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RGSZ1 and GAIP Regulate μ- but Not δ-Opioid Receptors in Mouse CNS: Role in Tachyphylaxis and Acute Tolerance

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In the CNS, the regulators of G-protein signaling (RGS) proteins belonging to the Rz subfamily, RGS19 (Gα interacting protein (GAIP)) and RGS20 (Z1), control the activity of opioid agonists at μ but not at δ receptors. Rz proteins show high selectivity in deactivating Gαz-GTP subunits. After reducing the expression of RGSZ1 with antisense oligodeoxynucleotides (ODN), the supraspinal antinociception produced by morphine, heroin, DAMGO ([D-Ala^2, *N*-MePhe^4,Gly-ol^5]-enkephalin), and endomorphin-1 was notably increased. No change was observed in the effect of endomorphin-2. This agrees with the proposed existence of different μ receptors for the endomorphins. The activities of DPDPE ([D-Pen^{2,5}]-enkephalin) and [D-Ala^2] deltorphin II, agonists at δ receptors, were also unchanged. Knockdown of GAIP and of the GAIP interacting protein C-terminus (GIPC) led to changes in agonist effects at μ but not at δ receptors. The impairment of RGSZ1 extended the duration of morphine analgesia by at least 1 h beyond that observed in control animals. CTOP (Cys^2, Tyr^3, Orn^5, Pen^7-amide) antagonized morphine analgesia when given during the period in which the effect of morphine was enhanced by RGSZ1 knockdown. Thus, in naive mice, morphine tachyphylaxis originated in the presence of the opioid agonist and during the analgesia time course. The knockdown of RGSZ1 facilitated the development of tolerance to a single dose of morphine and accelerated tolerance to continuous delivery of the opioid. These results indicate that μ but not δ receptors are linked to Rz regulation. The μ receptor-mediated activation of Gz proteins is effective at recruiting the adaptive mechanisms leading to the development of opioid desensitization.

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INTRODUCTION

The pertussis toxin-insensitive Gz transducer protein is regulated by a large number of receptors (Fields and Casey, 1997; Ho and Wong, 2001), leading to the inhibition of adenylyl cyclase activity (Wong *et al*, 1992) and the stimulation of K^+ channels (Jeong and Ikeda, 1998). μ-opioid receptors are described as regulating Gz proteins as well as Gi/o and Gq/11 proteins (Sánchez-Blázquez *et al*, 1995; Standifer *et al*, 1996). In periaqueductal gray matter (PAG) membranes, opioids activate Gz proteins via μ- but not δ-opioid receptors (Garzón *et al*, 1997a, b). Further, Gz proteins are linked to μ but not κ modulation of ERK activity in COS-7 cells (Belcheva *et al*, 2000). These differences are also observed in the production of antinociception. Agonists at μ receptors mainly activate the Gz proteins, while Go proteins are involved in δ-mediated supraspinal analgesia (see eg Garzón *et al*, 2000). Complicating matters, agonists display different affinities to μ receptors when coupled to Gi, Go or Gz proteins (Garzón *et al*, 1998; Stanasila *et al*, 2000; Massotte *et al*, 2002).

The Gz transducer protein shows features that greatly influence the quality of its regulatory activity on cellular effectors (Ho and Wong, 2001). In sharp contrast to Gαi/o subunits, which are rather ubiquitously expressed, the expression of Gαz subunits is limited to the retina, brain, adrenal medulla, and platelets (Fong *et al*, 1988; Matsuoka *et al*, 1988; Gagnon *et al*, 1991). Gαz is an excellent PKC and p21-PAK substrate, whereas Gαi/o, Gαq/11, Gα13, and Gαs are not (Lounsbury *et al*, 1993; Kozasa and Gilman, 1996; Wang *et al*, 1999). Gαi and Gαo hydrolyze bound GTP with a $t_{1/2}$ of about 10–20 s. However, Gαz is extremely inefficient ($t_{1/2} = 7$ min) (Casey *et al*, 1990; Wang *et al*, 1997). Therefore, in the absence of appropriate regulatory mechanisms, the synchronization of agonist-induced G-protein-coupled receptor (GPCR) activation with

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GαGTP-mediated regulation of target effectors hardly occurs. This action relies on the regulators of G-protein signaling (RGS) proteins that behave as GTPase-activating proteins (GAP). These bind receptor-activated GαGTP subunits and by accelerating the hydrolysis of GTP into GDP terminate Gα signaling at effectors. The action of RGS proteins of the R4 subfamily causes Gi/o protein signaling to terminate rapidly (0.1–1 s) (Koelle, 1997; Berman and Gilman, 1998). Deactivation of Gz proteins is achieved specifically by the Rz subfamily of RGS proteins (Glick *et al*, 1998; Wang *et al*, 1998; Ho and Wong, 2001). Rz proteins bind GαzGTP and increase the rate of hydrolysis by 200–400-fold, allowing the termination of signaling on a time scale of a few seconds (Glick *et al*, 1998; Wang *et al*, 1998). This shows that activated Gαz subunits are difficult to switch off after receptor activation unless Rz proteins promote GTP hydrolysis. The subsequent binding of the effector-inactive GαGDP subunits with $G\beta\gamma$ dimers refills the pool of receptor-regulated G-proteins needed by the agonist to transmit signals during its time in the receptor environment. Therefore, RGS proteins help to maintain the strong correspondence between agonist-induced receptor activation and the number of GαGTP subunits that regulate the effectors.

Besides the conserved domain of approximately 130 amino-acid residues that binds the GαGTP subunits and accelerates GTP hydrolysis (the RGS box), the majority of these proteins also contain other domains that permit their interaction with a range of signaling elements in regulatory pathways. The members of the R4 subfamily are proteins of about 20–24 kDa, except for RGS3 which has molecular weights of 61 and 103 kDa. RGS1, RGS2, RGS4, and RGS16 show an amphipathic helix at the N terminus that could serve as a membrane anchor, followed by the RGS box and a short C terminus. The members of the R7 subfamily, RGS6, RGS7, retinal RGS9-1, and RGS11, are proteins of about 50 kDa. The RGS9-2 isoform present in CNS is of 77 kDa. All are similarly organized. The regions flanking the RGS domain all contain a DEP domain (dishevelled, EGL-10, pleckstrin), the R7 homology domain (R7H), and the G-protein (heterotrimeric guanine nucleotide-binding regulatory protein) γ subunit-like (GGL) domain that binds to the $G\beta5$ protein but not to other $G\beta$ subunits (for a review, see Hollinger and Hepler, 2002). Members of the R12 subfamily show notable differences in size and the domains flanking the RGS box. RGS10 is a small protein of 21 kDa with a short N and C terminus. The 60 kDa RGS14 contains a short N terminus and a long C terminus in which are found a rap-binding domain (RBD) and a GoLoco homology domain. RGS12 (95 kDa) has the RBD and GoLoco homology domains in the C terminus. Also, its long N terminus comprises a phosphotyrosine-binding (PTB) domain plus a PDZ (PSD-95 (mammalian postsynaptic density protein), Dlg (drosophila disc-large protein), ZO-1 (a mammalian tight junction protein)) domain. The Rz subfamily includes RGS17 (Z2), Gα interacting protein (GAIP), RGSZ1, and retinal RET-RGS1 (Ross and Wilkie, 2000). The Gαi/o subunits are moderately acidic (pI 5.2–5.7) with a net negative charge at physiological pH. Gαz, however, has a pI of 7.6 and almost neutral charge in the cell environment. Rz is the only subfamily of RGS with a net negative charge at physiological pH (pI of about 5.4). This could facilitate their preferential interaction with Gαz subunits. Flanking the RGS box domain they have an N terminus with a heavily palmitoylated cysteine string motif involved in membrane targeting (De Vries *et al*, 1996), and a short C terminus of 11 or 12 amino-acid residues. GAIP also has an amphipathic helix at the N terminus and a PDZ binding motif at the C terminus (De Vries *et al*, 1998a; Ross and Wilkie, 2000). The PDZ-binding motif of the GAIP C terminus interacts specifically with the PDZ domain of GIPC (GAIP interacting protein C-terminus) (De Vries *et al*, 1998b). This CNS protein does not interact with other RGS proteins and, like other PDZ domain proteins, serves to cluster transmembrane receptors (and probably other proteins) with signaling molecules (Lou *et al*, 2001).

There is increasing evidence that certain RGS proteins show receptor selectivity (Xu *et al*, 1999; Wang *et al*, 2002). Thus, these regulatory proteins might determine the characteristics of the signals triggered by agonists acting at a given receptor. Previous CNS studies have demonstrated the suitability of antisense oligodeoxynucleotides (ODNs) directed towards the mRNA for promoting down-regulation of Gα subunits and RGS proteins (see eg, Garzón *et al*, 2000, 2001). This approach revealed a role for R4 and R7 proteins in the regulation of μ and δ receptor-mediated antinociception in mice (Garzón *et al*, 2001; Garzón *et al*, 2003; Sánchez-Blázquez *et al*, 2003). The R7 proteins facilitate the onset of tachyphylaxis during the time course of agonist effects as well as the development of tolerance to a single and adequate dose of agonists at μ-opioid receptors (Garzón *et al*, 2003; Sánchez-Blázquez *et al*, 2003). The present work addresses the role of the RGS proteins of the Rz subfamily and of GIPC on the activity of agonists at μ and δ receptors. Given the unique characteristics of Gz signaling, the role of Rz proteins in the onset, duration, and end of agonist effects, as well as in the processes that lead to opioid receptor desensitization, was also examined.

MATERIALS AND METHODS

Reduction of RGS and G-Protein Expression

Synthetic end-capped phosphorothioate (stated as *) antisense ODNs were prepared (Sigma-Genosys Ltd, Cambridge, UK). To reduce the synthesis of RGS proteins, the following ODN sequences were used (PrimerSelect, DNASTAR Inc., Madison, WI, USA): the 17 base ODN1 5′-C*T*GGCCCTGTGTGCT*G*T-3′ to nucleotides 175–191 and ODN2 5′-T*A*CGAGTCCCGGTGC*A*T-3′ to nucleotides 710–726 of the murine GAIP gene (NM_026446); the 18 base ODN1 5′-T*C*CACCAGAGGTGGTG*C*T-3′ to nucleotides 81–98 and ODN2 5′-C*A*TCACTTCAGCAGC C*G*G-3′ to nucleotides 328–345 of the murine GIPC gene sequence (AF089818); the 17 base ODN1 5′-T*T*CCG TCCGCTCAGA*T*C-3′ to nucleotides 127–143 and ODN2 5′-T*C*TCACTTCCGCCGC*A*C-3′ to nucleotides 161–177 of the murine RGSZ1 gene (AF191552; NM_021374); the 33 base ODN T*G*TAATCTCACCCTTGCTCTCTGCTGGGC CA*G*T to nucleotides 734–766 of the murine Gαz subunit gene (NM_010311) (Sánchez-Blázquez *et al*, 1995); and the 33 base ODN G*T*GGTCAGCCCAGAGCCTCCGGATGAC GCCC*G*A to nucleotides 477–502 of the murine Gαi2 subunit gene (NM_008138) (Raffa *et al*, 1994). These

sequences showed no homology to any other relevant cloned proteins (GeneBank database). Antisense ODN controls consisted of mismatched sequences in which five bases were switched without altering the remaining sequence: ODN2-GAIPM 5′-T*T*CGACTCGCG CTGC*T*T-3′, ODN1-GIPCM 5′-T*G*CACGAGAGCTGGAG*C*A-3′ and ODN2-RGSZ1M 5′-T*C*ACAGTTGCCCCGG*A*C-3′, and also of a random ODN (ODN-RD) (Sánchez-Blázquez *et al*, 1995).

The ODN solutions were made up in saline immediately prior to use. Animals were lightly anaesthetized with ether and injections were administered into the lateral ventricle as previously described (Sánchez-Blázquez *et al*, 1995; Garzón *et al*, 2000). Each ODN treatment was performed on a distinct group of mice according to the following 5 days schedule: 1 nmol on days 1 and 2, 2 nmol on days 3 and 4, and 3 nmol on day 5. Functional studies usually started on day 6. At the end of the ODN treatment, the mice were monitored for horizontal and vertical activity for periods of 10 min (Digiscan animal activity monitor system, Omnitech electronics, Columbus, OH, USA). Only ODNs causing no alteration to mouse motor performance were selected for the study. The lack of injury capacity of the ODNs used was routinely checked (Garzón *et al*, 2000, 2003).

Expression of RGSZ1

The RGSZ1 was generated by PCR using mouse brain cDNA as a template. The primers corresponding to the 5′ and 3′ ends of the coding region plus a 5′ restriction site for *Sal*I or *Not*I were 5′-ACTCTGGAGTCGACCGCACGGCCAAC-3′ and 5′-ATATATTTGCGGCCGCCTATGCTTCAACTG-3′ (the start codon was supplied by the expression vector, the stop codon is underlined). A 720 bp product was obtained and a glutathione *S*-transferase (GST) fusion protein of murine RGSZ1 (NM_021374) made by its insertion into the bacterial expression vector pGEX-4T-3 (Amersham Biosciences, #27-4801) between the *Sal*I and *Not*I sites. The vector was then introduced into *Escherichia coli* strain BL21(DE3) (Invitrogen, #C6060, Groningen, The Netherlands), and the protein expression was induced with 0.1 mM isopropyl-β-D-thiogalactoside (Amersham Biosciences, #27-3054) for 3 h at 37°C. The GST fusion proteins were purified with glutathione-Sepharose 4B (Amersham Biosciences, #27-4570) and cleaved with biotinylated thrombin (Novagen, #69672-3, Madison, WI, USA). The thrombin was removed by binding to streptavidin–agarose.

Production, Purification, and Characterization of Antibodies to RGSZ1

Antisera directed to the recombinant protein RGSZ1 were raised in New Zealand White rabbits (Biocentre, Barcelona, Spain) as previously described (Garzón *et al*, 1995). Anti RGSZ1 IgGs were purified by affinity chromatography using the recombinant RGSZ1 protein coupled to NHS-activated Sepharose 4 Fast Flow (17-0906-01, Amersham Biosciences, Barcelona, Spain), according to the manufacturer's instructions. The procedure has been described elsewhere (Garzón *et al*, 1995).

Detection of GAIP, GIPC, RGSZ1, Gαi2, and Gαz in Mouse Brain: Electrophoresis and Immunoblotting

At the end of ODN treatment, groups of mice were killed by decapitation and neural structures were collected. For each determination, structures from four mice were pooled and SDS-solubilized P2 membranes were resolved by SDS/polyacrylamide gel electrophoresis (PAGE) in 8 cm × 11 cm × 1.5 cm gel slabs (10–20% total acrylamide concentration/2.6% bisacrylamide crosslinker concentration). For immunodetection, 60 µg protein/lane were used for every neural structure. The separated proteins were then transferred to 0.2 µm polyvinylidene difluoride membranes (Bio-Rad). Polyclonal anti-GAIP, -RGSZ1, -GIPC, -Gαi2, and -Gαz were diluted typically 1 : 1000 in TBS-0.05% Tween 20 (TTBS) and incubated with the transfer membranes at 6°C for 24 h. The anti-GAIP N terminus (1–79) and the 4507 (GAIP 23–217) antibodies, generously provided by T Fischer (De Vries *et al*, 1998a; Fischer *et al*, 2000), the RGSZ1 antibody, the Gαi2 and the Gαz antibodies (Sánchez-Blázquez *et al*, 1995; Garzón *et al*, 1997b) were detected with a goat anti-rabbit IgG (H + L) horseradish peroxidase-conjugate antiserum (BioRad, #170-6515). While GIPC (Santa Cruz Biotech., Inc, sc-9650) antibody was detected with a donkey anti-goat IgG horseradish peroxidase-conjugate antiserum (sc-2020). Secondary antisera were diluted 1 : 5000 in TTBS, incubated for 3 h and revealed with the ECL + plus Western Blotting Detection System (RPN2132, Amersham Biosciences). Immunoblots were visualized with a ChemiImager IS-5500 (Alpha Innotech, San Leandro, California) and analyzed by densitometry (AlphaEase v3.2.2).

Solubilization of Membranes and Purification of Glycoproteins by Wheatgerm Lectin (WGL) Affinity Chromatography

Solubilization and WGL affinity chromatography were carried out at 4°C. Cerebral cortex membranes were resuspended in 2% Triton X-100 in buffer A (20 mM Tris.HCl, 1 mM EGTA, 4 µM leupeptin, 150 mM NaCl, 10 µM phenylmethylsulfonyl fluoride, 19 µg/ml soybean trypsin inhibitor, 50 µg/ml bacitracin, pH 7.5). The mixture was incubated at 4°C for 16 h with stirring, and then centrifuged at 100 000 × *g* for 1 h. The clear supernatant was applied at a rate of 1.5 ml/min to a WGL-Sepharose 4B column (AmershamPharmacia Biotech, #17-0444) previously equilibrated with 20 bed volumes of buffer A containing 1% Triton X-100, 1 mM $CaCl_2$ and 1 mM $MnCl_2$ (buffer B). The retained glycoproteins were then eluted with 0.25 M *N*-acetyl-D-glucosamine in buffer B, and were collected in siliconized tubes in fractions of 1 ml. After determining protein content, peak fractions were pooled and proteins were precipitated with dry ice-cold acetone for deglycosylation assays.

Deglycosylation of GAIP and RGSZ1

N-*glycosidase F assay*. The glycoproteins were resuspended and solubilized in 100 mM NaH_2P0_4, pH 7.7, 1 mM EDTA, 1% β-mercaptoethanol, 0.1% SDS, 1 mM dithiothreitol, to a final protein concentration of 4 µg/µl, and heated at 100°C

for 10 min. The solubilized material was supplemented with 0.65% octyltioglucoside to help remove the SDS from the proteins, and then incubated with *N*-glycosidase F (1 U/10 μg of protein) (Roche Diagnostics, 1 365 169, Mannheim, Germany) for 18 h at 37°C.

O-*glycosidase assay*. The glycoproteins were solubilized in 100 mM NaH_2P0_4, pH 6.8, 1 mM EDTA, 1 mM dithiothreitol, 0.5% SDS, to a final protein concentration of 2 μg/μl, and heated at 100°C for 10 min. The solubilized material was supplemented with 0.65% octyltioglucoside, EDTA 20 mM, and then incubated with *O*-glycosidase (1 U/20 μg of protein) (Roche, 1 347 101) for 18 h at 37°C. In both assays, samples were then concentrated, solubilized in Laemmli buffer, separated on a 10–20% SDS-polyacrylamide gel, blotted and the corresponding immunosignals were obtained.

RT-PCR

Total RNA was harvested from mouse brain structures using a single-step procedure (Ultraspec RNA isolation system, Biotecx Labs, Houston, TX, USA) based on the formation of RNA complexes with guanidinium molecules followed by isopropanol precipitation. The pellet was washed in 75% ethanol, dried, resuspended in 40 μl RNA storage solution (Ambion, Austin, TX, USA) and stored at −80°C until analysis. The yield of RNA was determined by UV spectrometry (260 nm). For every neural structure analyzed, 2 μg of total RNA were reverse transcribed using the RT-PCR First Strand Synthesis Kit (RETROscript, Ambion) with oligo (deoxythymidine) priming. cDNA synthesis was carried out at 42°C for 60 min. The PCR reactions were performed on 0.05, 0.1, and 0.2 μg total RNA in a final volume of 50 μl of the buffer solution containing 10 mM Tris.HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 125 μM of each dNTP, 0.5 μM of each primer, and 1 U of SuperTaq thermostable DNA polymerase (Ambion). cDNA primers (Sigma-Genosys) directed towards the murine genes were: GAIP (NM_026446), forward 5′-CCTCATG CACCGGGACTCGTA-3′ directed to base pairs 706–726, and reverse 5′-CTGTGGGCCTAAAGGGTGTTGTTC-3′ directed to base pairs 950–973 to yield a band of 268 base pairs; RGSZ1 (AF191552; NM_021374), forward 5′-ACGCCTGCTGCTTCTGTTGGT-3′ corresponding to base pairs 220–240, and reverse 5′-TTCATGAAGCGGGGATAG GAGTCT-3′ corresponding to base pairs 689–712, to yield a band size of 493 base pairs; GIPC (AF089818), forward 5′-GGCCGCACCTTCACCCTCAAAC-3′ to base pairs 661–682, reverse 5′-TCCCCGATGGCTCCCCAGACAT-3′ to base pairs 1010–1031, to yield a band of 371 base pairs.

A total of 31 amplification cycles were performed using a DNA Mastercycler (Eppendorf AG, Hamburg, Germany) in a thin-walled 0.5 ml PCR tubes (Ambion) according to the following protocol: one cycle of 94°C (1 min) followed by 30 cycles of 94°C (20 s) and 60°C (30 s). Final 3 min incubation at 72°C was allotted. To confirm the identity of amplified cDNA, PCR products were electrophoresed in 2.5% agarose gels with PCR markers 80–1000 (Biotools), and then incubated in SYBR gold solution (Molecular Probes #S-11494, Poortgebouw, Netherlands) for 20 min. DNA products were visualized with UV light (ChemiImager IS-5500).

Animals and Evaluation of Antinociception

Male albino mice CD-1 (Charles River), weighing 22–25 g, were housed and used strictly in accordance with the guidelines of the European Community for the Care and Use of Laboratory Animals (Council Directive 86/609/EEC). Animals were lightly anaesthetized with ether and all substances were injected into the right lateral ventricle in 4 μl volumes as previously described (Sánchez-Blázquez *et al*, 1995). The response of the animals to nociceptive stimuli was determined by the warm water (52°C) tail-flick test. Baseline latencies ranged from 1.5 to 2.2 s. Antinociception was expressed as a percentage of the maximum possible effect, MPE = 100 × (test latency−baseline latency)/(cutoff time (10 s)−baseline latency). For the study assays, groups of 10 mice received either a fixed dose or increasing doses of the opioid agonists and antinociception was assessed at various intervals. The doses of 10 nmol heroin and of 120 pmol DAMGO ([D-Ala^2, *N*-MePhe^4,Gly-ol^5]-enkephalin) were selected to match the analgesic effect of 10 nmol morphine, about 80% MPE. The endomorphins were used at doses producing in CD-1 mice maximal effects in the analgesic test (Sánchez-Blázquez *et al*, 1999b). In these CD-1 mice, the supraspinal analgesic effect of agonists at δ-opioid receptor is moderate (Sánchez-Blázquez and Garzón, 1993; Sánchez-Blázquez *et al*, 1993). The doses of 10 nmol DPDPE ([D-Pen^{2,5}]-enkephalin) and 10 nmol [D-Ala2] deltorphin II produce comparable analgesic effects. Statistical analysis of the results included analysis of variance (ANOVA) followed by the Student–Newman–Keuls test (SigmaStat, SPSS Science Software, Erkrath, Germany). The level of significance was set at $P<0.05$.

Production and Evaluation of Acute Tolerance to Morphine

A single i.c.v. injection of 3 or 10 nmol morphine (priming dose: PD) was used to produce acute tolerance (Garzón and Sánchez-Blázquez, 2001). Controls were given saline instead. A second i.c.v. dose of morphine (test dose: TD) was given 24 h later when the pretreatment (PD) had no effect on base line latencies. Analgesia was determined 30 min later by the tail-flick test. Acute tolerance was determined by the decrease of antinociceptive potency. Every treatment was performed on a different group consisting of 10 or 15 mice.

Induction and Assessment of Tolerance Upon Chronic Morphine Treatment

Groups of 15–20 mice were subcutaneously (s.c.) implanted with 10 ml/kg body weight of a suspension containing 50% saline (0.9% NaCl in distilled water), 42.5% mineral oil (Sigma #400-5), 7.5% mannide monooleate (Sigma #M-8546), and 0.1 g/ml morphine base (adapted from Sánchez-Blázquez *et al*, 1997). Development of tolerance was monitored by measuring the analgesic response to the s.c.-implanted chronic opioid and to a single i.c.v. dose of 10 nmol morphine. In mice not previously exposed to the opioid, this produced an effect of about 80% MPE in the tail-flick test.

Chemicals

The opioids used were: morphine sulfate (Merck, Darmstadt, Germany), DAMGO (Bachem, Bubendorf, Switzerland, H-2535), DPDPE (Bachem, H-2905), [D-Ala^2] deltorphin II (Bachem, H-8060), Cys^2, Tyr^3, Orn^5, Pen^7-amide (CTOP) (Bachem, H-2186), endomorphin-1 and endomorphin-2 (Tocris, Bristol, UK, #1055 & 1056), and heroin (Dirección General de Estupefacientes, Ministerio de Sanidad y Consumo, Spain).

RESULTS

RGSZ1, GAIP, and GIPC in Mouse Central Nervous System

The affinity-purified polyclonal antiserum raised against RGSZ1 immunoreacted with the recombinant protein (Figure 1a, lanes 1 and 2) and also with different glycosylated and nonglycosylated forms of neural RGSZ1 in mouse brain synaptosomal membranes (Figure 1a, lanes 3, 6, and 7). The antiserum labeled the higher bands in the fraction of glycosylated proteins derived from synaptosomal membranes of the cerebral cortex (Figure 1a, lane 7). The action of *N*- and *O*-glycosidase greatly reduced the apparent molecular weight of the RGSZ1-related immunosignals of the glycosylated fraction (Figure 1a, lanes 8 and 9). This observation indicates that in CNS RGSZ1 exists in different glycosylated forms (NetNGlyc 1.0 and NetOGlyc 2.0 Prediction Servers, Center for Biological Sequence Analysis). In the glycosylated fraction, the immunosignal corresponding to the lower band of about 27 kDa was not detected (Figure 1a, lane 7). The apparent molecular weight of this nonglycosylated form of this protein is coincident with that estimated for the amino-acid sequence of murine RGSZ1.

The antisera directed to GAIP also revealed the presence of glycosylated and nonglycosylated forms of this protein in mouse CNS (Figure 1b, lane 1, 2 and 3). The action of *N*- and *O*-glycosidases on the glycoprotein fraction increased the electrophoretic mobility of the GAIP-related bands (Figure 1b, lanes 4 and 5). The lower band of about 25 kDa coincides with the GAIP molecular weight and is a nonglycosylated protein (Figure 1b, lane 1 *vs* lanes 2 and 3). The GIPC antibody labeled a single band in synaptosomal membranes of mouse PAG (Figure 1c). The GIPC protein was not detected in the glycosylated fraction.

The PAG plays a major role in mediating the supraspinal analgesia of opioids when given by i.c.v. route (Yaksh *et al*, 1976). Thus, we have studied the presence of Rz/GIPC

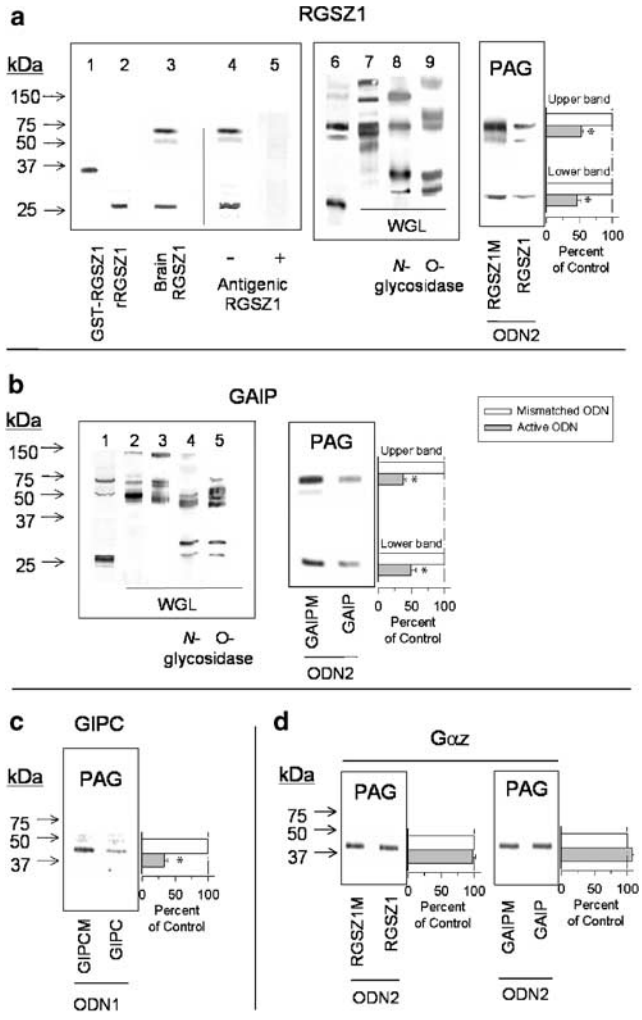

Figure 1 Detection of RGSZ1, GAIP, and GIPC proteins in mouse brain: efficacy of the ODN treatments. Characterization of the antibody to RGSZ1. (a) Lanes: 1, 30 ng GST-recombinant RGSZ1 fusion protein; 2, recombinant RGSZ1 (thrombin product); 3, RGSZ1-like immunoreactive material in 60 μg mouse PAG P2 fraction. Preincubation of the affinity-purified IgGs with 20 μg of recombinant RGSZ1 for 1 h at room temperature abolished the RGSZ1-like immunosignals associated with mouse PAG (this is indicated in lanes 4 and 5 which show IgGs incubated without and with antigenic RGSZ1, respectively). Glycosylated forms of RGSZ1. (a) Lanes: 6, 80 μg mouse cerebral cortex P2 fraction; 7, 60 μg of wheat germen lectin (WGL)-purified glycosylated proteins from mouse cerebral cortex P2 fraction; 8 and 9, action of *N*-glycosidase F and *O*-glycosidase on the WGL-purified material. Efficacy of ODN-RGSZ1 treatment. The efficacy of the 5-days treatment with the active ODN was assessed with antibodies directed to RGSZ1. Mice were killed on day 6. The PAG structures from four mice were pooled and about 60 μg SDS-solubilized P2 fractions resolved by SDS-PAGE (10–20% acrylamide/2,6% bisacrylamide) and Western blotted. Immunosignals were analyzed as described in Methods. ODN2-RGSZ1 is the active ODN and ODN2-RGSZ1M the mismatched variant. Glycosylated forms of GAIP. (b) Lane 1, 60 μg mouse cerebral cortex P2 fraction immunodetected with GAIP N-terminus (1–79); 40 μg of glycosylated proteins were immunodetected with anti-GAIP N-terminus (lane 2) and 4507 (GAIP 23–217) antibodies (lane 3); 4 and 5, action of *N*-glycosidase F and *O*-glycosidase on the WGL-purified material, revealed with GAIP N-terminus antibody. Efficacy of the ODN treatments on GAIP expression. ODN2-GAIP is the active ODN and ODN2-GAIPM the mismatched variant. Immunodetection of GAIP in PAG was performed as described for RGSZ1. (c) ODN-induced reduction of GIPC expression in PAG. ODN1-GIPC and ODN1-GIPCM are the active and mismatched forms, respectively. ODN treatment and immunodetection were performed as described for RGSZ1. (d) Lack of effect of ODN-induced RGSZ1 and GAIP knockdown on the expression of Gαz subunits in mouse PAG. Immunosignals were analyzed by densitometry (ChemiImager IS-5500, Alpha Innotech, San Leandro, California). Each bar is the mean ± SEM percentage change from the control of three to four independent determinations. *Significantly different from control protein levels—structures from mice injected with the mismatched sequence ODN, ANOVA–Student–Newman–Keuls test; $P < 0.05$.

proteins and the efficacious activity of the ODNs to temporarily reduce their levels in this neural structure. ODNs act mainly by translational arrest in neural tissues, and are responsible for very little transcript degradation (see Sánchez-Blázquez *et al*, 2003, and references therein). This seems to be due to the low RNase H activity of mammalian neural cells and cerebrospinal fluid (Wahlestedt *et al*, 1993; Wahlestedt, 1994). Therefore, the efficacy and selectivity of the ODN treatments were assessed with antibodies against RGSZ1, GAIP, and GIPC proteins. The active ODNs brought about reductions in RGSZ1-, GAIP- and GIPC-associated immunoreactivities of that observed for control PAG (Figure 1a, c). The knockdown of RGSZ1 or GAIP proteins did not change the expression of Gαz (Figure 1d), Gαo, or Gαi1/2 subunits involved in opioid cellular signaling (not shown).

RT-PCR assays showed amplification of the predicted sequences of RGSZ1, GAIP, and GIPC proteins in all the neural areas evaluated. The data, compared to the mRNA levels found for the cerebral cortex, showed some region-specific patterns for GAIP and RGSZ1 with a more uniform distribution for GIPC. The higher RGSZ1 mRNA signals were found in striatum, thalamus, PAG, and pons-medulla. GAIP mRNA showed differences in distribution throughout the CNS. Stronger signals were observed for hypothalamus, striatum, thalamus, cortex, and PAG, while fainter signals were seen for pons-medulla and spinal cord (Figure 2).

Influence of GAIP and GIPC on μ- and δ-Mediated Supraspinal Analgesia

Treatment with the selected ODNs did not alter the observed basal latencies in the tail-flick test, 1.5–2.2 s. Both active anti-GAIP ODNs produced comparable levels of protein knockdown and also identical results on the opioid-induced supraspinal analgesia. In animals treated with the active ODN1 and 2 to GAIP mRNA, the effects of morphine and heroin were slightly but significantly boosted at various intervals during the analgesic time course (Figure 3). For the sake of simplicity, data for ODN1-GAIPM and some of those for ODN1-GAIP are not presented. The apparent ED_{50}s (i.c.v. morphine) and 95% confidence limits in control mice treated with the mismatched ODN2-GAIPM were 3.50 (4.72–2.59), 8.56 (11.81–6.20), and >10 nmol/mouse when determined 30, 60, and 90 min after opioid administration. In GAIP knockdown mice treated with the active ODN2-GAIP, the morphine ED_{50} s at these intervals were 3.02 (4.16–2.18), 4.46 (6.02–3.30), and >10 nmol/mouse (Figure 6). This treatment also increased the activity of DAMGO over the entire time course (Figure 3). However, the analgesia of the endogenous μ-binding agonists endomorphin-1 and -2, and those of the selective agonists at δ-opioid receptors, DPDPE and [D-Ala^2] deltorphin II, were not altered by ODN-GAIP treatment (Figure 3). The ODN-induced impairment of GIPC brought about enhancements of morphine- and DAMGO-evoked analgesia that coincided with those observed in GAIP knockdown mice. Also, the action of the δ agonists remained unchanged in GIPC knockdown mice (Figure 4).

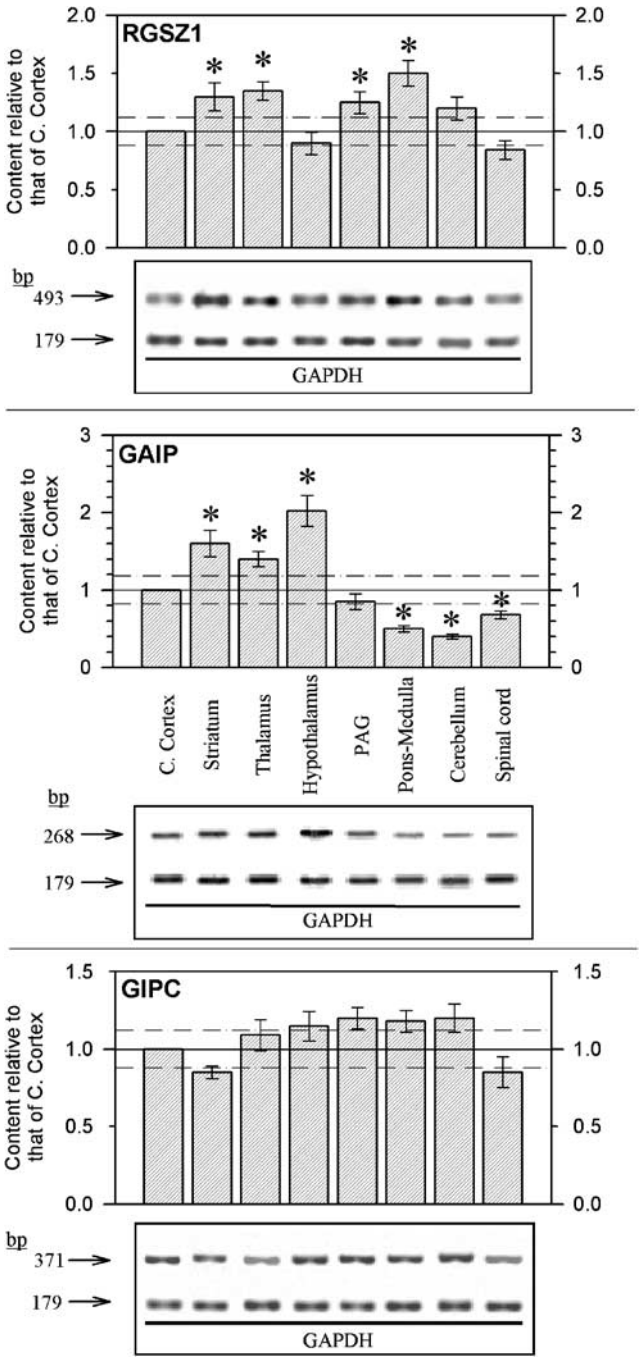

Figure 2 Detection of RGSZ1, GAIP, and GIPC mRNA in mouse brain. RT-PCR of RGSZ1, GAIP, and GIPC mRNA in different neural structures. The amplified products were 493, 268, and 371 bp for RGSZ1, GAIP and GIPC, respectively. The signals associated with these RGS mRNA were normalized with those obtained by amplification of a segment of 179 bp GAPDH (internal standard) (ChemiImager IS-5500, Alpha Innotech, San Leandro, California) and analyzed by densitometry (AlphaEase v3.2.2). Each bar is the mean ± SEM of three determinations. *Significantly different from the mRNA levels observed for cerebral cortex (arbitrary value of 1), ANOVA–Student–Newman–Keuls test (SigmaStat, SPSS Science Software); $P<0.05$.

Regulation of μ-Mediated Antinociception by RGSZ1: Its Role in Tachyphylaxis

The impairment of RGSZ1 expression led to an intense enhancement of morphine's efficacy to produce analgesia—

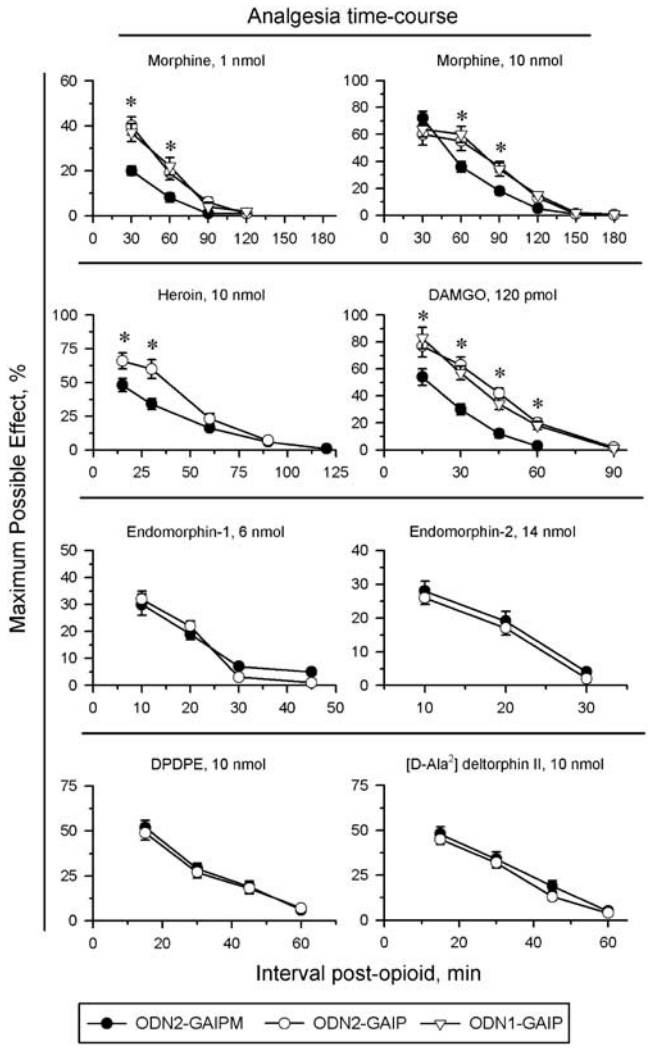

Figure 3 Effect of knockdown of GAIP on the time course of the analgesia evoked by μ- and δ-opioid receptor acting agonists. Animals with reduced levels of GAIP through ODN treatment were i.c.v.-injected with the opioids on day 6 (after 5 days ODN treatment). Analgesia was determined by the warm water 52°C tail-flick test at various intervals postinjection. Values are mean ± SEM from groups of 10–20 mice. *Significantly different from the group that had received the mismatched ODN.

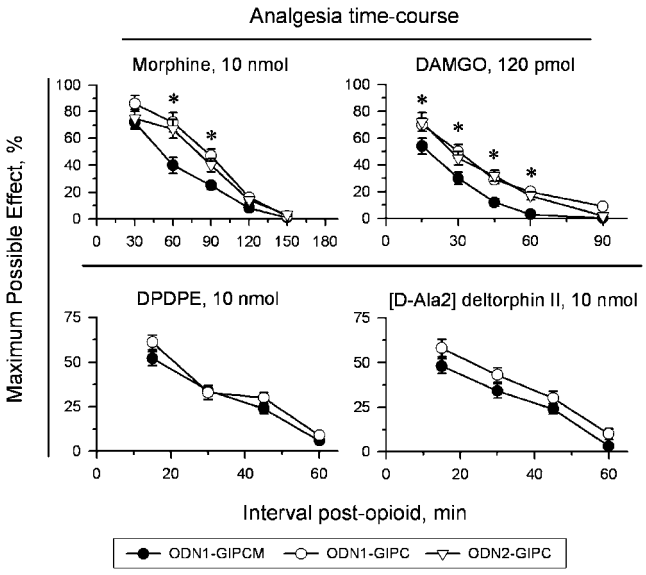

Figure 4 Knockdown of GIPC on the time course of the analgesia evoked by μ- and δ-opioid receptor acting agonists. Details as in the legend to Figure 3.

over two-fold at several points of the time course. Morphine analgesia was then observed for a significant longer period than in controls: the effect of 10 nmol morphine was extended for an additional 90 min (Figure 5). Treatment of the animals with the ODNs to RGSZ1 mRNA brought about a leftward shift of the opioid dose–effect curves constructed at various postopioid intervals (Figure 6). For the mismatched ODN2-RGSZ1M, the apparent ED50s (i.c.v. morphine) and 95% confidence limits in control mice were 3.42 (4.51–2.59), 9.10 (12.90–6.42), and >10 nmol/mouse when determined 30, 60, and 90 min after opioid administration. In RGSZ1 knockdown mice treated with the active ODN2-RGSZ1, the morphine ED_{50} s at these intervals shifted to 1.20 (1.45–0.99), 2.70 (3.51–2.08), and 4.61 (6.96–3.05) nmol/mouse (Figure 6). Comparable results were obtained with the ODN1-RGSZ1 and the corresponding mismatched ODN on morphine-evoked antinociceptive effect (not shown). The actions of heroin, DAMGO, and endomorphin-1 were also boosted in these RGSZ1 knockdown mice. No change of endomorphin-2 analgesia was observed. The antinociception of DPDPE and [D-Ala^2] deltorphin II was not altered by the RGSZ1 depletion.

To address whether the direct action of morphine at μ receptors was responsible for the extended response to the opioid in RGSZ1 knockdown mice, the antagonist CTOP was i.c.v.-injected 5 min before performing the analgesic test at 90, 120, 150, and 150 min post 10 nmol morphine. In this protocol, CTOP reduced the potency of the opioid at all these intervals (Figure 7). This result indicates that, in naive mice, cessation of the analgesic response to an acute dose of morphine occurs before the opioid is removed from the μ receptor environment.

Morphine-Induced Acute Tolerance in Rz and GIPC Knockdown Mice

The tolerance observed long after a single and adequate dose of an opioid—acute tolerance (see eg Garzón and Sánchez-Blázquez, 2001)—was investigated in mice with reduced levels of the GAIP, GIPC, and RGSZ1 proteins. Different groups of mice received the active ODNs. The corresponding mismatched ODN served as a control. All the control ODNs gave comparable results with this experimental protocol—ODN1-RGSZ1M is shown in Figure 8. After completing the 5 days of oligo treatment, half of the animals of every group received a priming dose of 10 nmol morphine. The rest were given saline instead. To detect the appearance of opioid acute tolerance, a test dose of 10 nmol morphine was i.c.v.-injected 24 h later into all mice. Antinociception was then evaluated 30 min later by the tail-flick test. The control group that received the priming dose and the test dose of morphine showed a marked

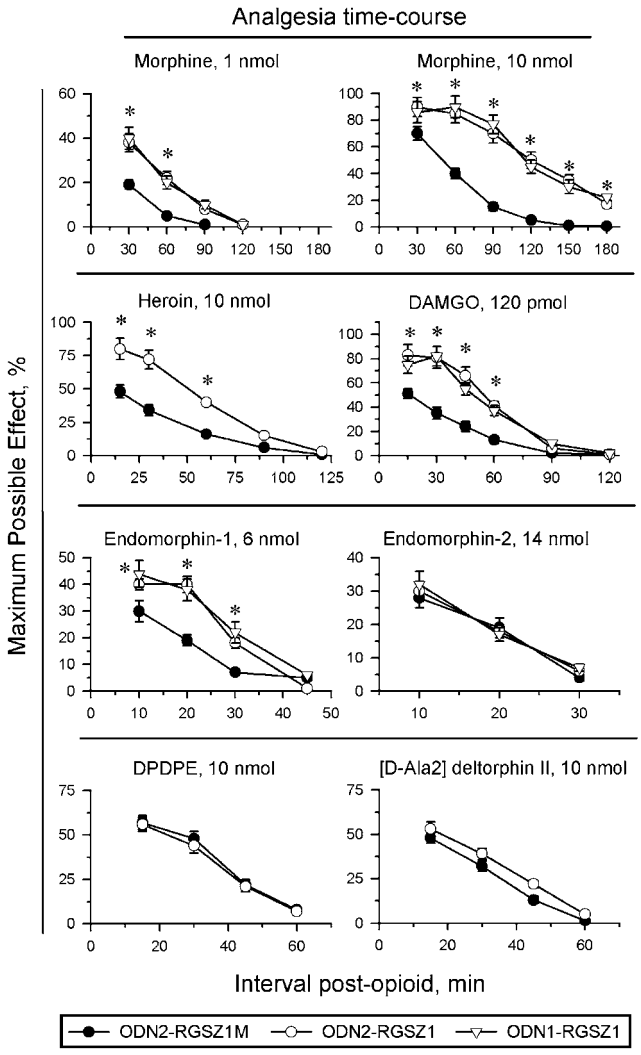

Figure 5 Effect of knockdown of RGSZ1 on the time course of analgesia evoked by μ- and δ-opioid receptor acting agonists. Details as in the legend to Figure 3.

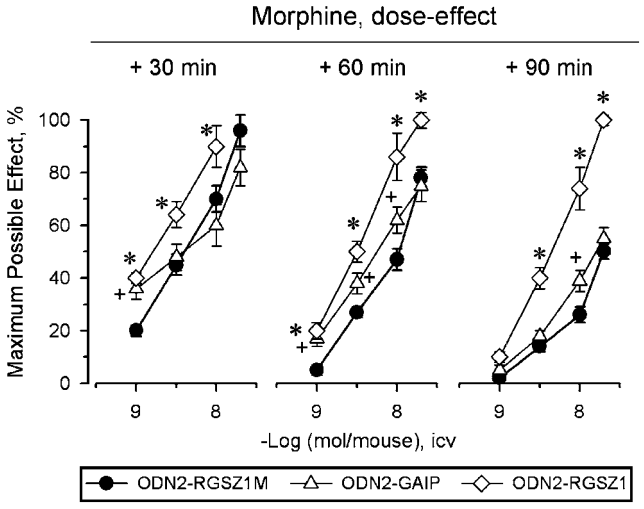

Figure 6 The influence of RGSZ1 and GAIP proteins on morphine-evoked analgesia. Animals with reduced levels of RGSZ1 and GAIP proteins through ODN treatment were i.c.v.-injected with various doses of morphine and analgesia determined at various intervals by the warm water 52°C tail-flick test. The effects of the opioid at 30, 60, and 90 min postinjection are presented in dose–effect curves. Values are mean ± SEM from groups of 10–20 mice. *, $^{+}$ Significantly different from the group that received the mismatched control.

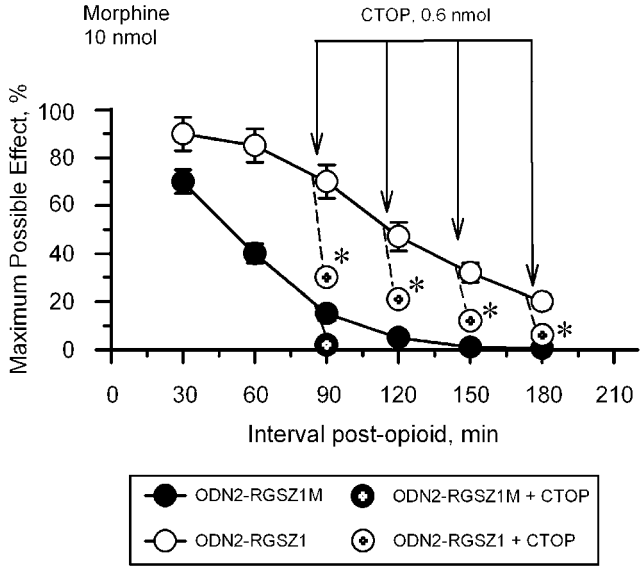

Figure 7 The effects of RGSZ1 on μ-mediated antinociception and its role in tachyphylaxis. Animals with reduced levels of RGSZ1 through ODN treatment were i.c.v.-injected with 10 nmol morphine on day 6 (after 5 days ODN treatment). Analgesia was determined by the warm water 52°C tail-flick test at various intervals postinjection The antagonist CTOP (0,6 nmol) was i.c.v.-injected into these mice 5 min before performing the analgesic test at 90, 120, 150, and 180 min post 10 nmol morphine. Values are mean ± SEM from groups of 10–20 mice. *Significant CTOP antagonist effect of morphine analgesia on the group treated with the active ODN2-RGSZ1, ANOVA–Student–Newman–Keuls test; $P < 0.05$.

impairment of the opioid analgesic effect. The impairment of GAIP, GIPC, or RGSZ1 proteins did not prevent morphine displaying strongly reduced activity (Figure 8a).

It should be noted that mice treated with the active ODN-GAIP or ODN-GIPC and which received saline instead of the morphine priming dose showed no potentiation of morphine test dose at the peak effect (30 min) compared to the mice in the mismatched ODN control group. However, at later time-course intervals, the analgesic activity of the opioid was effectively increased (Figures 3 and 4).

In this experimental paradigm of opioid acute tolerance, a tendency was observed for animals with impaired levels of GAIP or RGSZ1 proteins to show lesser analgesic responses to morphine than control mice treated with the mismatched ODNs (Figure 8a). To investigate whether increased activation of Gz-proteins facilitates the development of μ receptor-mediated acute tolerance, the mice were injected with a lower morphine priming dose of 3 nmol. ODN1-RGSZ1M-treated control animals showed no tolerance to 3 and 10 nmol morphine test doses evaluated 24 h later. However, acute tolerance to these doses of morphine developed in the RGSZ1 knockdown mice (Figure 8b).

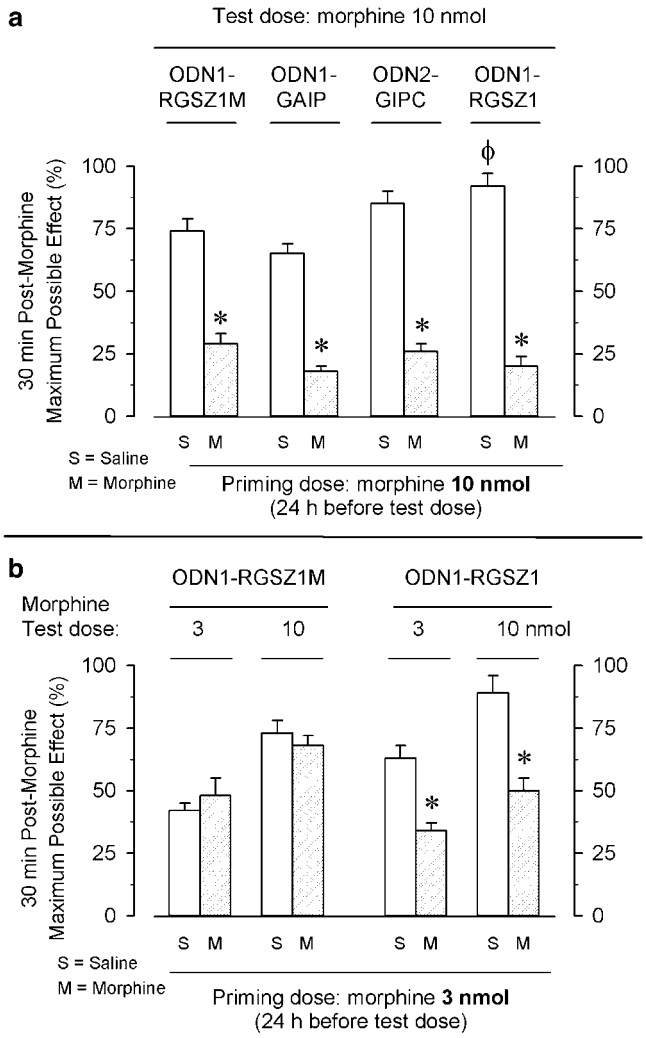

Figure 8 Role of RGSZ1, GAIP, and GIPC proteins on acute tolerance to a single dose of morphine. (a) Either saline or a priming dose of 10 nmol morphine was i.c.v.-injected into the mice that had received the active ODNs to RGSZ1, GAIP, and GIPC proteins or the control mismatched ODN. After 24 h, all groups received an i.c.v. test injection of 10 nmol morphine, and analgesia was evaluated after 30 min. Values are mean ± SEM. from groups of 10–20 mice. * Significantly different from the corresponding group injected with saline before the second dose of the opioid, ϕ from the group treated with the mismatched ODN and injected with saline before the second dose of the opioid. ANOVA–Student–Newman–Keuls test; $P<0.05$. (b) Saline or a priming dose of 3 nmol morphine was i.c.v.-injected to the mice that had received the active ODN to RGSZ1 proteins or the control mismatched ODN. The mice subjected to each ODN treatment were divided into two groups and the development of acute morphine tolerance was monitored by measuring the analgesic response 30 min after a single i.c.v. dose of 3 or 10 nmol morphine. * Significantly different from the corresponding group injected with saline before the second dose of the opioid, ANOVA–Student–Newman–Keuls test; $P<0.05$.

μ Receptor Activated Gz- and Gi2-Proteins in Morphine-Induced Acute Tolerance

In the production of supraspinal analgesia, the Gz- and Gi2-proteins are mostly activated by i.c.v.-morphine via μ-opioid receptors (see eg Garzón *et al*, 2000 and references therein). To address the role of these G-proteins in the development of acute morphine tolerance, mice were treated with active ODNs directed to the mRNAs coding for Gαz and Gαi2 subunits. Several studies report these antisense oligos to show selectivity and efficacy in reducing the coded proteins (Sánchez-Blázquez *et al*, 1995, 2001). The % of decrease in Gαi2- and Gαz-like immunoreactivity were for PAG 64 ± 4* and 47 ± 3*, respectively (values are the mean ± SEM from three independent experiments. * Significantly different for the ODN-RD group, ANOVA, Student–Newman–Keuls test, $P<0.05$) (Figure 9a). As previously reported, the depletion of either Gαz or Gαi2 subunits caused comparable reductions in 20 nmol morphine analgesic effects over the entire time course (Figure 9b) (Garzón *et al*, 2000). In the acute tolerance paradigm, morphine showed a reduced effect in controls (treated with a random ODN) and in Gαi2 knockdown mice. The impairment of Gαz blocked the development of acute tolerance to the analgesia of 20 nmol morphine (Figure 9c). Thus, the regulation of Gz proteins by μ receptors facilitated the appearance of opioid acute tolerance. The Gi2 proteins played a minor role to this μ receptor desensitizing effect.

RGSZ1 Knockdown and Tolerance to Chronic Morphine

The influence of RGSZ1 proteins in the development of tolerance to the continuous presence of morphine was investigated. Two groups of mice were prepared. One received the ODN2-RGSZ1; the other was given the corresponding mismatched ODN. After completing the oligo treatment, all mice were s.c.-implanted with an oily morphine pellet. Opioid levels of about 10–13 nmol/ml of serum and 5–8 nmol/g wet brain are reached for the first 12 h, but at 24 h these values are reduced by approximately 50% (Garzón and Sánchez-Blázquez, 2001). Within the first hour of continuous morphine treatment, the RGSZ1 knockdown mice showed a higher analgesic response than the control group. At later intervals, impairment of RGSZ1 proteins notably accelerated the reduction of opioid-induced analgesia (Figure 10a). In both groups of mice, the antinociceptive effect of the opioid was practically absent 8 h after implantation of the morphine pellet.

The analgesic efficacy of 10 nmol i.c.v. morphine, which produced about 80% of the MPE in ODN2-RGSZ1M-treated naive mice, was evaluated in animals undergoing 5 h of chronic morphine treatment. In ODN2-RGSZ1M-treated morphine-tolerant mice, i.c.v. morphine produced a moderate antinociceptive effect, about 40% MPE. The RGSZ1 knockdown morphine-tolerant mice showed a poorer response to i.c.v. morphine—about 15% MPE (Figure 10b). Thus, the impairment of RGSZ1 subunits (increased μ-mediated activation of Gz proteins) brought about an increased development of tolerance to morphine analgesic effects.

DISCUSSION

The RT-PCR assay showed the presence of RGSZ1, GAIP, and GIPC mRNA in mouse CNS. The levels of GIPC mRNA were similar in all areas studied. RGSZ1 and especially GAIP mRNA levels showed notable differences between areas. The affinity-purified antisera raised against RGSZ1 and GAIP recognized several forms of these proteins in synaptosomal

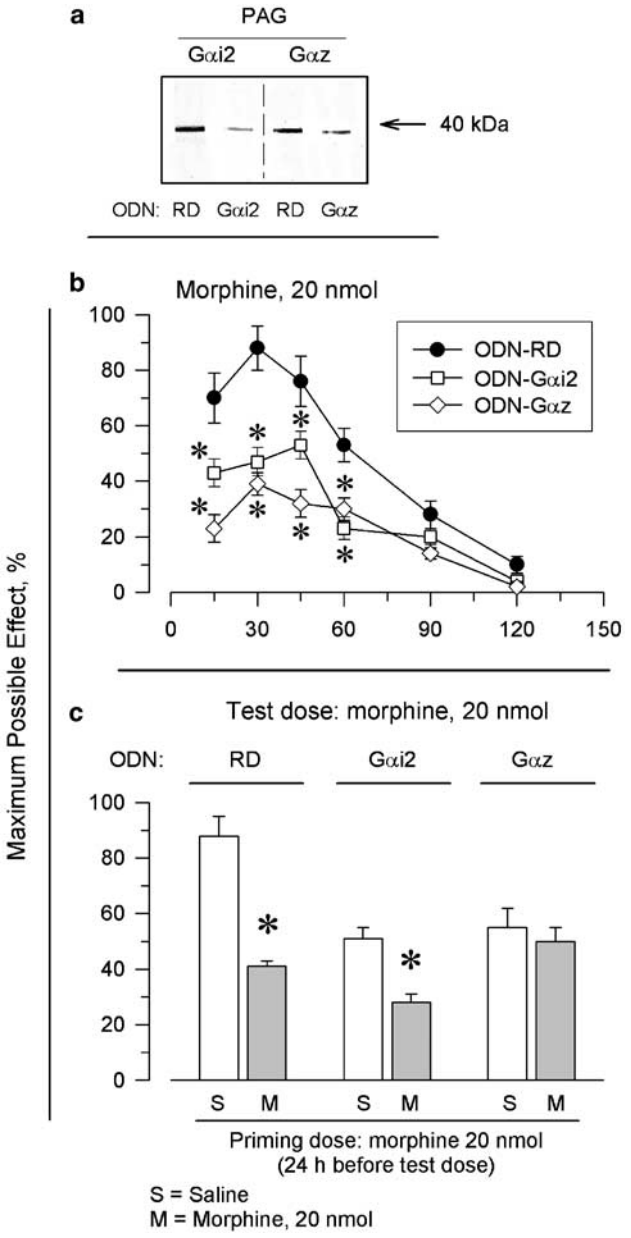

Figure 9 Role of Gαz and Gαi2 subunits on morphine analgesia and acute tolerance. (a) Efficacy of ODNs directed to Gαi2 and Gαz subunits. The control ODN-RD and the active ODNs were i.c.v.-injected to the mice for five consecutive days. On day 6, the mice were killed and PAG was obtained. The structures from four mice were pooled and about 60 μg/lane of SDS-solubilized P2 membranes were resolved by SDS-PAGE and electroblotted. Immunodetection and analysis of the signals were performed as described in Methods. (b) The time course of antinociception produced by 20 nmol morphine was analyzed in mice that had received ODN-RD or active ODNs to Gαi2 and Gαz subunits for 5 days. Values are mean + SEM from groups of 10–15 mice. * Significantly different from the group that received the random ODN, ANOVA–Student–Newman–Keuls test; $P < 0.05$. (c) Saline or a priming dose of 20 nmol morphine was i.c.v.-injected into the mice that had received the active ODNs to Gαi2 or Gαz subunits or the control ODN-RD. After 24 h, all groups received an i.c.v. test injection of 20 nmol morphine, and analgesia was evaluated after 30 min. Values are mean + SEM from groups of 10–15 mice. * Significantly different from the corresponding group injected with saline before the second dose of the opioid, ANOVA–Student–Newman–Keuls test; $P < 0.05$.

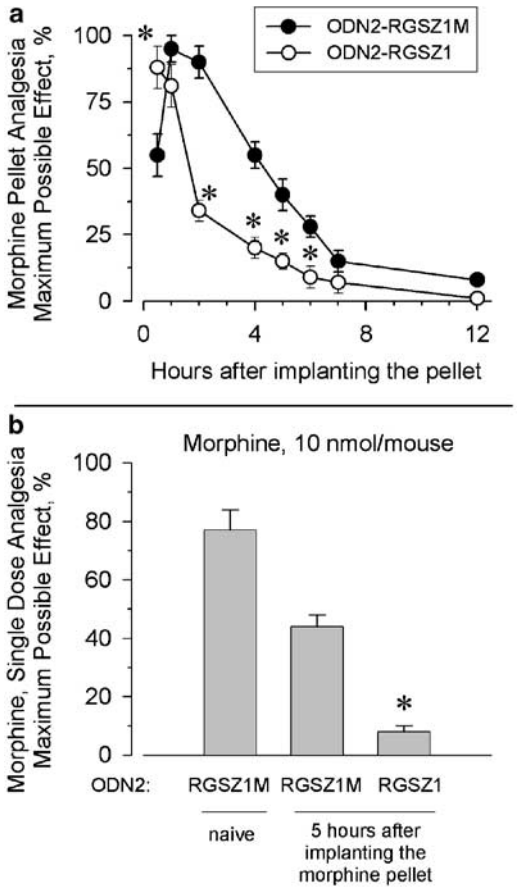

Figure 10 Influence of RGSZ1 knockdown on the development of tolerance to sustained chronic morphine. (a) Animals received the ODNs into the right lateral ventricle following a 5-day schedule. On day 6, the mice treated with the ODN2-RGSZ1 or the mismatched control ODN2-RGSZ1M were implanted at time zero with the oily pellet. Development of tolerance was monitored by measuring the analgesia produced by the release of the s.c.-implanted opioid (a), and by a single i.c.v. dose of 10 nmol morphine (b). In mice not previously exposed to the opioid, this dose produced an effect of about 80% MPE in the tail-flick test. This is indicated in the panel b with the bar corresponding to naive mice. Values are the mean + SEM from groups of 15–20 mice. * Significantly different from the group that had received the mismatched control, ANOVA–StudentNewman–Keuls test.

membranes of mouse cerebral cortex and PAG. Immunoreactive bands were found at 27 and 24 kDa, coinciding with the deducted molecular weights for RGSZ1 and GAIP, and also at about 50, 75, and 150 kDa. Rz proteins show several potential sites for *N*- and *O*-linked glycosylation as well as for serine, threonine, and tyrosine phosphorylation (Center for Biological Sequence Analysis). Deglycosylation studies revealed that bands of high molecular weight represent different degrees of glycosylation and probable conjugation with various phosphorylation states. Reductions in the levels of RGSZ1 yielded notable increases in the potency and especially in the duration of supraspinal analgesia elicited by μ receptor-binding opioids such as morphine, DAMGO, heroin, and endomorphin-1. Knockdown of GAIP promoted no change in endomorphin-1 antinociception and only moderately increased the potency of the other μ-binding agonists. The impairment of GIPC also enhanced morphine

and DAMGO analgesia. The finding that endomorphin-2 effects were not changed after impairment of either GAIP or RGSZ1 can be explained in that endomorphins show different G-protein activation profiles (Sánchez-Blázquez *et al*, 1999b; Massotte *et al*, 2002) after binding to different types (subtypes) of μ-opioid receptor (Sánchez-Blázquez *et al*, 1999a; Sakurada *et al*, 1999). Thus, the antinociceptive activity of endomorphin-2 does not involve substantial activation of Gz proteins. The knockdown of RGSZ1, GAIP, or GIPC promoted no significant change in the supraspinal analgesic effects of the δ-binding agonists DPDPE and [D-Ala^2] deltorphin II.

GAIP deactivates Gαi/o/z but not Gα12/14/s subunits (De Vries *et al*, 1996; Watson *et al*, 1996), while the regulation of Gαq by GAIP is not very efficient at all (Huang *et al*, 1997). The regulatory action of GAIP on G-proteins might depend on its binding to GIPC. GAIP, GIPC, and TrkA are coprecipitated in a complex that could link the G-protein and receptor tyrosine kinase pathways (Lou *et al*, 2001). The association of GAIP with GIPC to act as GAP on Gα subunits is also suggested by the results obtained on opioid analgesia. Other RGS proteins also require associated proteins to act as GAP or to regulate signaling pathways. The members of the R7 subfamily of RGS proteins and Gβ5 subunits are always found as dimers in the CNS, indicating that this association is required to deactivate GαGTP subunits (Snow *et al*, 1998; Zhang and Simonds, 2000). These R7/β5 complexes might also function as G$\beta\gamma$ dimers, forming receptor-regulated heterotrimers with Gα subunits (Sondek and Siderovski, 2001). In *in vitro* assays, GAIP and RGSZ1 have shown similar efficiencies in deactivating GαzGTP subunits (Wang *et al*, 1998)—they are both about 100-fold more efficacious than RGS4 (Hepler *et al*, 1997). However, GAIP is much less activated by cellular magnesium concentrations than RGSZ1. Therefore, in neurons, GAIP would act primarily on activated Gα subunits other than Gαz (De Vries *et al*, 1995, 1996; Wang *et al*, 1998). Among the Gαi/o subunits, GAIP prefers the GTP form of Gαi3 (De Vries *et al*, 1996). It also interacts strongly with Gαi1 and Gαo, but only does so weakly with Gαi2 (Woulfe and Stadel, 1999). Thus, GAIP shows low efficacy in deactivating neural Gαz and Gαi2 subunits—key G-proteins in the production of μ-opioid supraspinal analgesia (Garzón *et al*, 2000). This feature, plus its low expression in brain (De Vries *et al*, 1995; Grafstein-Dunn *et al*, 2001), probably accounts for the moderate enhancements of μ opioid effects observed in GAIP knockdown mice.

RGSZ1 is mostly expressed in the striatum, thalamus, PAG, and pons-medulla (Wang *et al*, 1998; this work) and is associated with the membrane fraction (Wang *et al*, 1997; this work). RGSZ1 is at least 20-fold more selective for activated Gαz than for Gαi/o family members, and has a lower affinity for Gαq and Gαs than for Gαi/o (Wang *et al*, 1998). RGSZ1 GAP selectivity and efficacy at deactivating Gαz subunits increases about four-fold at cellular magnesium concentrations (Wang *et al*, 1998). Although RGSZ1 shows little GAP activity towards Gαi/oGTP, its GAP activity on Gαz is competitively inhibited by these subunits (Wang *et al*, 1997). In the CNS, Gi/o proteins are by much more abundant than Gz-proteins. Therefore, the binding of RGSZ1 to these activated Gαi/o subunits might serve as a regulatory mechanism. RGSZ1 inhibition by Gαi/o subunits would then increase the cellular effects mediated by activated Gαz subunits. The data on opioid antinociception suggest that RGSZ1 is linked to regulation of μ but not δ receptors. The relative abundance of RGSZ1 in different brain areas, and its high selectivity and efficiency at deactivating Gαz subunits, probably accounts for the notable enhancement of μ receptor-mediated opioid effects observed in RGSZ1 knockdown mice.

The members of the Rz subfamily bind with similar affinities to activated GαzGTP and the transition state GαzGDP.P forms, but not the GαzGDP form, which cannot regulate effectors (Wang *et al*, 1997). In contrast, RGS of the R4 and R7 subfamilies bind GαGDP.P much better than GαGTP (Berman *et al*, 1996; Hunt *et al*, 1996; Posner *et al*, 1999). The extreme N-terminal helix of Gαz is crucial for interaction with Rz proteins (Tu *et al*, 1997; Wang *et al*, 1998). This interaction is not observed for Gαi1 and RGS4 (Tesmer *et al*, 1997) and could account for the binding of GαzGTP and not just of GαzGDP.P to Rz proteins. RGS proteins attempt to synchronize the variations in agonist-induced receptor-activation and the number of Gα-GTP subunits that regulate effectors. Upon the disappearance of the extracellular messengers, RGS proteins play a key role in turning off their effects. RGSZ1 binding the GαzGTP and GαzGDP.P forms with almost equal affinities might prevent a fraction of opioid-activated GαzGTP subunits from reaching and regulating their effectors. In addition, as GαzGTP subunits require a long time to switch into the transition state, RGSZ1 would need to bind the GαzGTP form at the effectors. These actions accelerate the development of agonist tachyphylaxis at μ receptors. Thus, by lowering the efficacy of GαzGTP deactivation after RGSZ1 knockdown, opioid analgesia was potentiated in this work and its duration notably elongated after a single administration of a μ opioid agonist. The impairment of RGSZ1 would permit a large fraction of GαzGTP subunits to reach and regulate their effectors. After they spontaneously acquire their GαzGDP.P transition state, they can also be deactivated by RGS4 and RGS10 proteins (Berman *et al*, 1996; Hunt *et al*, 1996; Hepler *et al*, 1997). The enhanced opioid analgesic effects observed in RGSZ1 knockdown mice were sensitive to CTOP antagonism, indicating the presence and binding of the μ agonists to the opioid receptors. Therefore, in naive mice, the action of RGSZ1 on receptor-activated GαzGTP subunits causes μ opioid effects to disappear before the agonists are removed from the receptor environment.

The potency and duration of the effects initiated at agonist-bound receptors depends on the continuous and efficient action of RGS proteins in the reconstitution of the pool of receptor-regulated heterotrimeric G-proteins. When Gα subunits cease to regulate effectors, the interval elapses, before the receptor regulates the reformed G-proteins, negatively influence the potency of the agonist. This is particularly so for members of the R7 subfamily whose inefficient action on GαGTP subunits causes a progressive draining of the receptor-regulated G-protein pool (see Sánchez-Blázquez *et al*, 2003 and references therein). Therefore, the action of certain RGS proteins appears to be responsible for attenuating and abbreviating agonist effects—that is, tachyphylaxis (see Garzón *et al*, 2001, and references therein). At least two mechanisms are involved in

RGS regulation of the amplitude and duration of agonist effects. One involves RGS GAP function and abbreviates the period during which GαGTP subunits regulate their effectors. The other involves RGS retention of GαGTP or GαGDP subunits, which prevents them from acting on effectors or binding to $G\beta\gamma$ dimers to reform the G-protein. Consequently, the downregulation of members of the neural R7 subfamily of RGS proteins (RGS6, RGS7, RGS9-2, and RGS11), or of the associated $G\beta5$ protein, reduces μ-opioid tachyphylaxis (Garzón *et al*, 2001, 2003; Sánchez-Blázquez *et al*, 2003). This is also observed after impairment of RGSZ1, and to a minor extent after impairment of GAIP (present work). The R7 proteins participate in opioid tachyphylaxis mostly by retaining Gα subunits, whereas the deactivation of GαzGTP subunits to reduce their regulation of effectors is probably the mechanism by which Rz proteins contribute to this effect.

If desensitization promoted by a single dose of agonists should last for days rather than hours, then the term acute tolerance would describe it better than tachyphylaxis. Interestingly, R7 or $\beta5$ knockdown mice show no acute tolerance to morphine (Garzón *et al*, 2001; Sánchez-Blázquez *et al*, 2003), although this does develop in RGSZ1, GAIP, and GIPC knockdown mice (present work). Thus, the interesting phenomena of opioid tachyphylaxis and acute tolerance can apparently be modulated in different ways. The experimental impairment of RGSZ1 function increased Gz regulatory activity and enhanced the potency and duration of acute agonist effects at μ-opioid receptors. In these circumstances, acute tolerance was observed in response to morphine doses that were ineffective at inducing this in control mice. Morphine-induced acute tolerance diminished after reducing the levels of Gαz subunits, but not after impairing Gαi2 subunits. These results indicate that Rz deactivation of μ-opioid activated Gαz subunits reduces the incidence of this phenomenon. In the presence of chronic morphine, where the deactivating control of GαzGTP subunits is impaired by knockdown of RGSZ1, tolerance developed more quickly. This negative feedback mechanism dampening the continuous action of morphine on μ receptors probably involves the recruitment of GRK-mediated phosphorylation and the inactivation of μ-opioid receptors. Therefore, the direct enhancement of μ-mediated Gz signaling by RGSZ1 knockdown, or indirect enhancement after Gαi2 knockdown and the subsequent increase in μ-opioid receptor regulation of Gz proteins, both intensify the action of adaptive mechanisms leading to acute or chronic tolerance to opioids.

In summary, RGSZ1 and GAIP regulate the activity of opioids at μ but not δ receptors in CNS. RGSZ1, by reducing the action of activated Gαz subunits on their cellular effectors, accelerates tachyphylaxis at μ receptors. The signals originated after activation of Gz-proteins play an important role in the development of long-term tolerance to these opioids.

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