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μ-Opioid receptor desensitization by β-arrestin-2 determines morphine tolerance but not dependence

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Morphine is a powerful pain reliever, but also a potent inducer of tolerance and dependence. The development of opiate tolerance occurs on continued use of the drug such that the amount of drug required to elicit pain relief must be increased to compensate for diminished responsiveness^{1–3}. In many systems, decreased responsiveness to agonists has been correlated with the desensitization of G-protein-coupled receptors. *In vitro* evidence indicates that this process involves phosphorylation of G-protein-coupled receptors and subsequent binding of regulatory proteins called β-arrestins^{4,5}. Using a knockout mouse lacking β-arrestin-2 (βarr2^{-/-}), we have assessed the contribution of desensitization of the μ-opioid receptor to the development of morphine antinociceptive tolerance and the subsequent onset of physical dependence. Here we show that in mice lacking β-arrestin-2, desensitization of the μ-opioid receptor does not occur after chronic morphine treatment, and that these animals fail to develop antinociceptive tolerance. However, the deletion of β-arrestin-2 does not prevent the chronic morphine-induced up-regulation of adenylyl cyclase activity, a cellular marker of dependence, and the mutant mice still become physically dependent on the drug.

We have shown previously⁶ that morphine-induced antinociception is enhanced and prolonged in mice lacking β-arrestin-2. This perpetuation of morphine analgesia suggests that mice lacking β-arrestin-2 may be resistant to the desensitization of the morphine signal. As specific inhibitors of desensitization do not exist, this genetically altered animal model provides a means to potentially abrogate desensitization of the μ-opioid receptor (μOR) in response to morphine. Thus, we examined the regulation of the μOR in relationship to the development of morphine tolerance and dependence in βarr2^{-/-} mice.

An acute challenge with a high dose of morphine induces acute morphine antinociceptive tolerance 24 h after the initial challenge. In this scheme, mice are assessed for their nociceptive response

latencies after a moderate dose of morphine (10 mg per kg, subcutaneously (s.c.)) 24 h after receiving an injection of either saline or 100 mg per kg morphine. Indicative of antinociceptive tolerance, wild-type mice exhibited a roughly 50% reduction in morphine responsiveness if they had received morphine, as compared to saline, the day before (Fig. 1a). The βarr2^{-/-} mice maintained the same degree of responsiveness to morphine, however, whether they had been treated with saline or morphine on the previous day. It therefore seemed that the βarr2^{-/-} mice did not develop acute antinociceptive tolerance to morphine.

As, in the clinical setting, tolerance to morphine's analgesic properties usually develops over continued use of moderate levels of the drug, we evaluated the analgesia provided after daily administration of morphine. Mice were injected daily with morphine, and paw-withdrawal latencies were recorded (Fig. 1b). Although the wild-type littermates had significantly diminished responsiveness to the drug by day 5, the knockout mice continued to experience as much antinociception on day 5 to day 9 as on day 1. To characterize this resistance to morphine antinociceptive tolerance in the βarr2^{-/-}

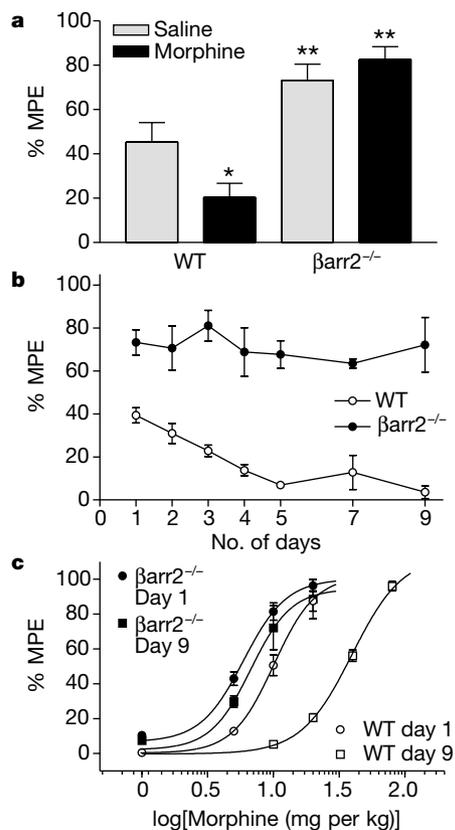


Figure 1 Lack of morphine antinociceptive tolerance in βarr2^{-/-} mice. **a**, Acute tolerance. Wild-type (WT) and βarr2^{-/-} mice were treated with either saline or morphine (100 mg per kg, s.c.). After 24 h, mice were treated with morphine (10 mg per kg, s.c.) and hot-plate-response latencies (56 °C) were recorded. Data are presented as the mean ± s.e.m., n = 7–8. Asterisk, P < 0.05 versus WT + saline; two asterisks, P < 0.05 versus WT, Student's *t*-test. **b**, Chronic tolerance. Mice were treated daily with morphine (10 mg per kg, s.c.); antinociception was assessed 30 min after the injection on the days indicated. Means ± s.e.m. are shown. P < 0.0001, WT versus βarr2^{-/-}; two-way analysis of variance (ANOVA). **c**, Chronic tolerance. Dose–response curves were determined using a cumulative dosing scheme on day 1 and day 9. On day 1, both genotypes were treated with 1, 5, 10 and 20 mg per kg, s.c. On day 9, βarr2^{-/-} mice (n = 6–12) were again challenged with this same dose scheme, whereas WT (n = 7–14) were cumulatively treated with 10, 20, 40 and 80 mg per kg, s.c. and βarr2^{-/-}. Means ± s.e.m. are shown. ED₅₀ (50% effective dose) values were calculated by nonlinear regression analysis (GraphPad Prism); 95% confidence intervals: day 1: WT, 10.1 (8.4–12.1); βarr2^{-/-}, 5.9 (5.0–7.0); day 9: WT, 39.6 (34.0–46.1); βarr2^{-/-}, 6.7 (4.8–9.3).

mice, we examined the effectiveness of morphine in a dose-response scheme, comparing responsiveness on day 1 to that on day 9. Notably, the β arr2^{-/-} mice did not experience a shift in their sensitivity to morphine after chronic daily administration, whereas the wild-type mice experienced a significant rightward shift in efficacy after continued treatment (Fig. 1c).

Both the maintenance of morphine responsiveness in the β arr2^{-/-} mice during chronic administration and the proposed role of β -arrestin-2 in desensitization of G-protein-coupled receptors suggest that a loss of receptor desensitization may underlie the resistance to the development of tolerance. As the analgesic actions of morphine are mediated predominantly through the μ OR⁷⁻⁹, we focused on the desensitization state of this receptor in the β arr2^{-/-} mice. To evaluate

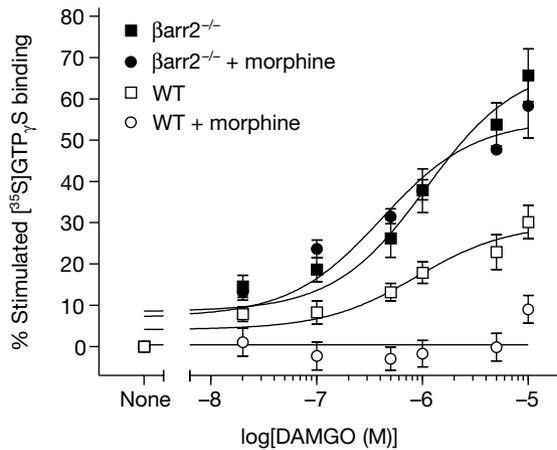


Figure 2 [³⁵S]GTPγS binding to brainstem membranes from β arr2^{-/-} and WT mice before and after chronic morphine treatment. After 5 d of chronic morphine (10 mg per kg, s.c. per day) brain regions were dissected from WT and β arr2^{-/-} mice. Data were analysed by nonlinear regression (GraphPad Prism) and are presented as the mean \pm s.e.m. of at least three experiments performed in triplicate in which WT and β arr2^{-/-} brain regions were assayed simultaneously. In the absence of agonist stimulation, basal [³⁵S]GTPγS binding was WT, 2,049 \pm 91 c.p.m.; β arr2^{-/-}, 1964 \pm 113 c.p.m. The curves were compared by one-way ANOVA followed by a Bonferroni post-hoc test (GraphPad Prism). β arr2^{-/-} and β arr2^{-/-} + morphine curves do not differ but are significantly greater than WT and WT + morphine curves ($P < 0.001$). WT + morphine differs from WT ($P < 0.001$).

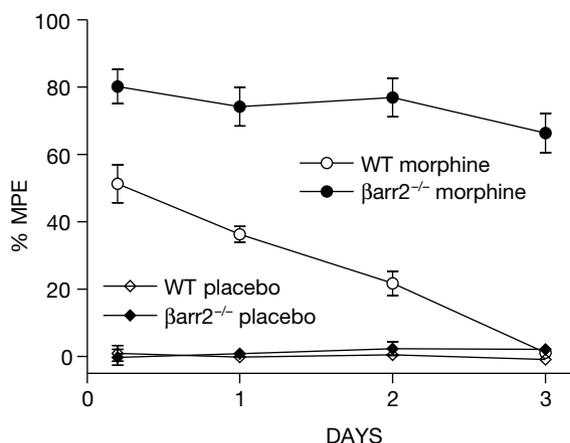


Figure 3 Lack of antinociceptive tolerance in β arr2^{-/-} mice after 72 h of morphine-pellet implantation. Morphine or placebo pellets were implanted subcutaneously and the degree of antinociception was determined by measuring latency of hot-plate (56 °C) responses as in Fig. 1. WT ($n = 8-12$) and knockout ($n = 7-9$) animals were analysed in parallel during the same experiment. Means \pm s.e.m. are shown. WT + morphine versus β arr2^{-/-} + morphine, $P < 0.0001$, two-way ANOVA).

the contribution of this state of the μ OR to the development of behavioural tolerance, we examined the ability of the receptor to couple to G protein on stimulation with the μ OR-selective agonist [*D*-Ala₂, (MePhe)₄, Gly₅-ol]-enkephalin (DAMGO) after chronic administration of morphine. In mice treated only with saline, there is already a difference in the extent of DAMGO-stimulated G-protein coupling in brainstem membranes between the two genotypes, as indicated by [³⁵S]GTPγS binding (Fig. 2). The elevated [³⁵S]GTPγS binding in the β arr2^{-/-} mice is similar to that reported in periaqueductal grey membranes from naive wild-type and β arr2^{-/-} mice⁶. After 5 d of chronic morphine treatment, when wild-type mice experienced substantial nociceptive tolerance, mice were killed and brain regions were used for biochemical studies. The coupling of the μ OR with G proteins was maintained in brainstem membranes from β arr2^{-/-} mice after chronic morphine treatment, whereas the receptors were uncoupled in the membranes from the chronically treated wild-type mice (Fig. 2). Similar results were also obtained in periaqueductal grey membranes (data not shown). The number of μ ORs, as assessed by [³H]naloxone binding⁶ in brainstem-membrane preparations from these same tissues did not decrease after treatment, nor did they vary between genotypes (wild type + saline, 49 \pm 7; wild type + morphine, 57 \pm 3; β arr2^{-/-} + saline, 54 \pm 9; β arr2^{-/-} + morphine, 53 \pm 1 fmol per mg protein). Similar results were also obtained in a separate set of brainstem-membrane-binding experiments using the μ OR-selective agonist, ³H-DAMGO (data not shown).

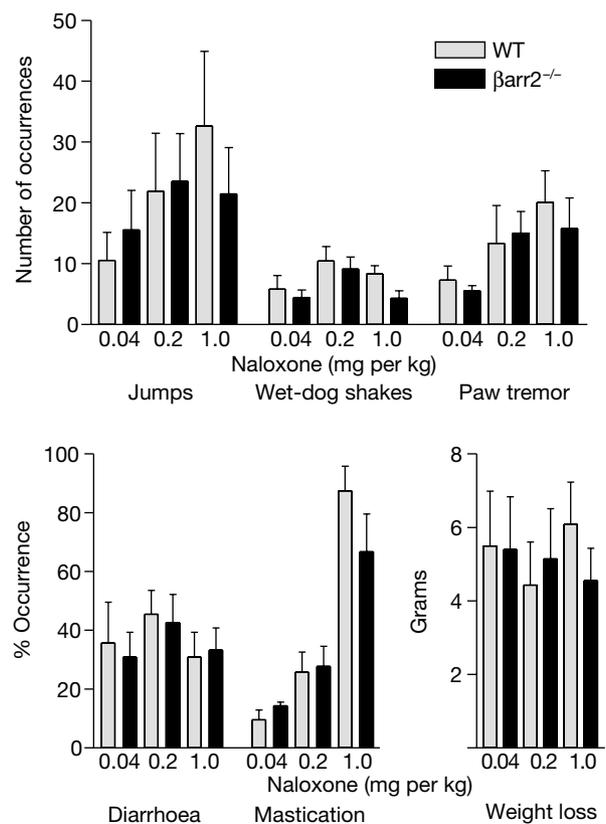


Figure 4 Naloxone-precipitated withdrawal after 72 h of morphine pellet implantation. Naloxone (0.04, 0.2 or 1.0 mg per kg, i.p.) was administered, and the number of jumps, wet-dog shakes and paw-tremor events were counted over 30 min. Occurrence of diarrhoea and mastication was noted as present or absent in six 5-min intervals and normalized according to a maximum of six possible (0 = 0%, 6 = 100%). The weight of each animal was determined before and 30 min after the naloxone injection. There were no significant differences ($P < 0.05$, Student's *t*-test) between the two genotypes. Data are presented as the mean \pm s.e.m.; WT, $n = 7-11$; β arr2^{-/-}, $n = 7-9$. Different sets of animals were used for each dose of naloxone.

To study further this loss of the development of morphine tolerance in the β arr2^{-/-} mice, we adopted a model of chronic morphine treatment in which the mice were continuously supplied with the drug. Subcutaneous implantation of sustained-release morphine pellets (75 mg) into the mice promotes the development of tolerance and also allows the daily measurement of antinociception. This approach is a powerful means for assessing both tolerance and dependence in rodents^{10–13}. Although wild-type littermates experienced a marked decline in responsiveness over 72 h of uninterrupted morphine, the β arr2^{-/-} mice maintained consistent nociceptive latencies (Fig. 3). The parental mouse strains (129/SvJ and C57/BL6) also developed tolerance to morphine in each of the models of chronic treatment (daily injection or pellet implantation) in a manner similar to the wild-type littermates (data not shown). Thus, whether tolerance to morphine is induced acutely, after a single high-dose injection, or chronically, by maintaining constant levels of the drug (morphine pellets) over several days, blocking μ OR desensitization by inactivating β -arrestin-2 eliminates morphine antinociceptive tolerance.

Prolonged exposure to morphine evokes the development of physical dependence to the drug. Tolerance to morphine is often viewed as the gateway to the development of physical dependence¹⁴. As the β arr2^{-/-} mice do not become tolerant to morphine in this scheme, we determined whether they would become dependent on the drug. Opiate dependence was determined by assessing withdrawal responses after administration of the opiate antagonist, naloxone, after 72 h of pellet implantation^{10–12}. Notably, although the β arr2^{-/-} mice did not develop morphine tolerance, they still experienced withdrawal after administration of naloxone (0.04, 0.2 or 1.0 mg per kg, intraperitoneally (i.p.)), which indicates that these mice, like their wild-type littermates, had become dependent on morphine during the 72-h treatment (Fig. 4). There were no significant differences discernable between the genotypes at any of the doses of naloxone.

The cellular mechanisms that lead to opiate dependence are mostly unknown, but the most observed correlative biochemical adaptation is an upregulation of the adenosine-3',5'-monophosphate (cyclic AMP) pathway, most notably in the upregulation of adenylyl cyclases^{1–3,14–16}. Therefore, we evaluated adenylyl cyclase activity in striata from mice treated in the daily chronic morphine scheme (mice in which [³⁵S]GTP γ S binding and receptor levels were

assessed in brainstem membranes; see Fig. 2). After 5 d of chronic treatment, there was a significant increase in basal and forskolin-stimulated adenylyl cyclase activity in both the wild-type and β arr2^{-/-} mice, as compared with saline-treated controls (Fig. 5). The observed increase in adenylyl cyclase activity, a hallmark of opiate dependence, in the β arr2^{-/-} mice indicates that these mice may experience biochemical adaptations in the chronic presence of morphine similar to those occurring in wild type. Although the β arr2^{-/-} mice do not become tolerant to morphine, they clearly become dependent on it, as assessed both behaviourally and biochemically.

Chronic opiate treatment results in functional uncoupling of the μ OR and its G protein in both cell culture^{17–19} and animal models^{14,20,21}. Although studies in transfected cell cultures have implicated phosphorylation by GRKs (G-protein-coupled receptor kinases) and binding of β -arrestins in μ OR desensitization, the contribution of these regulatory elements to the physiological regulation of the μ OR^{22–24}, particularly by morphine, and to the development of tolerance has not been previously tested directly *in vivo*. The use of the β arr2^{-/-} mouse, in which the desensitization process is impaired by a specific genetic manipulation, provides direct evidence in an animal model for the essential contribution of the β -arrestin-2 molecule to the desensitization of the receptor, and to the development of tolerance, after chronic exposure to morphine. Besides the desensitization of the receptor, several downstream adaptive responses to chronic morphine treatment have been observed including increased expression of the transcription factors, CREB and Δ FosB, in certain brain regions and most prominently, an upregulation of the cAMP pathway^{1–3,14,25}. Loss of β -arrestin-2 does not interfere with this cellular aspect of chronic morphine exposure, as both genotypes experience withdrawal and exhibit an increase in adenylyl cyclase activity.

Our results shed light on the relationship between opiate receptor desensitization in response to an agonist such as morphine and the development of both tolerance to and physical dependence on the drug. We have shown that abrogating μ OR desensitization abolishes the development of antinociceptive tolerance to acute or chronic morphine. Moreover, our findings—that in the absence of tolerance to morphine physical dependence persists—indicate that these two phenomena are dissociable, and that their underlying biochemical mechanisms are different. □

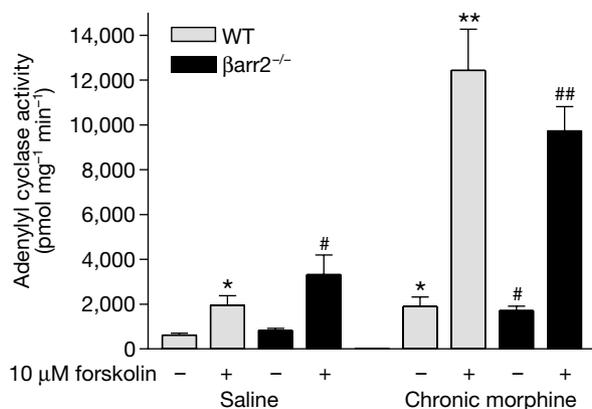


Figure 5 Adenylyl cyclase activity in WT and β arr2^{-/-} mice after chronic morphine treatment. Adenylyl cyclase activity was assessed in membranes prepared from striata of WT and β arr2^{-/-} mice after 5 d of chronic morphine treatment (10 mg per kg, s.c., per day). Data represent the mean \pm s.e.m. of experiments performed in four striata from four animals of each genotype assayed in triplicate in parallel experiments. Asterisk, $P < 0.05$ versus untreated WT; two asterisks, $P < 0.01$ versus all WT; hash, $P < 0.05$ versus untreated β arr2^{-/-}; two hashes, $P < 0.02$ versus all β arr2^{-/-}; Student's *t*-test. There were no significant differences between the genotypes under the same treatment conditions.

Methods

Mutant mice

Wild-type and knockout mice were derived from crossing (over eight generations) heterozygous β arr2 C57BL6/129SvJ animals as described⁶. β arr2^{+/-} and β arr2^{-/-} mice used in this study were age-matched, 3–5-month-old male siblings weighing between 20 and 30 g. In all experiments, wild-type littermates served as controls for the β arr2^{-/-} mice and both genotypes were evaluated simultaneously. Experiments were conducted in accordance with the NIH guidelines for the care and use of animals and with an approved animal protocol from the Duke University Animal Care and Use Committee.

Acute tolerance

Acute antinociceptive tolerance was induced as described²⁶. In each antinociception assay, nociceptive latencies were assessed as the response time to the hot plate (56 °C). The 'response' was defined by the animal either licking his fore- or hindpaws or flicking his hindpaws. In these studies, the most prominent response was forepaw licking. To avoid tissue damage, an artificial maximum time for exposure was imposed, which prevented the animal from contact with the plate for greater than 30 s. Data are reported as the maximum possible effect (% MPE), which was determined by accounting for each individual's basal response as well as the imposed maximum cutoff time using the following calculation: $100\% \times [(drug\ response\ time - basal\ response\ time) / (30\ s - basal\ response\ time)] = \% MPE$. Twenty-four hours after the 100 mg per kg morphine treatment, both genotypes had returned to their basal nociceptive latencies.

Chronic daily morphine treatment

In this study, two groups of mice were treated. The first group, containing wild-type ($n = 6$) and β arr2^{-/-} ($n = 6$) mice were treated daily (between 15:00 and 16:00) with morphine (10 mg per kg, s.c.) and antinociception was assessed 30 min later on each day for the first 5 d. The second group, containing wild-type ($n = 7$) and β arr2^{-/-} ($n = 6$) mice were treated daily (between 15:00 and 16:00) with morphine (10 mg per kg, s.c.), and

antinociception was assessed 30 min after the injections on every odd day for 9 d. Dose-dependent responses were determined in the second group on day 1 and on day 9 using a cumulative dosing model^{6,27}.

[³⁵S]GTPγS binding

Brain regions were dissected and immediately frozen in liquid nitrogen and were stored at -80 °C for less than 1 week before use. [³⁵S]GTPγS (1,250 Ci mmol, NEN, Boston, MA) binding was assessed as described^{6,28}. Briefly, samples were placed on ice and homogenized by polytron in membrane preparation buffer (50 mM Tris pH 7.4, 1 mM EDTA, 3 mM MgCl₂) and crude membranes were prepared by centrifugation at 20,000g for 15 min at 4 °C. Membranes were washed and pelleted twice in this buffer. Membranes were resuspended in assay buffer (50 mM Tris-HCl pH 7.4, 100 mM NaCl, 3 mM MgCl₂, 0.2 mM EDTA) containing 10 μM GDP, and the samples (20 μg protein per tube) were incubated in 50 pM [³⁵S]GTPγS. [³⁵S]GTPγS binding to isolated brainstem membranes was determined after a 2-h stimulation (30 °C) with 50–10,000 nM DAMGO. Reactions were terminated by rapid filtration over GF/B filters (Brandel, Gaithersburg, MD) using a Brandel cell harvester. Filters were washed three times with ice-cold 10 mM Tris-HCl pH 7.4 and then counted in a liquid scintillation counter. The per cent stimulated [³⁵S]GTPγS binding was calculated by dividing unstimulated [³⁵S]GTPγS binding into each agonist-stimulated point.

Morphine pellet implantation

Sustained-release morphine pellets (75 mg) and comparable placebo pellets were provided by the National Institute on Drug Abuse. Pellets were implanted subcutaneously under chloral hydrate anaesthesia and secured by sutures. Mice were kept unrestrained in cages and provided freely with food and water. Nociceptive latencies were measured 4, 24, 48 and 72 h after the surgery.

Adenylyl cyclase activity

Adenylyl cyclase activity was assessed by measuring conversion of [³²P]α[ATP (31 Ci mmol⁻¹; NEN) to [³²P]cAMP by column elution following forskolin stimulation. Forskolin-stimulated adenylyl cyclase activity was determined in striata taken from mice that had been treated daily with saline or morphine for 5 d. Samples were homogenized by polytron in membrane preparation buffer (10 mM Tris pH 7.4, 5 mM EDTA) and crude membranes were prepared by centrifugation at 20,000g for 15 min at 4 °C. Membranes were washed and pelleted twice in this buffer and then were resuspended in assay buffer (75 mM Tris-HCl pH 7.4, 15 mM MgCl₂, 2 mM EDTA, 500 μM IBMX). Membrane protein aliquots (10 μg per tube) were assayed for 10 μM forskolin-stimulated adenylyl cyclase activity in a final volume of 50 μl, for 5 min at 25 °C as described^{29,30}.

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Tyrosine-kinase-dependent recruitment of RGS12 to the N-type calcium channel

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γ-Aminobutyric acid (GABA)_B receptors couple to G_o to inhibit N-type calcium channels in embryonic chick dorsal root ganglion neurons¹. The voltage-independent inhibition, mediated by means of a tyrosine-kinase pathway², is transient and lasts up to 100 seconds. Inhibition of endogenous RGS12, a member of the family of regulators of G-protein signalling, selectively alters the time course of voltage-independent inhibition. The RGS12 protein, in addition to the RGS domain, contains PDZ and PTB domains³. Fusion proteins containing the PTB domain of RGS12 alter the rate of termination of the GABA_B signal, whereas the PDZ or RGS domains of RGS12 have no observable effects. Using