1 receptor and the PH-EGFP in a 5:1 molar ratio to guarantee that cells containing the PIP2 sensor also contain the Gq coupled receptor mAChR-M1. As shown in Figure 5C and D YM-254890 blocks the mAChR-M1 mediated PIP2 hydrolysis at the plasma membrane. These results taken together suggest that the Gi/o but not the Gq pathway is involved in the increased PPF observed in RGS2(-/-) neurons.

To obtain further evidence that RGS2 mediates its effect via the Gi/o but not the Gq pathway, we analyzed the effect of the RGS2 mutant (N149A) on the Gi/o and Gq pathway in Xenopus oocytes and on synaptic transmission in neurons. The corresponding amino acid in RGS4 (N128A), which belongs to the same RGS subfamily, has been shown to determine its affinity towards the G protein α subunits (Posner et al., 1999). Since RGS2 has a low potency as a GAP in the Gi/o pathway but a high potency as an inhibitor of the Gq pathway (Heximer et al., 1999; Heximer et al., 1997), we introduced the point mutation N149A into RGS2 under the assumption that the reduced affinity for both pathways would eliminate the effect of RGS2 on the Gi/o but would still be able to block the Gq pathway. To characterize the effect of the mutant RGS2 on the two signaling pathways, we made use of the Xenopus oocyte expression system. We and others have demonstrated that RGS2 accelerates the deactivation kinetics of the G protein inward rectifying potassium (GIRK) channel when the channels were activated by the M2 muscarinic acetylcholine receptor (mAChR-M2), a Gi/o coupled receptor (Doupnik et al., 1997; Herlitze et al., 1999; Mark et al., 2000a).
In addition, it was shown that activating the Gq coupled pathways in Xenopus oocytes via GPCRs activates an endogenous Ca\textsuperscript{2+} activated Cl\textsuperscript{−} current. The activation of this Cl\textsuperscript{−} current is blocked by RGS2 and other RGS proteins and has been suggested that the RGS effect is due to the inhibitory effect of the RGS on the Gq pathway (Mark et al., 2000a; Saugstad et al., 1996). To evaluate if the RGS2 mutant was still capable of inhibiting the Gq pathway we coexpressed RGS2 wild-type or RGS2(N149A) together with GIRK1/4 channels and P\textsubscript{2}Y\textsubscript{2} receptors. P\textsubscript{2}Y\textsubscript{2} receptors, when expressed in Xenopus oocytes, activate both the Gi/o as well as the Gq pathway (Mosbacher et al., 1998). Activation of the Gi/o pathway leads to GIRK channel activation, while stimulation of the Gq pathway activates the endogenous Ca\textsuperscript{2+} activated Cl\textsuperscript{−} channels, which can be measured as an outward current (see arrow in Figure 6A, second trace). Since very little outward K\textsuperscript{+} current is detected, due to the inward rectifying properties of the GIRK channel, most of the outward current is mediated by the Cl\textsuperscript{−} channel. Coexpression of RGS2 (1:2 dilution) or RGS2(N149A) (1:2 dilution) suppressed completely the Ca\textsuperscript{2+} activated outward current, suggesting that both the wild-type and the RGS2 mutant inhibit the Gq pathway (Figure 6A and B). Even upon further dilution, the RGS2(N149A) mutant (1:20) was still able to reduce the Gq activated Cl\textsuperscript{−} current significantly, but not completely (Figure 6B). The same effects were observed in our previous study when RGS2 was diluted 1:40 (see (Mark et al., 2000a)). We next analyzed the effect of the RGS2 mutant for its capability to accelerate the deactivation kinetics of GIRK channels, once they are activated via the Gi/o pathway. We coexpressed the RGS2 proteins together with
GIRK1/4 subunits and the mAChR-M₂. Activation of the mAChR-M₂ via application of 10 μM ACh leads to an increase in GIRK current. Once ACh is washed out the GIRK channel deactivates. The deactivation time depends on the termination of the G protein cycle and is accelerated by RGS proteins including RGS2 (Herlitze et al., 1999; Mark et al., 2000a). As shown in Figure 6C and D the GIRK channel deactivation is faster in the presence of RGS2 but is slowed in the presence of RGS2(N149A). The slowing of the GIRK channel deactivation can be explained by a dominant-negative effect of RGS2(N149A) on the Gi/o pathway, since RGS2(N149A) may compete for binding to the Gαi/o proteins with the endogenous RGS proteins in Xenopus oocytes.

The experiments described above suggest that RGS2(N149A) is still capable of blocking the Gq pathway but has lost its GAP activity on the Gi/o pathway, where it now acts as a dominant negative mutant. Thus, if RGS2 mediates its function on short term synaptic plasticity via the Gi/o pathway we should not be able to depress PPF with this mutant in RGS2(-/-), but should be able to induce PPF in the wild-type neurons due to the dominant negative effect of the mutant. In contrast, if RGS2 mediates its effect via block of the Gq pathway we should be able to reduce PPF in the knock-out neurons, but should see no effect on the wild-type neurons. Exogenous expression of RGS2(N149A) in neurons from RGS2(-/-) mice revealed no effect on PPF, but PPF was increased in wild-type neurons, providing additional evidence that the RGS2 mutant acts on the Gi/o pathway.
pathway in a dominant negative way (Figure 6E and F) and that RGS2 influences synaptic transmitter release at the presynaptic terminal.

Non-L-type Ca\(^{2+}\) channels exhibit increased G protein modulation in hippocampal neurons from RGS2 (-/-) mice

The results suggest that the basal activity of G proteins of the Gi/o family is higher in RGS2(-/-) mice, since PTX inhibits the activation of this G protein family via ADP-ribosylation of the G protein \(\alpha\) subunit. The increased activity of the G protein is probably due to the reduced level of RGS proteins which in turn prolong the activation of the Gi/o protein cycle. This predicts a higher concentration of active G protein subunits (i.e. \(G_{\alpha_{ii/o}}\) or \(G_{\beta_{\gamma}}\) subunits) within the cell and the presynaptic terminal. We have shown that presynaptic Ca\(^{2+}\) channels are modulated directly via G protein \(\beta_{\gamma}\) subunits leading to the inhibition of the channels (Herlitze et al., 1996b; Ikeda, 1996b). In addition \(G_{\beta_{\gamma}}\) subunits mediate presynaptic Ca\(^{2+}\) channel inhibition at the Calyx of Held (Kajikawa et al., 2001). The voltage dependent inhibition of the Ca\(^{2+}\) channels can be released by high positive prepulses, a process defined as prepulse facilitation (Elmslie et al., 1990). The amount of prepulse facilitation provides an indication of the degree to which the channels are modulated by G protein \(\beta_{\gamma}\) subunits (Zamponi and Snutch, 1998). We therefore tested if the prepulse facilitation of somatic non-L-type channels (mainly P/Q- and N-type channels) would be increased in the RGS2(-/-) neurons, suggesting increased levels of active Gi/o proteins. In the
presence of TTX to block Na\(^+\) channels and DHPs to block L-type channels, somatic Ca\(^{2+}\) currents were elicited by two test pulses to the same test potentials. Before the second test pulse a high positive prepulse was elicited to release G protein modulation (Figure 7B). The peak current ratios of the currents before and after the prepulse were compared to determine the amount of facilitation. Indeed, prepulse facilitation was increased from 1.0 to 1.8 in RGS2(-/-) neurons (Figure 7B and 7C). To further verify this result we compared the IV relationship of the non-L-type Ca\(^{2+}\) currents in the neuronal cultures. As predicted for G protein-modulated channels the peak amplitude of the Ca\(^{2+}\) current was shifted by 4 mV to more depolarized potentials with no change in the reversal potential (Figure 7D and F). This most likely reflects the slower opening of the channel and/or a shift in the voltage dependence of activation to more depolarized potentials. Both effects have been attributed to the modulation of voltage gated Ca\(^{2+}\) channels by G protein \(\beta\gamma\) subunits (Herlitze et al., 1996b; Ikeda, 1996b). In addition the average amplitude of the peak Ca\(^{2+}\) currents were reduced in the RGS2(-/-) neurons (Figure 6F); thereby supporting the increased inhibition of the Ca\(^{2+}\) channels by G\(\beta\gamma\) subunits.

The results suggest that RGS2 regulates Ca\(^{2+}\) influx through voltage gated Ca\(^{2+}\) channels into presynaptic terminals, by reducing the basal activity of Gi/o protein family.
Botulinum toxin A decreases the release probability and induces PPF of both RGS2 knock-out and wild-type hippocampal neurons

It has been shown that G\(\beta\gamma\) inhibits synaptic transmission in the lamprey neurons downstream of Ca\(^{2+}\) entry (Blackmer et al., 2001) (Figure 7A). Increased G\(\beta\gamma\) protein levels may therefore in addition to modulating Ca\(^{2+}\) influx, also act directly at the presynaptic vesicle release machinery. More detailed studies showed that G\(\beta\gamma\) most likely interferes with vesicle fusion by binding to SNAP-25 (Blackmer et al., 2005), thereby causing a reduction in transmitter release. In those experiments the specific cleavage of SNAP-25 by BoNT-A prevented additional transmitter/G\(\beta\gamma\) mediated synaptic inhibition. Therefore in our system if G\(\beta\gamma\) is acting on SNAP-25 to prevent transmitter release, partial cleavage of SNAP-25 in neurons from RGS2 knock-out mice should not significantly increase PPF as would be expected in the wild-type mice (Young, 2005). We therefore incubated the wild-type and RGS2(-/-) autaptic hippocampal neurons with BoNT-A for 3 h prior to the experiment, which leads to a 50-60% reduction in the SNAP-25 protein levels (Figure 7I and J) and compared the PPR of neurons incubated without BoNT-A. As expected, application of BoNT-A increased the PPR from 1.02 to 1.23 in wild-type mice (Figure 7G and H). Interestingly, an increase in PPR from 1.17 to 1.34 was also observed in the RGS2(-/-) neurons (Figure 7G and H). The PPR in RGS2(-/-) neurons treated with BoNT-A was significantly larger than the PPR in wild-type neurons, revealing that cleavage of SNAP-25 is affecting synaptic transmission of RGS2(-/-) and wild-type neurons in a similar way.
fashion. This suggests that inhibition of synaptic transmission by Gβγ is mediated via inhibition of presynaptic Ca^{2+} channels and not via binding to the SNARE complex. However, since it is not possible to completely cleave SNAP-25 without blocking transmitter release, the experiments have to be interpreted carefully in respect to variations in SNAP-25 cleavage and to the possibility that the remaining SNAP-25 is the target of Gβγ action.
Discussion

We demonstrate here for the first time that one of the small RGS family members RGS2 regulates synaptic strength. In the presence of RGS2, transmitter release probability is high, while in the absence of RGS2 synaptic transmitter release is reduced. Our data help to explain the effects previously observed in the RGS2 knock-out mice (i.e. reduced synaptic activity (Oliveira-Dos-Santos et al., 2000)), give mechanistic insight into the action of RGS2 on synaptic function and point to an importance of regulating RGS2 expression during neuronal activity for regulating neuronal circuits and behavior. The physiological consequences and the molecular mechanisms of RGS2 controlling synaptic transmitter release are discussed below.

Physiological consequences of RGS2 expression

RGS2 knock-out mice reveal increased anxiety and reduced male aggression, which is correlated with a reduction in synaptic transmission in CA1 hippocampal neurons (Oliveira-Dos-Santos et al., 2000). Further studies of the RGS2 knock-out mice revealed an important function for RGS2 in blood pressure control (Tang et al., 2003). Genetic dissection of trait loci also suggests that RGS2 plays an important role in anxiety (Yalcin et al., 2004). These studies suggest that RGS2 is involved in regulating neuronal circuits underlying autonomic nervous system regulation and animal behavior. Our study provides an explanation of
how RGS2 can modulate the synaptic output in the brain, namely that high
expression levels of RGS2 increase, while low expression levels decrease
synaptic strength. Since RGS2 is an immediate early gene, which is up-regulated
very efficiently during activity dependent processes, at least in certain brain areas
(Burchett, 2005; Burchett et al., 1998; Ingi et al., 1998), our results suggest that
the level of RGS2 proteins within a certain neuronal population will define their
synaptic efficacy via regulating the basal activity of G proteins of the Gi/o family.

Mechanistic insight into the function of RGS2 in neurons

Our initial finding, i.e. that PPF is increased in the absence of RGS2, suggested
a reduction in the synaptic release probability. A reduction in the release
probability can be caused by altering several processes during transmitter
release which involves Gi/o and/or Gq coupled receptor pathways. Classical
presynaptic inhibition is mediated by GPCRs which couple to the PTX sensitive
Gi/o proteins, where the Gβγ subunits inhibit presynaptic Ca^{2+} channels and
reduce Ca^{2+} influx during potential changes at the synaptic terminal (Herlitze et
al., 1996b; Ikeda, 1996b; Kajikawa et al., 2001). We found in the RGS2 knock-
out mice that non-L-type channels (i.e. mostly the presynaptic channel types N
and P/Q) in hippocampal neurons reveal a stronger voltage dependent G protein
inhibition in comparison to wild-type neurons, suggesting that the level of G
protein βγ subunits in the RGS2(-/-) neurons is higher. An increased basal activity
of the Gi/o pathway in neurons would explain the elevated Gβγ protein levels.
This basal activity is normally very low as has been estimated by the tonic inhibition of Ca\(^{2+}\) currents at the presynaptic terminal of the Calyx of Held (Cuttle et al., 1998). In fact, increased constitutive activity of GPCRs has been shown to play a role in causing diseases such as cancer, cardiac hypertrophy and hypertension (Seifert and Wenzel-Seifert, 2002). Our data also correlate well with the recent finding that increased expression of RGS4 in striatal cholinergic interneurons decrease the mAChR-M4 mediated Ca\(^{2+}\) channel inhibition (Ding et al., 2006).

G protein \(\beta\gamma\) subunits act on several synaptic effector proteins besides the presynaptic Ca\(^{2+}\) channels. It has been shown that G\(\beta\gamma\) interacts with several components of the synaptic release machinery (Jarvis and Zamponi, 2001) and that G\(\beta\gamma\) can lead to decreased transmitter release downstream of Ca\(^{2+}\) entry (Blackmer et al., 2001) most likely via the direct binding to the SNARE protein SNAP-25 (Blackmer et al., 2005; Blackmer et al., 2001; Gerachshenko et al., 2005). This conclusion was drawn based on the fact that BoNT-A, which cleaves SNAP-25, prevented the serotonin (G\(\beta\gamma\)) mediated synaptic inhibition. In contrast, BoNT-B, which specifically cleaves synaptobrevin, did not prevent serotonin's effect on transmission. We therefore tested the possibility that the increased concentration of free G\(\beta\gamma\) in the presynaptic terminal would occlude effects of BoNT-A, as would be expected if G\(\beta\gamma\) inhibits transmitter release via binding to SNAP-25. Since partial cleavage of SNAP-25 further reduced transmitter release in the RGS2(-/-) neurons in comparison to wild-type neurons, our results suggest
that the reduction of transmitter release by increased Gi\textsubscript{\beta\gamma} levels is mediated primarily by reducing Ca\textsuperscript{2+} influx through presynaptic Ca\textsuperscript{2+} channels. Another possibility for RGS2 function at the presynaptic terminal is that the Gi/o pathway may inhibit adenylate cyclase (for example via GABA\textsubscript{B}-receptors (Sakaba and Neher, 2003)) leading to a decrease in cAMP levels. Indeed, RGS2 has been described to inhibit the activity of certain adenylyl cyclase isoforms and thus reduce cAMP levels (Kehrl and Sinnarajah, 2002). Decreased cAMP levels and inhibition of adenylyl cyclase in the presynaptic terminal results in attenuation of vesicle recruitment to the RRP, an effect which is mediated by the cAMP dependent guanosine exchange factor (cAMP-GEF) and not by PKA. PKA by itself seems to play a role in synaptic transmission by increasing or maintaining the vesicle pool size (RRP and SRP (slowly releasable pool), an effect which is counteracted by the Ca\textsuperscript{2+}/calmodulin dependent protein phosphatase calcineurin (Nagy et al., 2004). However, we did not observe any alterations in synaptic vesicle cycling, suggesting that RGS2 inhibits synaptic transmitter release by modulating Ca\textsuperscript{2+} entry into the presynaptic terminal.

RGS2 has the capacity to accelerate Gi/o and to antagonize Gq pathways (Kehrl and Sinnarajah, 2002), but it has been suggested to have a greater affinity for G\textsubscript{\alpha}q than for G\textsubscript{\alpha}i/o proteins (Heximer et al., 1999). Therefore recent studies have concentrated on the effects of RGS2 on modulating Gq coupled pathways in heterologous expression systems and intact tissues, where RGS2 for example regulates the Ca\textsuperscript{2+} oscillation, adaptation, and excitability of pancreatic acini via
regulating intracellular IP$_3$ levels (Wang et al., 2004). It was therefore surprising to observe that the loss of RGS2 protein and over-expression of RGS2 in neuronal hippocampal cultures seems to act via the Gi/o pathway rather than the Gq pathway, at least in the presynaptic terminal. Neither blockers of the Gq pathway nor the point mutations within the RGS2 which abolish its effects on the Gi/o pathway while maintaining modulation of the Gq pathway were effective in rescuing RGS2 deficiencies. On the other hand PTX was sufficient to block the PPF effect in RGS2(-/-) mice and the RGS2(N149A) mutant acted as a dominant negative mutant for RGS2 function by increasing the PPF. These results suggest that the major targets of RGS2 within the presynaptic terminal are GPCRs which couple to the Gi/o pathway. This conclusion is also supported by the fact that vesicle recycling in RGS2(-/-) mice is normal, since several second messengers within the Gq pathway including diacylglycerol (DAG) and PKC have been described to modulate synaptic transmitter release at the level of vesicle priming and recycling (Morgan et al., 2005; Nagy et al., 2004; Rhee et al., 2002). Therefore, increased Gq signaling in the absence of RGS2 would have been expected to cause increased PLC activation, increased DAG synthesis and increased Munc13 activation, resulting in faster vesicle priming and less depression during high frequency trains. In contrast, inhibition of Gq pathways by RGS2 would have been expected to increase depression. In both cases the time course of vesicle recycling should be altered. This was however not the case, pointing again to RGS2 acting primarily on the Gi/o rather than the Gq pathway during short term synaptic plasticity.
In summary, we show that RGS2 regulates synaptic output. The importance of this finding lies in the underestimated effects of RGS2 on modulating the Gi/o pathway, the important function of RGS2 in regulating the basal activity of the Gi/o pathway within a neuron, and in the possibility that up-regulation of RGS2 in certain neuronal circuits will most likely modulate synaptic strength.

Acknowledgement: We thank Drs. E.S. Deneris and L.T. Landmesser for reading the manuscript and Dr. B. Roth for the YM-254890, Dr. S. Mumby for the RGS4 antibody and Dr. M. Wilson for the SNAP-25 cDNAs. This work was supported by National Institutes of Health Grants NS0447752 and NS42623 to S.H.
Experimental Procedures

cDNA Constructs, Viral Production and Infection and cell culture: Mouse RGS2, 4, 5, 8 cDNA (Herlitze et al., 1999; Mark et al., 2000b), mouse RGS7 (Accession number BC051133), G protein $a_{1,3,o}$ and $Gb_2$ (Herlitze et al., 1996b) and mouse $Ga_q$ (Accession number M55412, gift from Dr. M. Simon (Pasadena, CA) and SNAP-25A and B (gift from Dr. M. Wilson, Albuquerque, NM) were cloned into mammalian expression vectors (pcDNA variants) for expression in HEK293 cells. RGS2 cDNA was also cloned into the SinRep(nsP2S726)dSP-EGFP virus vector and for the initial recordings in rat into pSFV. For localization studies the RGS2 was inserted into pEYFP-N1 and pEYFP-C1 (Clontech). The point mutation RGS2(N149A) was introduced into RGS2 using an overlap extension PCR method (Herlitze and Koenen, 1990; Ho et al., 1989). The point mutation was confirmed by DNA sequencing and the cDNA carrying the mutation was cloned into SinRep(nsP2S726)dSP-EGFP and pBF1 (oocyte expression). PH-EGFP was a gift from Dr. T. Meyer (Stauffer 1998 343). Sindbis pseudovirions were prepared according to Invitrogen's instructions (Sindbis Expression System) and as published in our recent publication (Li et al., 2005). For RGS2 overexpression and rescue experiments, cultured neurons were infected with 50 µl of sindbis pseudovirions containing the cDNAs of RGS2 or RGS2(N149A) constructs. Recordings were performed up to 24 h post infection. Micro-island and continental cultures of hippocampal neurons were prepared
according to a modified version of published procedures from mouse or rat pups (P0-3) (Bekkers and Stevens, 1991; Wittemann et al., 2000).

**Immunocytochemistry, Imaging and Western blot:** Continental hippocampal cultures were prepared as described above and transfected with RGS2-YFP by using the Ca\(^{2+}\) phosphate method (Park et al., 2004). 24 h after transfection, neurons were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100 in PBS. Anti-GFP (Molecular Probes) and anti-synaptobrevin-2 (SYSY) antibodies were used to label RGS2 and the synaptic marker synaptobrevin-2. Neurons were incubated with the primary antibody overnight at 4°C, then washed, and then incubated with Alexa 488- and Alexa 568-conjugated secondary antibody (Molecular Probes) for 30 min at room temperature. Cells were embedded in Prolong Gold antifade (Molecular Probes). For the calculation of the number of synapses and synapsin/MAP2 staining ratio low density hippocampal cultures were prepared, fixed and stained as described above. Anti-synapsin I (Invitrogen) and anti-MAP2 (Sigma) antibodies were visualized with Texas-Red and Alexa 488-conjugated secondary antibodies. Images were acquired with a Zeiss LSM 410 or 510 confocal microscope and analyzed by using VOLOCITY software (Improvision). Western blots from 14 day old hippocampal neurons from wild-type and RGS2(-/-) mice were performed according to published procedures (Wittemann et al., 2000). Antibodies were from Santa Cruz (G\(_{\alpha_1}\), G\(_{\alpha_13}\), G\(_{\alpha_2}\), G\(_{\alpha_{q/11}}\), G\(_{\beta_2}\)) and RGS4 antibody was a gift from Dr. S. Mumby, Southwestern University, TX, USA. For monitoring the Gq-
coupled receptor activation with the PH-EGFP constructs, HEK293 cells were transfected in a 5:1 molar ratio with mAChR-M1 and PH-EGFP. 24 h after transfection cells were continuously perfused with extracellular recording solution. GFP fluorescence was monitored on the Zeiss LSM 510 inverted confocal microscope at RT using a 63 x air objective. Pictures were taken every 5 sec and analyzed using VOLOCITY software (Improvision). 100 µM Mch (acetyl-B-methylcholine chloride, Sigma) was applied within 2 min and the average fluorescence intensity in a defined cytoplasmic region was normalized to the average intensity before the application of the drug ($F_0 (F/F_0)$).

**In Situ Hybridization**: In situ hybridization was performed as described previously by Schaeren-Wiemers and Gerfin-Moser (Schaeren-Wiemers and Gerfin-Moser, 1993) and described in the supplemental material.

**Real Time Quantitative PCR**: Total RNA was subtracted from 14 DIV cultured neurons with RNeasy® Mini Kit (Qiagen Inc.) and purified with on-column DNase digestion using RNase-Free DNase Set (Qiagen Inc.). For RT-PCR, $1\mu$g of RNA was used for reverse transcription with Advantage® RT-for-PCR Kit (BD Biosciences) to generate $100 \mu$l cDNA. $3\mu$l of the final RT product was used for real time PCR for RGS2, 5, 7 and 8 and 18S RNA. Real time PCR quantification was performed on iCycler Iq™ Detection System (Bio-Rad) with CYBR® Green assay (Bio-Rad), and DNA fragments of RGS2, 5, 7 and 8 and 18S RNA were amplified with primer pairs given in the supplemental material. The PCR
reactions used a modified 2-step profile with initial denaturation for 3 min at 95°C; 40 cycles of 95°C 15 sec and 57°C 25 sec. For internal control, 3 µl of 1:100 diluted RT product was used for the 18S reaction with every run so that the 18S cyber threshold (Ct) value was reported in the same range of those of the RGS2 fragments to give more accurate comparison. Relative gene expression data was analyzed with 2-ΔΔCT method (Livak and Schmittgen, 2001).

**Electrophysiology and Data Analysis:** For EPSC measurements currents were elicited by a 2 ms long test pulse to 10 mV and recorded and analyzed as published previously (Wittemann et al., 2000). Sucrose solution and various extracellular [Ca²⁺]o solutions were applied directly onto the recorded neurons by using a fast-flow perfusion system (ALA Scientific Instruments). The EPSC and RRP charge was calculated by integrating the currents elicited by the single action-potential or the sucrose application. The Ca²⁺ response curves were fitted according to the Hill equation: EPSC = EPSC_maximal/(1+(EC₅₀/[Ca²⁺]o)^Hill coefficient). The fit values from each curve were used to normalize the dose response curves. The mEPSCs amplitude histograms were plotted with 0.5 pA bins and were fitted with a Gaussian function. Miniature EPSC analysis was performed manually using Mini Analysis Program (Synaptosoft) with an amplitude threshold of 5 pA. 100 ng/ml PTX (Sigma), 10 ng/ml YM-254890 (gift from Dr. Brian Roth) and 0.2 nM BoNT-A (Sigma) were applied 24 h, 18 h and 3 h, respectively, prior to recordings onto the neuronal cultures. Two microelectrode voltage clamp recordings were performed as extensively described in our recent paper (Mark et
al., 2000a) and given in the supplemental material. Recording solutions for EPSC measurements and non-L-type channel recordings are described (Li et al., 2005) and given in the supplemental material. Statistical significance throughout the experiments was tested with ANOVA by using IGOR software unless otherwise indicated. Standard errors are given as mean ± SEM.

All experiments were approved by the Institutional Animal Research Facility.
Figures

Figure 1
Figure 1. RGS2 expressed in hippocampal neurons targets to synaptic sites and regulates short-term synaptic plasticity.

(A) RGS2 mRNA is expressed in neurons from wild-type hippocampal cultures but not in cultures from RGS2(-/-) mice. In situ hybridization of neurons from low-density hippocampal cultures of wild-type mice (upper panel) and RGS2(-/-) mice (lower panel). Nuclei of neurons were visualized with DAPI staining (left). RGS2 mRNA was detected with a DIG-labeled probe and visualized with an Alexa546-coupled anti-DIG antibody (scale bar is 10 μm).

(B) Exogenously expressed RGS2-YFP reveals somato-dendritic staining and colocalization with the presynaptic marker synaptobrevin 2 at synaptic sites. (left), Fluorescence patterns of neurons from low density hippocampal cultures infected with RGS2-YFP reveal a punctuate staining. (middle), Hippocampal neurons were stained with an anti-synaptobrevin 2 antibody and visualized with an Alexa546-coupled secondary antibody. A punctuate staining similar to that seen with RGS2-YFP was observed. (right), Overlay of the left and middle picture demonstrates that RGS2-YFP is partially colocalized with the presynaptic marker synaptobrevin 2 as indicated in the yellow staining (scale bar is 25 μm).

(C to E) Comparison of the paired-pulse ratio (PPR) from autaptic cultures of rat (D), mice and RGS2(-/-) mice (E) in the absence and presence of exogenously expressed RGS2. Single neuron autapses were voltage clamped at a holding potential of –60 mV. EPSCs were evoked by pairs of 2 ms depolarizing pulses to 10 mV (50 ms interpulse interval (20 Hz)) every 2 s. Representative sample
traces of the summary data in D and E are shown in panel (C). PPR was calculated as the ratio of the second peak EPSC amplitude to the first one.

(D) Exogenously expressed RGS2 significantly reduced the 20 Hz PPR (depression) compared with wild-type controls (**P<0.005).

(E) RGS2(-/-) cultures show significantly increased PPR (facilitation) compared with wild-type controls (**P<0.005), and PPR is reduced in RGS2(-/-) and wild-type neurons, when RGS2 is exogenously expressed (*P<0.05).
Figure 2
Figure 2. Cultured hippocampal neurons from RGS2(-/-) and wild-type mice have comparable G protein expression and make comparable amounts of synaptic contacts.

(A) Western blot from 14 day old wild-type (left) and RGS2(-/-) (middle) cultured hippocampal neurons. The expression of the following endogenous proteins was evaluated with antibodies: G\textsubscript{\alpha1}, G\textsubscript{\alpha3}, G\textsubscript{\alpha5}, G\textsubscript{q/11}, G\textsubscript{\beta2} and RGS4. Loading control was a-tubulin. As a positive control for the antibody we transfected HEK293 cells with cDNAs for the indicated proteins (right).

(B) Real time quantitative PCR reveals that neither the absence nor the overexpression of RGS2 (lower panel) changes the relative gene expression of RGS5, 7 and 8. Data are represented relative to the expression of RGS2 in the wild-type neurons. The experiments were performed with three independent neuronal cultures in duplicates (n=6). A 1:100 dilution of sample was used for 18S as an internal control.

(C) Examples of low density hippocampal neurons (14 days in culture) from wild-type (upper) and RGS2(-/-) (lower) mice stained with MAP2-antibody (green) and synapsin I-antibody (red). (Middle) Magnification of the indicated region within the neuron (left) and (right) 3D reconstruction of dendritic arbors from 0.36 \( \mu \text{m} \) z-stacks reveal synaptic contacts between the postsynaptic MAP2 labeled neuron and the presynaptic site labeled by synapsin I.

(D and E) The number of synaptic contacts (D) (i.e. the number of synapsin I punctuates per 10 \( \mu \text{m} \) MAP2 stained dendrite) and the synapsin I/MAP2 staining
ratio (E) (i.e. pixel area stained by synapsin I/MAP2) is comparable between neurons from wild-type and RGS2(-/-) mice.

(F) Comparison of the EPSC amplitudes between autaptic hippocampal neurons from wild-type and RGS2(-/-) mice of the first and second EPSC elicited by two 2 ms long test pulses to +10 mV separated by 50 ms. The EPSCs elicited by the first pulse are significantly different, while the second EPSCs between wild-type and RGS2(-/-) are not, indicating that functional synaptic contacts are sufficiently and comparably formed in both cultures.
Figure 3
Figure 3. RGS2 regulates the probability of synaptic vesicle release and Ca\(^{2+}\) dependence of transmitter release.

(A) (left) Examples of EPSCs evoked by 2 ms depolarizing pulses from -60 mV from wild-type (upper) and RGS2(-/-) (lower) neurons and (right) of the hypertonically mediated release of quanta from the same neuron upon application of 500 mM sucrose for 4 s.

(B) Probability of synaptic vesicle release was evaluated by calculating the ratio of release evoked by the action-potential to that evoked by hypertonic sucrose. In autaptic neurons from RGS2(-/-) mice, the vesicular release probability is significantly reduced compared to wild-type controls (*P<0.05).

(C and D) Varying external Ca\(^{2+}\) and Mg\(^{2+}\) concentrations from 1 to 8-10 mM and 8-10 to 1 mM, respectively, were applied using an amplifier controlled perfusion system.

(C) Example EPSC traces of wild-type (with extracellular [Ca\(^{2+}\])\(_o\) 1 mM, 4 mM, and 8 mM) and RGS2(-/-) neurons (with extracellular [Ca\(^{2+}\])\(_o\) 1 mM, 6 mM, and 10 mM).

(D) EPSC amplitudes were normalized to the maximal response determined by the free dose response fit for the single experiment. In the absence of RGS2 the midpoint of the curve is shifted to the right, indicating that RGS2(-/-) autapses need higher external Ca\(^{2+}\) concentrations to obtain the same synaptic response as the wild-type ones.
Figure 4
Figure 4. Synaptic vesicle recycling as well as spontaneous release properties are not altered in hippocampal neurons from RGS2(-/-) mice.

To evaluate whether RGS2 affects synaptic vesicle recycling two approaches were applied. (A and B) The amount and time course of depletion of the RRP as well as the recovery of the RRP following activity is not different between RGS2(-/-) cultures and wild-type controls. The depletion was determined by measuring the recovery of the EPSC amplitude at varying time points following depletion induced by a 30 stimuli train at 20 Hz. Example traces are shown on top. In A (lower), the EPSCs were normalized to the largest EPSC during the 20 Hz stimuli train. In B (lower), the recovered EPSCs were normalized to the first EPSC in the 20 Hz stimuli train.

(C and D) The refilling of the RRP was also measured by applying paired pulses of hypertonic solution (500mM sucrose, each for 4s) with varying interpulse intervals (1 s, 4 s, 7 s, 10 s, 13 s, 30 s, and 60 s). Example traces are shown in C. The second response was normalized to the first response and the quantified data from wild-type and knock-out cultures are shown in D. Again, there was no significant difference between the recovery rates of neurons from wild-type and RGS2(-/-) mice.

(E) Examples of mEPSCs recorded in the presence of 200 nM TTX from wild-type and RGS2(-/-) autaptic hippocampal cultures. The analysis indicated that the amplitude distribution (mean values: wild-type 11.5 ± 0.1 pA (4510 events, n=32), RGS2(-/-) 11.4 ± 0.1 pA (6441 events, n=37)) (F) and the frequency (G)
(mean frequency) and (H) (interevent interval cumulative fraction plot)) of spontaneous vesicle release were not significantly altered in the absence of RGS2, suggesting that postsynaptic properties did not change in neuronal cultures from RGS2(-/-) mice. Statistical significance of the interevent interval cumulative fraction plot and the mEPSC amplitude distribution was evaluated with a Kolmogorov-Smirnov 2 sample test (p>0.1).
Figure 5
Figure 5. RGS2 regulates synaptic plasticity through PTX-sensitive pathways.

Single neuron autapses were voltage clamped at a holding potential of –60 mV. EPSCs were evoked by pairs of 2 ms depolarizing pulses (10 mV) at 50 ms interpulse intervals (20 Hz) every 2 s.

(A) Example traces of wild-type and RGS2(-/-) neurons in the presence and absence (left) of PTX (middle) and YM-254890 (right) elicited by a two pulse 20 Hz stimulation.

(B) PTX (Gi/o blocker) pretreatment (100 ng/mL, 24 hrs) abolished the increased 20 Hz PPR in RGS2(-/-) autapses (**P<0.005), while YM-254890 (Gq blocker) pretreatment (10 ng/mL, 18 hrs) had no significant effect.

(C and D) Monitoring of intracellular Gq-pathways activation by PH-EGFP.

(C) Confocal images of HEK293 cells transfected with mAChR-M1 and PH-EGFP before (left) and after (right) application of 100 µM Mch. Images are shown as negative contrast images. Black circles show the cytoplasmic area which was used for comparing the fluorescence intensity.

(D) Time course of fluorescence ratio changes (F/F₀) within the cytoplasm during 100 µM Mch application in the presence (lower) and absence (upper) of YM-254890 (10 min and 18 hrs pretreatment 10 ng/mL).
Figure 6. The RGS2 mutant N149A, which is able to affect the G_q pathway but not the G_{i/o} pathway, is unable to rescue the increased PPR in RGS2(-/-) autapses but acts as a dominant negative mutant in wild-type neurons.

(A and B) Both RGS2 and RGS2(N149A) inhibit the G_q pathway.

(A) Example voltage ramp traces of GIRK1/4 currents elicited from Xenopus oocytes coexpressing the mAChR-M_2, P_2Y_2-R, P_2Y_2-R and RGS2 and P_2Y_2-R and RGS2(N149A) by application of 10 μM ACh to activate the mAChR or 10 μM ATP to activate the P_2Y_2-R. Note, the P_2Y_2-R activates not only the G_{i/o} (as the mAChR-R does) but also the G_q pathway. This becomes evident in the large Ca^{2+} activated Cl^{-} current (arrow, outward current). This outward current is absent when RGS2 or RGS2(N149A) are coexpressed with the P_2Y_2-R, since the G_q pathway is blocked by the RGS2 proteins.

(B) In the presence of RGS2 or RGS2(N149A) mutant the P_2Y_2-R mediated outward current is drastically reduced or absent, suggesting that the G_q pathway is inhibited. Values from outward currents are the largest outward current detected after application of ATP and were measured at +40 mV.

(C and D) RGS2 but not RGS2(N149A) is able to accelerate the Gi/o pathway.

(C) Example traces measured at -60 mV of GIRK1/4 currents recorded from Xenopus oocytes by activation of mAChR-M_2 coexpressed with or without RGS2 or RGS2(N149A). GIRK deactivation time is accelerated in the presence of RGS2 and slowed in the presence of RGS2(N149A), indicating that RGS2 but not RGS2(N149A) accelerates the Gi/o pathway.
(D) Comparison of deactivation time constants, derived from a single exponential fit of the deactivation curve as shown in C.

(E and F) Exogenous expression of RGS2(N149A) in RGS2(-/-) autaptic cultures did not rescue PPR, but increased the PPR in wild-type neurons, suggesting that RGS2(N149A) can act as a dominant negative mutant on the Gi/o pathway.

(E) Example EPSC traces of RGS2(N149A) exogenously expressed in wild-type and RGS2(-/-) neurons elicited by a two pulse 20 Hz stimulation.

(F) Comparison between the PPRs of wild-type and RGS2(-/-) neurons exogenously expressing RGS2 or RGS2(N149A).
Figure 7

[Diagram and data presentation]
Figure 7. G protein mediated Ca^{2+} channel inhibition is increased in hippocampal neurons from RGS2(-/-) mice, while BoNT-A has similar effects on the release probability and PPR of neurons from wild-type and RGS2 knock-out mice.

(A) A diagram to show that increased G\betaγ levels in the presynaptic terminal may reduce transmitter release by inhibition of presynaptic Ca^{2+} channels or by binding to SNAP-25 to interfere with vesicle fusion. BoNT-A partially cleaves SNAP-25, which causes PPF in hippocampal neurons.

(B and C) Ca^{2+} currents were elicited from a holding potential of -60 mV by a 10 ms test pulse to +5 mV. After 2 s a 10 ms prepulse to +100 mV was applied, and a second 10 ms test pulse to +5 mV was elicited after stepping back for 10 ms to -60 mV. Facilitation ratios were determined by dividing the peak current of test pulse 2 by the peak current of test pulse 1.

(B) Examples of traces recorded from wild-type and RGS2(-/-) neurons indicating that, in the absence of RGS2, G protein modulation of the Ca^{2+} channels is increased.

(C) Diagram of the quantified Ca^{2+} current facilitation ratios indicate again that in the absence of RGS2 G protein modulation of Ca^{2+} currents is increased (**P<0.0005).

(D) Example current traces (IV curve) of non-L-type currents from wild-type and RGS2(-/-) neurons elicited by a 500 ms voltage ramp from -60 to +90 mV. The
comparison of the traces reveals a positive shift in the peak inward current with no change in the reversal potential.

(E) Diagram of the averaged peak currents of currents elicited by the voltage ramp demonstrates that the non-L-type Ca\(^{2+}\) currents are reduced in the absence of RGS2 (*P<0.04).

(F) Diagram of the voltage at which the peak current appears during the voltage ramp. The diagram shows that the peak current is shifted to more positive potentials in RGS2(-/-) neurons (*P<0.007).

(G and H) Application of BoNT-A increases PPR of both wild-type and RGS2(-/-) neurons suggesting that the main action of G\(\beta\gamma\) in reducing transmitter release is via inhibition of presynaptic Ca\(^{2+}\) channels. (G) Examples of EPSC traces of wild-type and RGS2(-/-) neurons in the presence and absence of BoNT-A elicited by a two pulse 20 Hz stimulation.

(H) Comparison of the paired-pulse ratio (PPR) from autaptic cultures of wild-type mice and RGS2(-/-) mice in the presence or absence of BoNT-A. Single neuron autapses were voltage clamped at a holding potential of –60 mV. EPSCs were evoked by pairs of 2 ms depolarizing pulses (10 mV) (50 ms interpulse interval (20 Hz)) every 2 s. PPR was calculated as the ratio of the second peak EPSC amplitude to the first one. In the presence of BoNT-A in RGS2(-/-) as well as wild-type cultures PPR (facilitation) is significantly increased (*P<0.05; **P<0.01).

(I) Western blot analysis of endogenous SNAP-25 from wild-type and RGS2(-/-) hippocampal cultures before and after BoNT-A (0.2 nM) treatment for 1 and 3 h.
α-Tubulin was used as a loading control. As a positive control for the antibody we transfected HEK293 cells with cDNAs for SNAP-25 A and B.

Quantification of the relative amount of SNAP-25 after 3 h of BoNT-A treatment. After 3 h of BoNT-A treatment SNAP-25 protein is reduced by 50-60% when compared to control protein levels in wild-type and RGS2(-/-) neurons.
Supplemental Figure

A

RGS2-YFP

RGS2-Ab

B

WT
RGS2-Ab

WT + RGS2
RGS2-Ab

WT + RGS2
GFP (not fused)

C

RGS2(-/-)
RGS2-Ab

RGS2(-/-) + RGS2
RGS2-Ab

RGS2(-/-) + RGS2
GFP (not fused)

D

WT

RGS2(-/-)

plus RGS2

E

HEK293

Brain

Hippocampal cultures

HEK293

(-/-) MOK WT WT (-/-) RGS2-YFP

Han et al. (2006) Supplemental Figure
Supplemental Figure

The RGS2 antibodies are unspecific but recognize RGS2.

A) HEK293 cells were transfected with RGS2-YFP (pEYFP-N1). The RGS2 antibody (middle) recognizes the RGS2-YFP fusion protein. YFP fluorescence is shown on the left. (right) DIC image of the transfected cells reveal that for non-transfected cells on the right side of the image the fluorescent signal of RGS2-antibody in HEK293 cells is small. Z-stack images (0.1μm stacks) were taken on a upright Leica DMLFSA.

B and C) RGS2 antibody recognizes unspecific proteins in the wild-type and RGS2(-/-) neurons (left). There were no differences in the amount of protein staining between the knock-out and wild-type cultures (see D). Infection of these cultures with RGS2 SinRep(nsP2S726)dSP-EGFP virus allow for visualization of the infected neurons via GFP fluorescence (not fused to RGS2) (right) and indicates that the RGS2 protein concentration is increased after virus infection (middle). Note that these pictures were taken on the Zeiss LSM 510 Meta system, with the same software settings (e.g. exposure times and gains).

D) Calculation of fluorescent signal given by the RGS2 antibody for non-infected neurons and RGS2 infected neurons. The fluorescent signal increased significantly after RGS2 infection. No differences in the fluorescent signal between knock-out and wild-type cultures could be detected.

E) Performance of the RGS2 antibody in Western blots. (Left)Western blots of brain extracts from RGS2(-/-) and wild-type mice (middle lane control non-transfected HEK293 cells). (middle) Western blots of hippocampal cultures from
RGS2(−/−) and wild-type mice. (right) Western blot of HEK293 cells transfected with RGS2-YFP (pEYFP-N1).

Method for A-C. 12-24 h after transfection or viral infection, cells were fixed with 4 % paraformaldehyde and permeabilized with 0.2 % Triton X-100 in PBS. Anti-RGS-2 (Abcam) antibody was used to label RGS2 (1:500 dilution). Neurons were incubated with the primary antibody overnight at 4°C, then washed, and then incubated with Alexa 568-conjugated secondary antibody (Molecular Probes) for 30 min at room temperature. Cells were embedded in Prolong Gold antifade (Molecular Probes).
Supplemental Methods

In Situ Hybridization:
The probes for mouse RGS2 were isolated using the following primer pair: 5’ ATGCAAAGTGCCATGTTCTGGCT 3’ and 5’ TTCTGCCCAGAGCTGCGCTTCCTCA 3’. The PCR product was cloned into pCR4-TOPO (Invitrogen) and confirmed by sequencing. Digoxigenin-labeled sense and antisense probes were synthesized using an in vitro transcription kit (Roche) according to the manufacturer’s instructions.

Primer Pairs used in Real Time Quantitative PCR:

RGS2 forward: TGATTGCCCAAAAATATCCAA
RGS2 backward: GGGCTCCGTGGTGATCTG
RGS5 forward: GGGAAATTCTCCTCCAGAAGC
RGS5 backward: GAAGCTGGCAAATCCATAGC
RGS7 forward: GCAGTGGAGGACCTGAAGAG
RGS7 backward: CGTCCTGGTTCCTTCACATT
RGS8 forward: CTCTCCACGGAAGAAGCAAC
RGS8 backward: GCCTTGGTGACTAGCTTTGC
18S forward: AAACGGCTACCACATCCAAG;
18S backward: CCTCAATGGATCCTCGTTA.
Specificity of RT-PCR products was documented with gel electrophoresis and resulted in a single product with desired length. The melt curve analysis showed that each primer pair had a single product-specific melting temperature. All primer pairs have at least 95% of PCR efficiency, as reported from the slopes of the standard curves generated by iQ software (Bio-Rad, version 3.1). Relative gene expression data was analyzed with 2-ΔΔCT method (Livak and Schmittgen, 2001).

**Recording solutions:**

For EPSC measurements, the recording solution contained in mM, extracellular: 172 NaCl, 2.4 KCl, 10 HEPES, 10 glucose, 4 CaCl₂, and 4 MgCl₂ (pH 7.3); intracellular: 145 K⁺ gluconate, 15 Hepes, 1 K⁺-EGTA, 4 Na-ATP, and 0.4 Na-GTP (pH 7.3).

For non-L-type channel recordings in cultured hippocampal neurons, the following solutions were used: Internal solution (mM), 120 N-methyl-D-glucamine, 20 TEACl, 10 HEPES, 1 CaCl₂, 14 phosphocreatine (Tris), 4 Mg-ATP, 0.3 Na₂GTP, 11 EGTA, pH 7.2, with methanesulfonic acid; for external solution (mM), 145 TEA, 10 HEPES, 10 CaCl₂, 15 glucose, pH 7.4, with methanesulfonic acid. In addition, 1 µM TTX (Sigma) and 5 µM nimodipine (Sigma) were added to the external solution to block voltage-dependent Na⁺ channels and L-type Ca²⁺ channels.
mRNA Synthesis, Xenopus Oocytes Preparation, and Two Microelectrode Voltage Clamp Recordings:

For recordings of GIRK currents in Xenopus oocytes GIRK 1 and 4, RGS2 and RGS2(N149A) and mACHR-M2 and P2Y2-R were cloned into pBF1 as described (Mark et al., 2000a). Plasmids were linearized 3’ after the poly A stretch and mRNA was synthesized using a mRNA synthesis kit (Ambion, Inc.) according to the manufacturer protocol. For the set of experiments respective cRNAs were synthesized at the same day and under the same conditions. For determining the cRNA concentration the fluorescent intensity of the synthesized cRNA band was compared to the fluorescent intensity signal of the RNA ladder containing 0.5μg/3μl for each RNA band (Gibco BRL; 0.24-9.5 Kb RNA ladder). cRNAs were diluted to the same concentration (100ng/μl for GIRK and RGS constructs; 200ng/μl for GPCRs) and referred to as the 1:1 or 2:1 dilution respectively, which were stored at -80°C. These solutions were used for further dilutions. Throughout the experiments Xenopus oocytes were injected with a 1:10 dilution of GIRK1/4, a 2:1 dilution of the GPCR, a 1:2 dilution for RGS2 or 1:2 or 1:20 dilution for RGS2(N149A). Xenopus oocytes were removed and prepared for mRNA injection as previously described (Mark et al., 2000a). Oocytes were injected with a 50 nl volume containing the cRNAs and incubated at 19°C in OR2 solution (5 mM HEPES pH7.3, 82.5 mM NaCl, 2.5 mM KCl, 1 mM Na2HPO4, 0.5 g/l polyvinylpyrolidone, 1 mM MgCl2, 1 mM CaCl2, 0.1% penicillin/streptomycin). 24 h after injection oocytes were incubated for 30 min in OR2 containing collagenase type 1A (0.5 mg/ml, Sigma). The follicle layer was removed the next
day manually using tweezers. Currents were measured using a standard-two microelectrode voltage clamp set up 72-96 h after mRNA injection. Oocytes were placed into a continuous flow chamber with a solution exchange rate of approximately 1 sec and perfused with 90 mM K+ modified frog Ringer’s solution (10 mM HEPES pH7.3, 27.5 mM NaCl, 90 mM KCl, 1.8 mM CaCl2). Oocytes were voltage clamped at 0 mV for 20 ms, then a 960 ms long voltage ramp from -100 to +50 mV was applied which then stepped back to 0 mV for 20 ms. This 1 sec long protocol was repeated 100 times. Transmitters (10 μM ACh and 10 μM ATP) were typically applied after 10 sec for 30 sec and then washed out. Time constants for deactivation curves were calculated at -65 mV and were determined with a single exponential fit. The Ca2+ activated Cl− current (outward current) was measured at +40 mV. Here the largest outward current, which was elicited by 10 μM ATP, was used for analysis. Voltage and current electrodes had a resistance of 0.1-0.6 MΩ and were filled with 3 M KCl. Currents were sampled at 1 KHz using Pulse++ software and analyzed on a Macintosh computer using software (Igor and Pulse++).
CHAPTER 3

RGS2 REGULATES SEROTONERGIC SIGNALING IN MOUSE BRAIN
Abstract

Regulators of G protein signaling proteins (RGS) modulate G protein signals throughout the brain. RGS2, one of the small members of the RGS family has been suggested to be involved in modulating anxiety like behaviors. Emotional behaviors including anxiety are regulated in particular by the transmitter serotonin. We therefore analyzed if the loss of RGS2 protein in the mouse brain will alter the properties of serotonergic neurons in mid/hindbrain regions containing serotonergic neurons, which project into neuronal circuits involved in anxiety, i.e. amygdala, hippocampus and cortex. Our results reveal that while RGS2 is highly expressed in the serotonergic system early in development, the loss of RGS2 within the mouse brain does not result in changes in the gross morphology of the serotonergic transmitter system. However, loss of RGS2 leads to changes in the physiological properties of the neurons, including changes in membrane potential and spontaneous firing. These effects are mediated by increased G protein activity in these neurons. Our results suggest that RGS2 regulates precisely the firing and output of serotonergic neurons in the brain, which gives an explanation for the anxious phenotypes of RGS2 (-/-) mice.
Introduction

RGS proteins terminate G protein signals. Among the RGS family RGS2 plays a prominent role in the brain. RGS2 expression has been shown to be rapidly up-regulated in various brain regions like the hippocampus by excitatory stimuli (Ingi et al., 1998). These results suggest a regulatory role of RGS2 in synaptic transmission and synaptic plasticity that is further underlined by the fact that RGS2 knock-out mice reveal a decrease in synaptic transmission related to increased anxiety and reduced male aggression of the mice (Han et al., 2006; Oliveira-Dos-Santos et al., 2000). In addition RGS2(-/-) mice are hypertensive. Hypertension involves RGS2 effects within the periphery but most likely also changes in higher order blood pressure regulation (Gross et al., 2005). Thus, the involvement of RGS2 in synaptic transmission and plasticity events in the brain is critical for both the physiology of neurons as well as for behavioral output.

Two major findings suggest that RGS2 affects anxiety. The first set of evidence came from the RGS2 knock-out mouse. Characterization of several behaviors of these mice suggested that RGS2(-/-)mice are more anxious, since they spent more time in the dark in comparison to wild-type litter mates, as evaluated with the light/dark preference test (Oliveira-Dos-Santos et al., 2000). The behavioral changes in the RGS2(-/-) mouse are supported by the fact that RGS2 has recently been identified as a quantitative trait for anxiety (Yalcin et al., 2004) and
variations within the RGS2 gene are expected to play a role for the development of anxiety in humans (Leygraf et al., 2006).

Anxiety like behaviors involve the inter-connection of multiple brain regions including the cerebral cortex, amygdala, hypothalamus and hippocampus (Bear et al., 2001). Short-term treatment of anxiety attacks and anxiety related disorders involve stimulation of the GABAergic pathways in the brain. Long-term treatment of anxiety involves the manipulation of the serotonergic transmitter system, suggesting that anxiety is in particular regulated by the transmitter serotonin. Several lines of evidence support the modulatory role of serotonin for the emotional state. For example, increase of serotonin in the brain is associated with reduced anxiety. Conversely, reduction of serotonin levels in the brain and a loss of the serotonergic transmitter system in mice causes strong anxiety (Gross and Hen, 2004; Hendricks et al., 2003). Genetic variations within the human 5HT-transporter (5HTT) gene linked to anxiety again point to the serotonergic system as a modulator of anxiety. Individuals that are homozygous for the short allele of the 5HTT gene, correlated with reduced activity of 5HTT are more anxious (Auerbach et al., 1999; Lesch, 2001). This finding is supported by the increased anxiety demonstrated in the 5HTT knock-out mouse (Holmes et al., 2003). Further evidence for the involvement of serotonin in anxiety comes from studies of G protein coupled receptors (GPCRs), which are activated by serotonin (i.e. 5HT-1A and 1B receptors). Application of 5HT-1A and -1B receptor agonists, mutations in the receptor genes, as well as 5HT receptor
knock-outs correlate with increased anxiety, when the receptors are blocked, malfunctioning or deleted. Conversely, anxiety is reduced when the receptors are stimulated (Nelson and Chiavegatto, 2001). Interestingly, 5HT-1A seem to be involved in the development of anxiety circuits, since conditional knock-out of the receptor 4 weeks after birth does not induce anxiety related phenotypes as seen with the permanent knock-out of the receptor. In addition, single nucleotide polymorphisms associated with anxiety have been identified in the human 5HT-1A gene (Strobel et al., 2003) and the mono-amino-oxidase A (MAOA) gene (Caspi et al., 2002), the enzyme which is involved in the metabolization of serotonin, dopamine and noradrenaline. Thus, all these findings suggest that serotonin is involved in modulating anxiety and anxiety related behavior.

The serotonergic transmitter system consists of only a small number of neurons, which project into all brain areas. The activity of the serotonergic transmitter system is regulated via transmitter release from local neurons and/or afferents to the raphe nuclei (hetero-regulation) and in particular via autoregulatory mechanisms arising from the serotonergic neurons themselves (auto-regulation). Auto- as well as hetero-regulation of the serotonergic system involves in particular GPCRs which couple to the Gi/o pathway. GPCRs coupling to the Gi/o pathway, such as 5HT-1A, -1B and -1D are found at presynaptic terminals and somato-dendritic areas within the serotonergic neurons and are therefore involved in the regulation of neuronal excitability and transmitter release. Overall, activation of these receptors will cause reduced transmitter release at the
presynaptic terminals and increased hyperpolarization accompanied with decreased firing rates and altered firing patterns at the somato-dendendritic sites. Interestingly, the Gq pathway seems to play a minor role in the serotonergic neurons for controlling the activity. We demonstrated in our recent publication (Han et al., 2006) that the basal activity of the Gi/o proteins is increased in the absence of RGS2. This raises the issue of how RGS proteins and in particular RGS2 regulate the pre- and postsynaptic events and what can be expected when RGS2 is expressed at high or low levels within the serotonergic system.

In order to address this question we crossed RGS2 (-/-) mice with mice, where the serotonergic system is light up by expression of YFP within the serotonergic system (Scott et al., 2005). Using electrophysiological, immunohistochemical and molecular biological techniques, we show that RGS2 is highly expressed early in development in the serotonergic system. The expression of RGS2 is not necessary for the gross development of the transmitter system but changes the physiological properties of the neuronal circuit when absent. Serotonergic neurons in B7 reveal a decrease in the spontaneous firing rate accompanied with a decrease in the membrane potential when RGS2 is absent, with no change in the action potential waveform or AP properties. The results are in agreement with our previous finding that the basal G protein activity of the Gi/o pathway is increased and reveal that the serotonergic output is reduced in RGS2 (-/-) mice in comparison to wild-type mice.
Results

**RGS2 is expressed in the serotonergic transmitter system**

mRNA of RGS2 has been detected in the dorsal raphe (DR), which contains serotonergic neurons (Grafstein-Dunn et al., 2001). In addition, using fluorescence activated cell sorting (FACS) of YFP expressing serotonergic neurons (Scott et al., 2005), followed by an Affimetrix gene array to detect differences in mRNAs levels in the serotonergic transmitter system, RGS2 but not RGS4 mRNAs, which belongs to the same RGS family, were found to be highly expressed within the YFP positive neurons (serotonergic neurons). To verify that RGS2 mRNA can be detected in serotonergic neurons we performed quantitative real time PCR and compared the mRNA levels to mRNA levels detected in cultured hippocampal neurons. As indicated in Figure 1 RGS2 mRNA was detected in YFP-positive serotonergic as well as hippocampal neurons, but not in hippocampal neurons from RGS2(-/-) mice, which we used as a negative control. Interestingly, while RGS4, the closest family member of RGS2, is expressed in higher mRNA levels in hippocampal neurons in comparison to RGS2, RGS4 mRNA could not be detected in YFP-positive serotonergic neurons. These findings suggest that RGS2 has a specific function in the serotonergic transmitter system.
RGS2 does not alter the development and overall morphology of the serotonergic transmitter system

In order to analyze whether RGS2 is involved in determining the development of the serotonergic transmitter system, we looked at the overall morphology of the developmental pattern of YFP positive serotonergic neurons. We compared neurons from wild-type and RGS2(-/-) mice during different developmental stages and serotonin positive nuclei. As indicated in Figure 2 we could not detect any differences in the gross morphology among different nuclei after birth (B5-B8, P0, Figure 2A) and in embryonic stage (E12-12.5 Figure 2B).

RGS2 determines the basal G protein activity in dissociated, serotonergic neurons

We recently demonstrated that non-L-type Ca\(^{2+}\) channel modulation in cultured hippocampal neurons from RGS2(-/-) mice is increased in comparison to wild-type neurons suggesting that basal G protein activity is increased. We therefore wanted to know whether this is also the case in serotonergic neurons. We dissociated hindbrain/midbrain slices containing YPF positive serotonergic neurons and recorded non-L-type Ca\(^{2+}\) channel (mostly Ca\(_v\)2 channels) modulation in these neurons. The voltage dependent G protein modulation of the Ca\(_v\)2 channel family is characterized by the fact that modulation can be relieved by depolarizing voltage pulses, a phenomenon known as pre-pulse facilitation.
(Elmslie et al., 1990). We evoked Ca\(^{2+}\) currents by 5 ms long test pulses to 5 mV from a holding potential of -60 mV and compared the peak current amplitude before and after application of a high positive pre-pulse to +100 mV. As indicated in Figure 3A and B, in the absence of RGS2 (RGS2(-/-)), the facilitation ratio which is defined by the peak current amplitude of the current elicited after the pre-pulse relative to the current amplitude of the current before the pre-pulse, is increased. This suggests that in the absence of RGS2 the G protein modulation of non-L-type channels is increased due to increased basal activity of the G proteins. Increased G protein activity should result in increased inhibition of Ca\(^{2+}\) channels and therefore smaller whole cell Ca\(^{2+}\) currents. To compare the peak current amplitude between serotonergic neurons from wild-type and RGS2(-/-) mice we applied 500 ms voltage ramps from -60 mV to +90 mV and compared the peak current amplitude of the inward current. As shown in Figure 3 C and D the peak current amplitude was reduced in RGS2(-/-) neurons when compared to wild-type neurons. A small but not significant increase in the voltage where the peak current occurs was also observed, as would be expected for a channel which is shifted in their voltage dependence of activation to more positive potentials. Since the peak current is dependent on the driving force and the amount of channels open, this value has to be interpreted carefully.

**RGS2 determines the resting membrane potential of serotonergic neurons**
The increased pre-pulse facilitation ratio of non-L-type Ca\(^{2+}\) channels in dissociated serotonergic neurons suggests that the basal activity of the G protein is increased. Increased G protein activity should modulate various ion channels within serotonergic neurons including G protein inward rectifier K\(^+\) channels, which are modulated by G proteins in a similar, i.e. membrane delimited way (Mark and Herlitze, 2000). We therefore analyzed and compared the resting membrane potential of serotonergic neurons in B7 nuclei between RGS2(-/-) and wild-type neurons and found that in the RGS2(-/-) neurons the resting membrane potential is more negative (Figure 4C). This suggests that GIRK channels are more activated due to the basal activity of the G protein in these cells. We next wanted to know if the shape and properties of the AP are altered in the absence of RGS2. The shape and properties of the AP are determined by various conductance including several Ca\(^{2+}\) (T-, N- and P/Q-type channels), K\(^+\) (A-type, Ca\(^{2+}\) activated, delayed and delayed-rectifiers) and Na\(^+\) conductance. However, we could not detected differences in the threshold, the amplitude, AP width and the afterhyperpolarization (AHP) between APs recorded from B7 YFP-positive neurons from RGS2(-/-) or wild-type neurons (Figure 4A and B).

**RGS2 determines the spontaneous firing of serotonergic neurons**

The midbrain raphe serotonin neurons elicit spontaneous APs with a regular, slow firing pattern (1-5 AP/s) (Aghajanian and Vandermaelen, 1982; Vandermaelen and Aghajanian, 1983). Since a decrease in the resting
membrane potential may predict a change in the firing of serotonergic neurons, we recorded the spontaneous firing of B7, YFP-positive neurons. As indicated in Figure 5A-C, 70-80 % of wild-type serotonergic neurons fire spontaneously with a frequency of 3.3 Hz. These neurons are silenced by the application of serotonin, but return to their spontaneous firing mode, once serotonin is washed out (Figure 5A). In contrast, only 40 % of YPF-positive, serotonergic neurons from RGS2(-/-) mice fire spontaneously (Figure 5B). In addition, the spontaneously firing neurons fire with a lower frequency (1.5 Hz) in comparison to wild-type neurons. These results suggest that RGS2 indeed regulates the spontaneous firing rate of serotonin neurons at least in the B7 nuclei, which is most likely related to the fact that K+ conductance including GIRK channels are more active due to the higher basal activity of the G proteins.
Discussion

We demonstrate here that RGS2 is expressed in the serotonergic system of mice and that RGS2 regulates the spontaneous firing and membrane properties of this transmitter system. The physiological consequences as well as future experiments are discussed below.

**RGS2 is expressed in the serotonergic transmitter system but does not determine its development**

mRNA of RGS2 has been detected in DR, which contain serotonergic neurons (Grafstein-Dunn et al., 2001). In addition, using fluorescence activated cell sorting (FACS) of YFP expressing serotonergic neurons (Scott et al., 2005), followed by an Affimetrix gene array to detect differences in mRNAs levels in the serotonergic transmitter system, RGS2 but not RGS4 mRNAs, which belongs to the same RGS family, were found to be highly expressed within the YFP positive neurons (serotonergic neurons). The gene array data could be verified by quantitative real time PCR (Figure 1). These findings suggest that RGS2 has a specific function in the serotonergic transmitter system. We therefore first analyzed whether RGS2 determines the development and the gross morphology of the serotonergic system using mice where the serotonergic system is visualized by YFP expression (Scott et al., 2005). We did not find any obvious differences between the serotonergic system of E12-12.5 or P0 mice in the absence or
presence of RGS2. We will next analyze if RGS2 determines the fine morphology of the serotonergic neurons. This is of particular importance since it has been shown that hippocampal neurons from RGS2(-/-) mice have reduced spine number in comparison to wild-type litter mates (Oliveira-Dos-Santos et al., 2000). We will therefore next analyze the precise morphology of YPF-positive neurons including spine number and shape in the B7 nuclei, to see if reduce spine number in this transmitter system may contribute to the anxiety phenotype observed in RGS2(-/-) mice.

**RGS2 controls the spontaneous firing of the serotonergic system**

Our major finding is that in the absence of RGS2, the spontaneous firing of serotonergic neurons is reduced. The activity of the serotonergic transmitter system is regulated via transmitter release from local neurons and/or afferents to the raphe nuclei (hetero-regulation) and in particular via autoregulatory mechanisms arising from the serotonergic neurons themselves (auto-regulation). The midbrain raphe serotonin neurons elicit spontaneous AP with a regular, slow firing pattern (1-5 AP/s) (Aghajanian and Vandermaelen, 1982; Vandermaelen and Aghajanian, 1983). Application of serotonin onto the neurons leads to a decreased firing rate. This autoregulation is mediated via 5HT-1A receptors, which are located somatodendritically (Aghajanian et al., 1972; Blier and de Montigny, 1987; VanderMaelen et al., 1986). Activation of the 5HT-1A receptor
causes hyperpolarization via opening $K^+$ channels (Aghajanian and Lakoski, 1984; Yoshimura and Higashi, 1985) and inhibition of high voltage activated (HVA) $\text{Ca}^{2+}$ channels ((Penington and Fox, 1994; Penington and Kelly, 1990). Interestingly, other 5HT receptors such as 5HT-1B, 5HT-2, 5HT-3, and 5HT-7 do not seem to play a role at the somtodendritic regulation of the firing rate of the serotonergic neurons (Pineyro and Blier, 1999), pointing to the important function of the Gi/o pathway in auto-regulation of the serotonergic system. At the presynaptic terminal 5HT-1B and 5HT-1D receptors have been described to be involved in the auto-regulation of the 5HT-release. Activation of these receptors leads to a decrease in the transmitter release most likely via inhibition of presynaptic $\text{Ca}^{2+}$ channels. Again these effects have been attributed to the Gi/o pathway (Pineyro and Blier, 1999). Thus, the increase of the Gi/o pathway activity in the serotonergic system should reduce synaptic output. This indeed seems to be the case. As indicated in Figure 5 the spontaneous firing rate of the serotonergic neurons is reduced in the absence of RGS2. This effect is accompanied by a decrease in the resting membrane potential in RGS2(-/-) neurons in comparison to wild-type neurons. Whether these effects are mediated by the increased activity of GIRK channels or whether other channel conductance plays a role has to be investigated. Additionally, we do not know at this point whether the increased basal activity of the G proteins, which leads to an increased G protein modulation of non-L-type $\text{Ca}^{2+}$ channels will play a role for the synaptic output, i.e. the presynaptic terminal. So far we did not observe any autaptic innervation of the serotonergic neurons itself via axon collaterals as
was suggested by earlier studies (Stamford et al., 2000). Therefore paired-recordings of either serotonergic neurons or serotonergic neurons and targets of 5HT neurons such as hippocampal neurons have to be performed in order to answer this question.

Another interesting question, which has to be addressed, is whether the change in resting membrane potential can be attributed to a specific receptor type such as the 5HT1A receptor or whether we have a general up-regulation of G protein activity. This is an interesting point since the serotonergic system is also controlled via hetero-regulation. The hetero-regulation of the serotonergic firing rate involves various transmitter systems. As expected the GABAergic input into the serotonergic neurons reduces neuronal firing, while glutamatergic input increases firing (Becquet et al., 1993; Levine and Jacobs, 1992). Other transmitters acting on GPCRs, rather than ion channels, such as noradrenaline (α2-adrenergic receptor (Gi/o) (Baraban and Aghajanian, 1980), dopamine (D2 receptors (Gi/o) (Ferre and Artigas, 1993; Ferre et al., 1994), substance P (Gq/11) (Kerwin and Pycock, 1979; Reisine et al., 1982), bombesin (neuromedin B receptor (Gq) (Pinnock et al., 1994) and cholecystokinin (CCKA receptor (Gi/o) (Boden et al., 1991) have also been described to modulate the firing of serotonin neurons. The activation of GPCR leads to an increase in the firing rate and not to a decrease as observed for serotonin. It is important to note that these effects seem to be mediated via activation of Gq as well as Gi/o pathways. This is in particular surprising for the activation of α2-adrenergic receptor and D2 receptors
which both couple to Gi/o pathways. Activation of the Gi/o pathway normally results in inhibition rather than activation of the neuronal firing (see for example (Ehrengruber et al., 1997; Li et al., 2005)). It has been suggested that one possible mechanism of action is via inhibition of a voltage activated K⁺ current (IA) (Aghajanian, 1985). This K⁺ current counteracts the depolarizing phase of the AP. However, this possibility has to be analyzed in more detail, since very little information is available about the G protein modulation of this current type via the Gi/o or Gq pathway.

Another interesting point is that the serotonergic neurons also contain Ih currents (hyperpolarization activated Na⁺/K⁺ current), which are modulated by cyclic nucleotides such as cAMP and cGMP (Toledo-Rodriguez et al., 2005). cAMP levels in particular are modulated over the Gs pathway and a recent paper by Roy et al. suggests that RGS2 also negatively regulates the Gs pathway (Roy et al., 2006). Therefore regulation of the Ih current in serotonergic neurons via RGS2 and the Gs pathway may set the resting membrane potential and input resistance and determine the pace-making activity of spontaneously firing serotonergic neurons.

In summary, we have shown that RGS2 is expressed in serotonergic neurons and that RGS2 regulates the firing of a transmitter system found to control emotion. The differences in anxiety observed between RGS2(-/-) and wild-type mice might be therefore related to the difference in the firing properties of
serotonin neurons. Expression of RGS2 specifically in 5HT neurons will shed light onto this question.
Experimental Procedures

Mouse Breeding for labeling RGS2(-/-) neurons with YFP

To study the function of RGS2 knock-out specifically in serotonergic neurons, we generated YFP labeled serotonergic neurons from RGS2(-/-) mice and wild-type controls. YFP is under the control of the ePet-1 promotor, which allows expression of YFP only in serotonergic neurons (Scott et al., 2005). Since no YFP(+/-) homozygous mice are available, we used heterozygous YFP(+/-) mice to cross with the RGS2(-/-) mice. We genotyped the first generation (F1) to obtain the heterozygous genes of both YFP and RGS2 (YFP(+/-) and RGS2(+/-)) for breeding. In the second generation, both YFP expressing RGS2 deficient mice (YFP(+/-) and RGS2(-/-)) and YFP expressing wild-type controls (YFP(+/-) and RGS2(+/-)) were obtained. Primers: YFP expression (5’-GAACTCCAGCAGGACCATGT-3’ and 5’-TATATCATGGCCGACAAGCA-3’); RGS2 wild-type allele (5’-CCGAGTTCTGTGAAGAAAACATTG-3’ and 5’-GGGACTCCTGGTCTCATGTAGCAT-3’); and RGS2(-/-) allele (5’-GCTAAAGCGCATGCTCCAGAC-3’ and 5’-GGCCCACATTACACGAACC-3’).

Real Time PCR

The experiments were performed with three independent neuronal cultures/preparations (n=3). A 1:100-300 dilution of sample was used for 18S. The following primer pairs were used: RGS2 forward/backward:
TGATTGCCAAAATATCCAA / GGGCTCCGTGGTGATCTG; RGS4 forward/ backward: AGAAATGGCTGAATCGTTG / CCTCTCTGGTGCAAGAGTCC; 18S forward/backward: AAACGGCTACCACATCCAAG / CCTCCAATGGATCCTC GTTA. Specificity of RT-PCR products was documented with gel electrophoresis and resulted in a single product with desired length. The melt curve analysis showed that each primer pair had a single product-specific melting temperature. All primer pairs have at least 95% of PCR efficiency, as reported from the slopes of the standard curves generated by iQ software (Bio-Rad, version 3.1). Relative gene expression data was analyzed with 2-DDCT method (Livak and Schmittgen, 2001).

**Histology and Imaging**

Newborn pups (P0) were decapitated and whole brains were fixed in 4% paraformaldehyde and PBS (pH 7.4) at 4°C. Fixation for embryos was 30 min. The fixed tissue was placed in 30% sucrose w/v and 1 x PBS (pH 7.4) overnight at 4°C, and subsequently mounted in OCT medium (Tissue-Tek) and stored at -80°C. Sections (20 μm) were obtained using a cryostat. Fixed tissue sections were dried after mounting onto SuperFrost Plus slides (Fisher Scientific) for 1 h. For serotonin staining, slides were washed in PBST (1x PBS, pH 7.4, 0.1% Triton X-100) for 15 min and subsequently blocked for 2 h in PBST, 2% normal serum. Slides were incubated in PBST, 2% normal serum, and rabbit polyclonal anti-5HT (1:10,000) (DiaSorin) overnight at 4°C. Slides were washed four times for 5 min
each at room temperature in PBST, followed by incubation with Texas Red goat anti rabbit serum (1:400) (Jackson Immuno Research) for 2 h in the dark. Slides were washed for a final time in PBST for 5 min at room temperature and mounted using ProLong antifade reagent (Molecular Probes). For EYFP visualization only, slides were re-hydrated using PBS and mounted using ProLong antifade reagent (Molecular Probes). Fluorescent images were collected on an Olympus Optical (Tokyo, Japan) BX51 microscope with a SPOT RT color digital camera (Diagnostic Instruments). Embryonic fluorescent images were obtained from 20 μm Z-stacks performed on the Zeiss LSM 510 inverted confocal microscope at room temperature using a 20x air objective. Post-processing of images was performed using Adobe Photoshop CS2 and Canvas™X.

**Physiology**

Acutely dissociated neurons: Serotonergic neurons were enzymatically isolated with modified version of dissociation techniques described previously (Swensen and Bean, 2003). Postnatal day 10-14 pups were anesthetized with avertitin (0.2 cc/g) and decapitated. Coronal slices (400 μm thick) through the dorsal raphe nuclei were prepared by using a VT1000S vibrotome (Leica) in ice-cold dissociation solution containing (in mM): 82 Na₂SO₄, 30 K₂SO₄, 5 MgCl₂, 10 HEPES, 10 glucose, pH 7.4 with NaOH. YFP signal was visualized using a DM LFSA fluorescent microscope (Leica). YFP positive slices were collected and incubated in 10 ml of room temperature dissociation solution containing 2.5mg/ml protease XXIII (pH 7.4), and subsequently incubated in a 30°C water-bath for 7-8
The slices were then washed in ice-cold dissociation solution containing 1 mg/ml bovine serum albumin and 1 mg/ml trypsin inhibitor and maintained on ice in dissociation buffer. Slices were withdrawn as needed and triturated with a fire-polished Pasteur pipette to liberate individual cells. Dissociated neurons were maintained in a Petri dish coated with collagen/laminin for 30-40 min or until settled. Recordings were made using a EPC-9 amplifier (HEKA). For calcium channel recordings in dissociated serotonergic neurons, the following solutions were used: Internal solution (mM), 120 N-methyl-D-glucamine, 20 TEA Cl, 10 HEPES, 1 CaCl2, 14 phosphocreatine (Tris), 4 Mg-ATP, 0.3 Na2GTP, 11 EGTA, pH 7.2, with methanesulfonic acid; for external solution (mM), 145 TEA, 10 HEPES, 10 CaCl2, 15 glucose, pH 7.4, with methanesulfonic acid. In addition, 1 µM TTX (Sigma) was added.

Brain Slices: Postnatal day 12-15 pups were anesthetized with avertin (0.2 cc/g) and decapitated. Coronal slices (270-300 µm thick) through the dorsal raphe nuclei were prepared by using a Leica VT10000S vibrotome in ice-cold dissecting solution containing (in mM): 124 NaCl, 2.5 KCl, 1.23 NaPhosphate monobasic, 3 MgSO4, 26 NaBicarbonate, 10 dextrose, 1 CaCl2, equilibrated with 95% O2/5% CO2. Slices were incubated at 30°C for 30 min and then maintained at room temperature until needed. On-cell or whole-cell recordings were made in B7 neurons visualized under IR-differential interference contrast and DM LFSA fluorescent microscope (Leica) with EPC-10 amplifier (HEKA). During recordings,
slices were superfused with artificial cerebrospinal fluid containing (in mM): 124 NaCl, 3 KCl, 1.23 NaPhosphate monobasic, 1.2 MgSO₄, 26 NaBicarbonate, 10 dextrose, 2.5 CaCl₂, equilibrated with 95% O₂/5% CO₂ and flowing at 2 ml per min. Patch electrodes (2-4 MΩ resistance) contained (in mM): 140 K-methylsulfate, 4 NaCl, 10 HEPES, 0.2 EGTA, 4 MgATP, 0.3 Na₃GTP, and 10 phosphocreatine. Membrane potentials reported were not corrected for liquid junction potentials. Statistical significance throughout the experiments was tested with ANOVA by using IGOR software. Standard errors are given as mean ± SEM.
Figures

Figure 1
Figure 1. RGS2 mRNA is detected in YFP positive, serotonergic neurons at early developmental stages.

Quantitative real time PCR (RT-PCR) of hippocampal neurons in culture and YFP positive serotonergic neurons. Data of the left and middle graphs are represented relative to the expression of RGS2 in cultured hippocampal neurons (21 dic) of wild-type mice. (Left) In hippocampal neurons RGS2 is detected in wild-type but not RGS2(-/-) neurons, while RGS4 is expressed at higher levels in both RGS2(-/-) and wild-type neurons. (Middle) In cell sorted YFP positive (Y+), serotonergic neurons from wild-type mice of embryonic day 12.5, mRNA from RGS2, but not RGS4 was detected. (Right) To compare the relative levels of RGS2 and RGS4 in the different neurons 18S RNA was used as internal control and normalized to the 18S RNA found in hippocampal neurons from wild-type mice.
Figure 2

A

wild-type

B6  B5

B7  B8

RGS2(-/-)

B6  B5

B7  B8

B7  B8

B

wild-type

RGS2(-/-)


Figure 2. The morphology of the serotonergic system at postnatal and embryonic stages is not altered in RGS2(-/-) mice.

(A) Coronal sections (P0, 20 μm) of midbrain showing EYFP fluorescence of B5-B8 raphe nuclei from wild-type (upper) and RGS2(-/-) (lower) mice. D, dorsal; V, ventral. Scale bar: 200 μm.

(B) Confocal images of wild-type (E 12.5) and RGS2 (-/-) (E12) embryonic sagittal sections (20 μm). EYFP fluorescence (green), 5-HT staining (red, stained with rabbit polyclonal anti-5HT and visualized with Texas Red-coupled secondary antibody), and overlay images are shown. D, dorsal; V, ventral; A, anterior; P, posterior. Scale bars: 100 μm.
Figure 3
Figure 3. The G protein modulation of Ca\textsuperscript{2+} channels is increased in serotonergic neurons of RGS2(-/-) mice in comparison to wild-type mice suggesting that the basal G protein activity is increased.

Hindbrain slices from 10-14 day old RGS2(-/-) and wild-type mice were acutely dissociated according to Raman and Bean (1999) (Raman and Bean, 1999). 

(A and B) Ca\textsuperscript{2+} currents were elicited from a holding potential of -60 mV by a 10 ms test pulse to +5 mV. After 2 s, a 10 ms prepulse to +100 mV was applied and a second 10 ms test pulse to +5 mV was elicited after stepping back for 10 ms to -60 mV. Facilitation ratios were determined by dividing the peak current of test pulse 2 by the peak current of test pulse 1. (A) Examples of traces recorded from wild-type and RGS2(-/-) serotonergic neurons indicating that in the absence of RGS2, the G protein modulation of the Ca\textsuperscript{2+} channels is increased. (B) Diagram of the quantified Ca\textsuperscript{2+} current facilitation ratios indicate again that in the absence of RGS2 G protein modulation of Ca\textsuperscript{2+} currents is increased in serotonergic neurons (**P<0.01).

(C) Example current traces (IV curve) of Ca\textsuperscript{2+} currents from wild-type and RGS2(-/-) neurons elicited by a 500 ms voltage ramp from -60 to +90 mV.

(D) Diagram of the averaged peak currents elicited by the voltage ramp demonstrates that the Ca\textsuperscript{2+} currents are reduced in the absence of RGS2 (*P<0.05).

(E) Diagram of the voltage at which the peak current appears during the voltage ramp. The diagram shows that the peak current is shifted to more positive potentials in RGS2(-/-) neurons, but not significantly.
Figure 4

A

Wild-Type

| 20 pA |

AP amplitude

AHP

Threshold

20 mV

200 ms

RGS2(-/-)

| 20 pA |

50% AP width at 50%

20 mV

2 ms

200 ms

B

<table>
<thead>
<tr>
<th></th>
<th>Wild-Type n=19</th>
<th>RGS2(-/-) n=12</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP Amplitude (mV)</td>
<td>76.2</td>
<td>76.4</td>
</tr>
<tr>
<td>Threshold (mV)</td>
<td>25.8</td>
<td>29.6</td>
</tr>
<tr>
<td>AHP (mV)</td>
<td>27.3</td>
<td>26.6</td>
</tr>
<tr>
<td>AP Width at 50% (ms)</td>
<td>1.78</td>
<td>1.67</td>
</tr>
</tbody>
</table>

C

\[ V_{resting} \text{ (mV)} \]

WT RGS2(-/-)

\[ -60 \text{ (15)} \]

\[ -76 \text{ (9)} \]
Figure 4. The membrane properties of YFP positive serotonergic neurons from hindbrain (DR) slices are different between RGS2(-/-) and wild-type mice.

(A) Response of YFP positive neurons (left, wild-type; right, RGS2(-/-)) to a 500 ms 20 pA depolarizing and hyperpolarizing current steps from holding potential at –60 mV. Blow-up figure shows the enlarged action potential waveform of the left figure. Parameters of the action potential (AP) waveform, AP amplitude, threshold, AHP (afterhyperpolarization), and AP width at 50%, are indicated.

(B) Summary table of the AP parameters from wild-type and RGS2(-/-) B7 neurons. Differences between all values are not significant.

(C) Diagram of the resting membrane potential ($V_{resting}$) indicated that serotonergic neurons from RGS2(-/-) mice are more hyperpolarized.
Figure 5
Figure 5. Spontaneous firing of serotonergic neurons in B7 was reduced in RGS2 (-/-) mice.

(A) Example traces of on-cell firing from a wild-type B7 neuron. Spontaneous firing is at 3-4 Hz, which was blocked completely by 5-HT (10 µg/ml) application. This blockade was recovered by washing for 30 min.

(B) Diagram of the quantified firing rate of wild-type (75%) and RGS2(-/-) (38%) B7 neurons.

(C) The spontaneous firing frequency of RGS2(-/-) B7 neurons is significantly reduced compared with wild-type B7 neurons. (* P< 0.05)
CHAPTER 4

DISCUSSION
Predictions for the modulation of ion conductance by RGS2 and the consequences for influencing the physiological properties and transmitter release of serotonergic neurons

G protein-mediated signaling pathways are involved in the regulation of neuronal excitability and transmitter release in the serotonergic system. GPCRs such as 5HT-1A, -1B and -1D are found at presynaptic terminals and somato-dendritic areas within serotonergic neurons. Since these GPCRs are coupled to Gi/o signaling pathway, activation of these receptors will cause reduced transmitter release at the presynaptic terminals and increased hyperpolarization accompanied with decreased firing rates and altered firing patterns at the somato-dendendritic sites. Interestingly, the Gq signaling pathways seem to play a minor role in the serotonergic neurons for controlling neuronal activity. We demonstrate in Chapter 1 that the basal activity of the Gi/o subunits is increased in the absence of RGS2. This finding raises the issue of how RGS2 regulates pre- and postsynaptic events and what can be expected when RGS2 is expressed at high or low levels within the serotonergic system.

RGS proteins accelerate both the onset and decay of G protein mediated signals. This suggests that RGS proteins are essential for the precise physiological signaling events such as synaptic transmission in the CNS, which involves G protein coupled receptor cascades and ion channels. Several studies demonstrate the G protein modulation of presynaptic Ca\textsuperscript{2+} channels of the N-, P/Q- and R-type (Jarvis and Zamponi, 2001). These studies revealed that RGS
accelerates the onset and offset of transmitter mediated inhibition of presynaptic Ca$^{2+}$ channels and also point to a role of RGS in altering the amount of inhibition for the presynaptic Ca$^{2+}$ channels (Ding et al., 2006; Mark et al., 2000b). In my studies, I found by characterizing RGS2(-/-) autaptic synapses in cultured hippocampal neurons as well as serotonergic neurons that the basal G protein activity of the Gi/o pathway is increased (Figure 7 in Chapter 2). This causes increased inhibition of presynaptic Ca$^{2+}$ channels and a reduction in synaptic transmitter release. Thus at the presynaptic terminal, I predict that the transmitter release of serotonergic neurons is modulated via the RGS2 protein accelerating/terminating the Gi/o pathway. High levels of RGS2 attenuates the function of 5HT-1B and 5HT-1D receptors, which are located at the presynaptic terminal leading to less inhibition of presynaptic Ca$^{2+}$ channels and increased synaptic transmission. In contrast, synaptic transmitter release in RGS2(-/-) neurons is reduced, due to the higher basal activity of the Gi/o pathways accompanied by the increased inhibition of presynaptic Ca$^{2+}$ channels (Figure 1 in Chapter 2). To directly answer this question, electrophysiological recordings should be performed in the serotonergic brain slices from both wild-type and RGS2(-/-) mice. An auto-inhibitory circuit in the DR would be an ideal system to study the synaptic transmission of serotonergic neurons. The DR contains a high density of serotonergic neurons, which underlie auto-inhibition. Auto-inhibition has been suggested to be mediated via axon collaterals (recurrent axons) leading to recurrent inhibition of the serotonergic neurons (Aghajanian and Lakoski, 1984; Li et al., 2001; Park et al., 1982; Pineyro et al., 1996). Presynaptic
receptors, 5HT-1B and 5HT-1D receptors, on the presynaptic terminal of axon collaterals modulate the transmitter release through the Gi/o pathway. This circuit in fact resembles the autaptic neuronal circuit used in our original study. If RGS2 modulates transmitter release at the presynaptic terminal of the recurrent axons, we would expect increased paired-pulse ratio or induction of paired-pulse facilitation (PPF) in the serotonergic RGS2(-/-) neurons in comparison to wild-type neurons. Also, we would expect that this effect is mediated by an increase in the G protein modulation within the neuronal circuit involving PTX-sensitive Gi/o pathways, which would be consistent with my finding from the cultured hippocampal neurons. Although my study in Chapter 2 demonstrated that RGS2 down-regulates the basal activity of Gi/o proteins, in other neuronal systems such as the serotonergic system this regulation could be a GPCR-specific effect. Therefore, selective 5HT receptor agonists and antagonists could be used to address whether the effect is mediated via a specific 5HT receptor, i.e. 5HT-1B or 5HT-1D or both receptor types or if other transmitter types are involved.

My study also reveals changes on membrane properties and neuronal firing of serotonergic neurons in RGS2(-/-) mice in comparison to neurons from wild-type mice (Figure 5 in Chapter 3). This suggests that the properties of somatodendritic ion channels are altered in the serotonergic RGS2(-/-) neurons. For example, GPCRs activating the Gi/o pathway at the somatodendritic site act on K⁺ conductance, such as the G protein coupled inward rectifying K⁺ channel (GIRK) and also on Ca²⁺ conductance (Caᵥ2 Ca²⁺ channels). GIRK currents play
a role in regulating neuronal excitability in somatodendritic areas. Activation of GPCRs coupling to the Gi/o pathway leads to activation of this channel type followed by hyperpolarization of the cell membrane accompanied with a decrease in the firing frequency (Ehrengruber et al., 1997; Li et al., 2005; Mark and Herlitze, 2000). Increased levels of RGS2 will therefore reduce the G protein activation of GIRK channel, while deficiency of RGS2 will increase GIRK channel activity, therefore regulating the membrane potential and action potential firing. This in fact, could be the mechanism underlying the reduced membrane potential observed in serotonin neurons from RGS2(-/-) mice in comparison to wild-type neurons. Regulation of Ca_2^+ channels via Gi/o pathways and RGS proteins have recently been described to modulate the firing pattern and firing frequency of striatal cholinergic interneurons (Ding et al., 2006). Ca^2+ influx through the voltage gated Ca^2+ channels, which are activated during AP firing, can lead to the activation of Ca^2+ activated K^+ channels accompanied with changes in the firing rate and spike frequency adaptation. The reduced influx of Ca^2+ ions through Ca_2^+ channels and increased efflux of K^+ ions through GIRK channels during increased G protein modulation predicts the hyperpolarization of the resting membrane potential and the alteration of the firing rate. Therefore, increased G protein modulation of GIRK channels and/or voltage gated Ca^2+ channels in the serotonergic RGS2-/- neurons could be the explanation for the reduced firing rate and hyperpolarized resting membrane potential which was described in Chapter 2 (Figure 4).
In summary, activation of GPCRs coupling to the Gi/o pathway will lead to a decrease in neuronal activity either by presynaptic inhibition or postsynaptic effects involving hyperpolarization. High level of RGS2 proteins will reduce Gi/o mediated signaling and increase serotonin release, conversely low level of RGS2 will leave the Gi/o signaling pathway more active and will therefore decrease serotonin release.

**Putative effects of RGS2 on neuronal morphology**

One possible mechanism of how RGS2 could alter neuronal circuits underlying behavior is by changing the morphology of the neurons. In my study, no significant defects were found in the gross morphology of serotonergic system in RGS2-/- mice. However, morphological alterations of synaptic structure have been observed in RGS2 deficient mice. In the RGS2 knockout mouse, the numbers of neurons in several brain areas, including hippocampus, brain stem, cerebellum and cortex, were calculated and no apparent change was identified (Oliveira-Dos-Santos et al., 2000). However the density of apical and basilar spines was significantly reduced in CA1 hippocampal areas, suggesting a role of RGS2 in spine formation and perhaps neuronal development. Since spine density correlates with synapse number and synaptic activity, reduced spine density in RGS2(-/-) mouse was attributed to reduced neuronal activity in the hippocampal region. More direct evidence supporting the regulatory role of RGS2 in synaptic development was obtained in PC12 cells (Heo et al., 2006). RGS2 was found at the termini of neurites in differentiated PC12 cells. Exogenous
expression of RGS2 significantly enhanced growth factor-induced neurite outgrowth. In contrast, RGS2 specific siRNA treatment suppressed the neurite outgrowth. The mechanism underlying this effect is that a short region within the N-terminus of RGS2 directly interacts with tubulin and enhances the polymerization of microtubules. This study may give an explanation for the morphological phenotype in RGS2 deficient mice in the CA1 neurons. Given the fact that a variety of stimulations such as electroconvulsive seizures, haloperidol, cocaine, and LTP increase the level of RGS2 mRNA rapidly and transiently in the brain, up-regulated expression of RGS2 might in turn promote the development of neurites in various brain regions.

Besides this direct effect of RGS2 on regulating polymerization and stabilization of microtubules, RGS2 could also affect synaptic development through indirect pathways in various brain regions, for example the serotonergic system. Serotonin is found to be involved in shaping the morphology of dendrites including spine formation as well as dendritic length and branching (Faber and Haring, 1999; Haring and Yan, 1999; Mazer et al., 1997; Norrholm and Ouimet, 2000; Okado et al., 1993; Wilson et al., 1998; Yan et al., 1997; Yang et al., 1998). Serotonin depletion, 5HT-1A receptor antagonist treatment, and tryptophan hydroxylase inhibitor treatment resulted in a significant and permanent reduction of dendritic spines (Faber and Haring, 1999; Haring and Yan, 1999; Mazer et al., 1997). Acute antidepressant treatment such as serotonin specific re-uptake inhibitors (fluoxetine or fluvoxamine), a
norepinephrine-specific re-uptake inhibitor (desipramine) can impact dendritic length and spine density (Norrholm and Ouimet, 2000). These data suggest that 5HT may play an important role in the normal increase and maintenance of synapses in developing and adult animals. My study on serotonergic neurons demonstrates that RGS2 deficiency induces higher calcium channel inhibition and more hyperpolarized cell membrane, which most likely affect the serotonergic output (Chapter 2). Therefore, decreased numbers of spines in the serotonergic system can be expected in the RGS2(-/-) mouse. Thus, investigating the spine morphology and spine number of the serotonergic system in the RGS2(-/-) mouse and comparing them to wild-type mice is important to investigate the possibility that long-term emotional changes are regulated via RGS2 mediated morphology changes in the neuron itself.

Expression of RGS2 or RGS2 mutant (RGS2N148A) specifically in the serotonergic system to elucidate the function of RGS2 in this transmitter system

The RGS2 knockout mice give us the opportunity to characterize the role of this protein for neuronal and system function. The drawback of this model is that the knockout of RGS2 throughout the animal cannot distinguish the role of RGS2 in a defined tissue or neuronal circuit. RGS2 is not only expressed in the serotonergic system but also in other brain areas such as the hippocampus, cortex, striatum, and several thalamic and hypothalamic nuclei (Grafstein-Dunn et al., 2001). Therefore, manipulating RGS2 expression levels in the serotonergic system will
help to demonstrate the circuit specific function of RGS2 and the relation to the behavioral output only within the serotonergic circuit. Specifically, RGS2 knock-out mice show two emotional phenotypes: increased anxiety in males and females and decreased aggression in males. Other behaviors analyzed, such as motorneuron function including motor balance and coordination, circadian activity and exploratory behavior were not altered (Oliveira-Dos-Santos et al., 2000). Anxiety related behaviors are in particular modulated by serotonin in the brain, and it is therefore likely that altered levels of RGS2 protein within the serotonergic system probably result in altered serotonin release and are involved in modulating this behavior. However, the possibility that other neuronal systems, such as the GABAergic, dopaminergic and/or noradrenergic systems could participate in modulating anxiety in RGS2/- mouse cannot be excluded. In addition, increased anxiety is normally correlated with increased aggression (Parmigiani et al., 1998). The RGS2 mice are different in this respect, since they are more anxious and less aggressive. Therefore, it is of great interest to determine which neuronal system downstream of the serotonergic system (e.g. amygdala, cortex or hippocampus) is regulated by RGS2 leading to changes in male aggression.

The ePet-1 enhancer identified by Dr. Deneris’s lab will provide us with the possibility to specifically increase or decrease the expression or functional levels of RGS2 in the serotonergic system of mice. We will generate Pet-1 driven transgenic mouse lines to express wild-type RGS2 proteins under wild-type or
RGS2(-/-) backgrounds. These transgenic mouse lines will demonstrate that the up- and down-regulation of RGS2 within the serotonergic system determines the function and G protein regulation of the transmitter system itself, thereby modulating its synaptic output. Creating the ePet-RGS2 mouse also allows us to express RGS2 only in the serotonergic system, when crossed with RGS2(-/-) mice. These mice will identify whether RGS2 expression solely in the serotonergic system is sufficient to rescue the behavioral changes of the RGS2 knock-out mice or if the expression of RGS2 in other transmitter systems and circuits will be necessary. It will also clarify if RGS2 expressed in the serotonergic system affects only anxiety or only aggression or both phenotypes. Thus, comparison of the behavioral phenotypes between the RGS2 transgenic mouse lines in wild-type or knockout backgrounds will dissect the relationship of neuronal circuits and certain behavior outputs.

Another interesting question, which arise is whether behaviors influenced by RGS2 involves the Gi/o, the Gq or both pathways. Increased RGS2 protein levels in neurons are expected to negatively regulate both Gi/o and Gq signaling pathways. To distinguish Gi/o from Gq-mediated signaling we will generate another mouse line, which expresses the dominant negative RGS2 mutant (RGS2N148A). This mutant has been characterized by us in both heterologous expression systems and hippocampal neurons. Specifically, the mutant inhibits the Gq pathway but is not able to accelerate and/or inhibit the described Gi/o effects on ion channels within the presynaptic terminal. Interestingly, this mutant
confers dominant negative effects on the Gi/o pathway on synaptic transmission and induces the same synaptic changes as the knock-out of RGS2. Expression of RGS2N148A will therefore allow us to specifically analyze the effect of the RGS2 on the Gi/o pathway in the serotonergic system of mice.

Transgenic expression of the RGS2 mutant within serotonergic neurons will then give us the possibility to determine if RGS2 acts specifically on only one G protein pathway or if RGS2 acts in general on multiple G protein pathways expressed in serotonergic neurons. For example, GPCR mediated presynaptic inhibition is down-regulated by RGS2 in hippocampal cultures. The question is whether this is a general effect or whether this effect is mediated through specific GPCR, such as 5HT1A or 5HT1B pathways in the serotonergic system. To address this question, specific blockers or agonists for only these receptor types could be applied and the effect of the agonists/antagonists on the G protein modulation of non-L-type Ca$^{2+}$ channels in the soma of the serotonergic system could be analyzed. With this approach specific RGS2/GPCR/effector signaling pathways could be identified. Finding specific RGS2 signaling pathways within the serotonergic system will have major importance for developing drugs to control serotonin release in diseases such as anxiety disorders.
Possible Therapeutic Uses of RGS2-Related Drugs

It has been very well demonstrated that RGS proteins negatively regulate and fine tune G protein signaling. Therefore, alteration of RGS expression levels will alter GPCR-mediated signals.

The major function of RGS is to accelerate the GAP activity and ultimately the GTP hydrolysis on the $\mathrm{G}\alpha$ subunit. This activity requires direct protein-protein interaction between RGS and the target $\mathrm{G}\alpha$ subunit. The crystal structure of RGS4 protein has revealed an interaction site called ‘A-site’ that could be used as a drug target. At this site, threonine182 of $\mathrm{G}\alpha_i$ is buried and interacts with five different residues in the RGS-box (Tesmer et al., 1997). An inhibitor could be designed to specifically fit in this pocket of the RGS-box to prevent or modulate the RGS-$\mathrm{G}\alpha$ interaction, leading to either an increase or a decrease in the G protein signal. Previous studies have demonstrated that expression of RGS-resistant $\mathrm{G}\alpha$ subunits in vivo or in vitro dramatically enhanced GPCR signaling once activated by agonists, suggesting a potential of role of RGS A-site inhibitors in potentiating agonist signaling. Other protein domains within RGS could be used to design RGS inhibitors. For example, RGS GAP activity could be regulated by other signaling molecules through a second interaction site such as the B-site. The B-site of the axin RGS-box is regulated by phosphatidylinositol-3,4,5-trisphosphate ($\mathrm{PIP}_3$) to inhibit the axin GAP activity and by calmodulin to increase the GAP activity (Ishii et al., 2001; Popov et al., 2000). Therefore, drugs targeting the regulatory sequence of RGS proteins could either be RGS inhibitors
or RGS activators. In contrast to the RGS inhibitor drugs, drugs that act as RGS activators will increase the RGS GAP activity and decrease the signaling through Gi/o, Gq or Gs pathways. Another possibility for drug design is to target other functional domains of the RGS proteins outside of the RGS-box. For example, RGS2 has been shown to specifically interact with the scaffolding protein spinophilin, which binds to the intracellular loop of several GPCRs by its non-conserved N-terminal domain (Wang et al., 2005). Interruption of this interaction would down-regulate the specific GPCR-mediated signaling.

Another important drug or scientific tool to develop would be tissue specific RGS2 drugs. RGS2 is widely expressed in mammalian tissues, including a variety of brain regions. Inhibition of RGS2 protein in a particular tissue could therefore greatly increase the action of GPCR agonists in that tissue. For example, the anxiety related behavior of RGS2(-/-) mice suggests that one of the main functions of RGS2 within the serotonergic system is to regulate anxiety-related circuits. A serotonin-specific RGS2 inhibitor would dramatically potentiate the anxiologic function of 5-HT or 5-HT agonists. Thus, exploiting the tissue-specific expression pattern and function of RGS proteins could provide the opportunity to increase the action of GPCR agonist drugs and decrease the possible side effects.
In summary, since the phenotypes related to the RGS2 knockout, i.e. anxiety and hypertension are clinically relevant, understanding the role of RGS2 in neuronal circuits involved in anxiety and central blood pressure control will be important for human health.
References


Auerbach, J., Geller, V., Lezer, S., Shinwell, E., Belmaker, R. H., Levine, J., and Ebstein, R. (1999). Dopamine D4 receptor (D4DR) and serotonin transporter
promoter (5-HTTLPR) polymorphisms in the determination of temperament in 2-month-old infants. Mol Psychiatry 4, 369-373.


are G alpha(i) interaction sites with guanine nucleotide dissociation inhibitor Activity. J Biol Chem 276, 29275-29281.


phorbol ester- and diacylglycerol-induced augmentation of transmitter release is mediated by Munc13s and not by PKCs. Cell 108, 121-133.


Scheschonka, A., Dessauer, C. W., Sinnarajah, S., Chidiac, P., Shi, C. S., and Kehrl, J. H. (2000). RGS3 is a GTPase-activating protein for g(ialpha) and g(qalpha) and a potent inhibitor of signaling by GTPase-deficient forms of g(qalpha) and g(11alpha). Mol Pharmacol 58, 719-728.


