

# rG $\beta_1$ : A Psychostimulant-Regulated Gene Essential for Establishing Cocaine Sensitization

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Repeated doses of cocaine or amphetamine lead to long-lasting behavioral manifestations that include enhanced responses termed sensitization. Although biochemical mechanisms that underlie these manifestations currently remain largely unknown, new protein synthesis has been implicated in several of these neuroadaptive processes. To seek candidate biochemical mechanisms for these drug-induced neuroplastic behavioral responses, we have used an approach termed subtracted differential display (SDD) to identify genes whose expression is regulated by these psychostimulants. rG $\beta_1$  is one of the SDD products that encodes a rat G-protein  $\beta$  subunit. rG $\beta_1$  expression is upregulated by cocaine or amphetamine treatments in neurons of the nucleus accumbens shell region, a major center for psychostimulant effects in locomotor control

and behavioral reward. Antisense oligonucleotide treatments that attenuate rG $\beta_1$  expression in regions including the nucleus accumbens abolish the development of behavioral sensitization when they are administered during the repeated cocaine exposures that establish sensitization. These treatments fail to alter acute behavioral responses to cocaine, and they do not block the expression of cocaine sensitization when it is established before oligonucleotide administrations. Full, regulated rG $\beta_1$  expression is a biochemical component essential to the establishment of a key consequence of repeated cocaine administrations, sensitization.

**Key words:** PCR differential display; amphetamine; cocaine; G-protein; sensitization; gene regulation; addiction

Psychostimulants such as cocaine and amphetamine act acutely to change behavior through actions at dopaminergic and other monoaminergic brain systems. Behavioral changes can also be induced by repeated psychostimulant administration. These can include tolerance, conditioned responses, and enhanced responses termed sensitization (Ellinwood and Cohen, 1971; Post and Rose, 1976). Alterations in activities in several brain extracellular, cytoplasmic, and nuclear signaling pathways have been described after repeated psychostimulant treatments (Nestler, 1992; Nestler et al., 1993; Hyman, 1996). Although the exact role that each of these biochemical events plays in any of the long-term behavioral responses to psychostimulants remains unknown, the ability of protein synthesis inhibitors to block psychostimulant-induced sensitization supports a requirement for regulated gene expression in this neuroadaptation to long-term psychostimulants (Karler et al., 1993; Sorg and Ulibarri, 1995).

The large individual and societal burdens imposed by chronic psychostimulant use underscore the importance of identifying psychostimulant-regulated biochemical mechanisms and defining the contributions that each makes to the drug-induced brain responses that underlie behavioral adaptations to psychostimulants. We now report that a strategy for identifying drug-regulated genes identifies a G-protein subunit whose full, regulated expression is

necessary for the development of psychostimulant-induced sensitization, a key animal model adaptation to psychostimulants.

## MATERIALS AND METHODS

**Animals, drug treatments, and RNA preparation.** Male Sprague Dawley rats (250–300 gm) were housed under a 12 hr light/dark cycle and given food and water *ad libitum*. Intraperitoneal injections of amphetamine (7.5 mg/kg), cocaine (50 mg/kg), or saline were given to some rats that were decapitated at various time intervals thereafter. Brains were removed rapidly, regions were dissected, and Poly (A<sup>+</sup>) RNA was extracted by using oligo-dT cellulose as described (Fastrack, Invitrogen, San Diego, CA). Total RNA was extracted from various brain regions using RNazol B (Tel-Test, Friendswood, TX). RNA was precipitated by isopropanol, washed with 75% ethanol, and dissolved in DEPC-treated water containing 1 mM EDTA, pH 7.0.

**Subtracted differential display (SDD).** Two micrograms of poly (A<sup>+</sup>) RNA prepared from striatum were used as a template for synthesis of single-stranded DNA. First-strand DNA synthesis was primed with 20 pmol of oligo-dT in 50 mM Tris-HCl buffer, pH 8.3, at 25°C, containing 0.5 mM dNTPs, 1 U/ $\mu$ l of RNase inhibitor, 75 mM KCl, 3 mM MgCl<sub>2</sub>, and MMLV reverse transcriptase, as described (First Strand synthesis kit, Clontech, Cambridge, UK). For subtraction, the single-stranded DNA prepared from *d*-amphetamine-treated striatal RNA was mixed with excess poly (A<sup>+</sup>) RNA prepared from striata of saline-treated animals. A 10-fold mRNA excess was calculated based on complete conversion of RNA to DNA in the reverse transcription reaction. The mixture was heat-denatured at 90°C for 5 min and then reannealed at 22°C for 12 hr in 10 mM Tris-HCl buffer, pH 7.4, containing 500 mM sodium chloride and 100 mM polyA (18 mer). The poly (A<sup>+</sup>) RNA-DNA complex and unhybridized poly (A<sup>+</sup>) RNA were precipitated using oligo-dT cellulose, and the unhybridized DNA remaining in the supernatant was concentrated by lyophilization.

Subtracted cDNAs were amplified by differential display PCR as described (Liang and Pardee, 1992) using anchor (5'-TTTTTTTTTTTTT(G/C)-3') and arbitrary primer (5'-GGACAGCTTC-3') sequences synthesized using a Millipore oligonucleotide synthesizer and purified using gel

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electrophoresis. PCRs were performed in 10 mM Tris-HCl buffer, pH 8.3 at 25°C, containing 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.001% gelatin, 0.2 mM dNTPs, 1 pM <sup>32</sup>S-dATP, 5 μM poly dT primer, 2.5 μM arbitrary primer, and 2.5 U/100 μl *AmpliTaq* DNA polymerase. Forty thermal cycles of 94°C for 30 sec, 40°C for 2 min, and 72°C for 1 min were followed by final extension at 72°C for 5 min.

Amplified radiolabeled cDNAs were separated on 6% polyacrylamide sequencing gels that were blotted, dried, and subjected to film autoradiography. DNA bands showing substantial intensity differences between the saline- and drug-treated striata were excised. DNA was eluted from excised gel slices by boiling in 50 μl water and ethanol precipitation. Precipitated DNA was redissolved in 10 μl H<sub>2</sub>O, and 4 μl of the DNA elute was reamplified in a 50 μl reaction volume using the same primer sets and thermal cycling parameters noted above, omitting radiolabeled precursors. Ten microliters of the reamplified product were analyzed by separation on 4% polyacrylamide gels and ethidium bromide staining. Two microliters of each reamplified PCR product were subcloned into the PCRII vector (Invitrogen). The plasmid DNAs were extracted and purified using Qiagen (Qiagen, Hilden, Germany) and subjected to sequencing using manual and automated methods. Sequences were compared using BLAST (National Library of Medicine, Bethesda, MD).

**Identification of a full-length rGβ<sub>1</sub> DNA.** Approximately 8 × 10<sup>6</sup> plaques from a size-selected rat brain cerebral cortex DNA library prepared in λZAP II (Stratagene, La Jolla, CA) were screened using a 340 bp *EcoRI* fragment of the SDD 1 product radiolabeled by random priming with <sup>32</sup>P-dATP as described (Boehringer Mannheim, Indianapolis, IN). Positively hybridizing plaques obtained from three cycles of plaque purification were analyzed preliminarily, and both strands of a 2.9 kb clone containing the full coding region of rGβ<sub>1</sub> were sequenced using manual and automated methods.

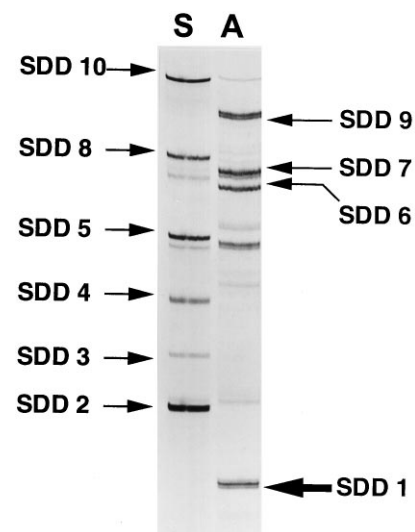
**RNA analyses.** Northern hybridization used 10 μg per lane of total RNA denatured by heating at 65°C for 15 min in 2.2 M formaldehyde and 50% (v/v) formamide, electrophoresed on 1% agarose gels containing 2.2 M formaldehyde, and blotted to Nytran membranes (Schleicher & Schuell, Keene, NH). Hybridization probes were gel-purified *EcoRI* fragments prepared from subcloned SDD products and radiolabeled with [<sup>32</sup>P]dCTP using random priming (Boehringer Mannheim). Blots were denatured by boiling and hybridized at 55°C overnight in a solution containing radiolabeled DNA, 50% formamide, 5× SSC buffer (1× SSC = 0.15 M NaCl, 15 mM sodium citrate, pH 7.0), 1× Denhardt's solution (0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone, 0.02% Ficoll), and heat-denatured sheared herring sperm DNA (100 μg/ml). Blots were washed three times with buffer containing 0.2× SSC and 0.1% SDS at 30°C and then subjected to autoradiography using film and phosphorimaging (Molecular Dynamics, Sunnyvale, CA).

For RNase protection analyses, cRNA probes were prepared from subcloned SDD products by *in vitro* transcription from the T7 promoters of pPCRII in the presence of [<sup>32</sup>P]CTP as described (MAXIscript kit, Ambion, Austin, TX). One nanogram of radiolabeled cRNA was mixed with 50 ng total RNA extracted from different brain regions in 50% deionized formamide, 100 mM sodium citrate, pH 6.4, 300 mM sodium acetate, pH 6.4, and 1 mM EDTA. The mix was denatured at 90°C for 5 min, and hybridization was performed at 45°C for 16–20 hr. The unhybridized labeled and unlabeled RNAs were removed by digestion with 5 U RNase A and 20 U RNase T1 at 37°C for 30 min. Undigested mRNA-cRNA complexes were precipitated by ethanol/0.3 M NaOAc, and pellets were redissolved in 80% formamide/0.1% xylene cyanol/0.1% bromophenol blue/2 mM EDTA, denatured by heating at 90°C for 10 min, and separated by electrophoresis on 4% polyacrylamide gels. Gels were dried, and results were analyzed by autoradiography using film and phosphorimaging (Molecular Dynamics).

**Analyses of rGβ<sub>1</sub> immunoreactivity.** rGβ<sub>1</sub> immunoreactivity was detected by a rabbit polyclonal anti-G-protein β<sub>1</sub> antibody directed against peptide sequences TLSQITNNI (kindly provided by Dr. W. F. Simonds, National Institute of Diabetes and Digestive and Kidney Diseases) that were identical to those found in rGβ<sub>1</sub>.

For immunohistochemistry, 40 μm frozen sections were prepared from brains of rats killed by perfusion with 4% paraformaldehyde under pentobarbital anesthesia 4 hr after treatment with cocaine (50 mg/kg, i.p.). Immunoreactivity detected by the primary antibody was visualized by a nickel chloride-enhanced ABC system (Vectastain Elite). Intensity of staining of the most densely immunoreactive nucleus accumbens neurons was rated independently by two observers unaware of drug pretreatments.

For Western analyses, protein was extracted from brain regions and



**Figure 1.** SDD of cDNAs from striata of control and amphetamine-treated rats. Gel autoradiography of the PCR differential display products amplified from subtracted cDNA prepared from the striata of rats killed 4 hr after saline (*S*) or amphetamine (*A*) (7.5 mg/kg, i.p.) injections. SDD was performed as described in text. The band containing SDD 1 corresponding to rGβ<sub>1</sub> is indicated by the bold arrow.

separated on SDS-PAGE gels, transferred to nitrocellulose membranes using electroblotting, and stained with the polyclonal antibody diluted 1:1000 with detection using an enhanced chemiluminescence system (Amersham, Arlington Heights, IL).

**Anti-rGβ<sub>1</sub> oligonucleotide treatment.** Male Sprague Dawley rats (300 gm) were anesthetized with pentobarbital and implanted with 22 gauge guide cannulas with tips 1 mm dorsal to the left lateral cerebral ventricle. Oligonucleotides were injected on alternate days beginning 5 d after guide cannula implantation. Two microliters of artificial CSF containing 10 μg rGβ<sub>1</sub> antisense (5'-TTCACCTCATCT-TCACGTC) or missense (5'-ATCGCTCGTCATCTCGTC) oligonucleotides were injected intracerebroventricularly using Hamilton syringes fitted through the guide cannulae. Daily intraperitoneal injections of 20 mg/kg cocaine were begun 6 hr after the fourth oligonucleotide injections or at other times as indicated and continued for 5 d (see Figs. 7, 8). During behavioral test sessions, animals were habituated to the apparatus for 50 min, injected with cocaine, and monitored for 150 min to assess locomotion. Locomotor responses to cocaine injections were measured using an Auto-Track system (Columbus Instruments, Columbus, OH) and analyzed for "distance traveled" by detection of breaks in infrared beams placed at 2.4 cm intervals.

## RESULTS

### Identification of differentially expressed genes including a G-protein β subunit homolog

SDD PCR identified a number of mRNAs whose expression was altered in rat striatum 4 hr after treatment with *d*-amphetamine (7.5 mg/kg, i.p.) (Fig. 1). SDD 1 was one of more than 10 regulated, subcloned SDD cDNAs (Fig. 1, arrow) identified by this approach. SDD 1 displayed a 340 bp sequence homologous to the bovine transducin β subunit (81%) and to the cDNA encoding the human G-protein β<sub>1</sub> subunit (81%) (Fong et al., 1987), but only 70% identity to a rat sequence previously reported as rat G-protein β subunit (GenBank accession no. L29090).

Regulated expression of a G-protein could provide a readily understandable candidate mechanism for neuronal adaptations to previous drug exposures. We therefore used the 340 bp sequence to identify cDNAs in a rat cerebral cortex λZapII library; sequences from three cDNAs matched SDD 1. One of the cDNAs was a 2.9 kb species that encoded 27 bp of 5' untranslated

1 GATTTCGGCAGGTTGAAAGCACTGA**GACGTGAAGATGAGTGAA**CTTGACCAGTGCCTGGCAGGAGCCGAGCAACTGAAGAACCAAAT  
M S E L D Q L R Q E A E Q L K N Q I 18

91 AGAGATGCCCGTAAAGCATGTGCAGATGCGACTCTTTCTCAGATCACAAACAACATTGATCCAGTGGGAAGAATCCAAATGCGAACCCAGG  
R D A R K A C A D A T L S Q I T N N I D P V G R I Q M R T R 48

181 AGAACACTGAGGGGGCATCTGGCAAGATTTATGCCATGCACTGGGGCACAGACTCAAGGCTCCTTGTGTCAGCGCTCTCAGGATGGGAAA  
R T L R G H L A K I Y A M H W G T D S R L L V S A S Q D G K 78

271 CTCATCATCTGGGACAGCTATACCACAAACAAGTTTCATGCCATCCCTCTGCGCTCCTCTGGGTCTGACCTGTGCATACGCTCTCTCT  
L I I W D S Y T T N K V H A I P L R S S W V M T C A Y A P S 108

361 GGGAAATATGTGGCCTGTGGTGGCCTGGATAACATCTGCTCCATTTACAATCTGAAAACCTCGTGAAGGAAATGTGCGTGTGAGTCGTGAG  
G N Y V A C G G L D N I C S I Y N L K T R E G N V R V S R E 138

451 CTGGCAGGACATACAGGTTATCTATCCTGTTGCCGTTTCTGGATGACAATCAGATAGTTACCAGTCTGGAGACACCACATGTGCCCTG  
L A G H T G Y L S C C R F L D D N Q I V T S S G D T T C A L 168

541 TGGGACATCGAGACCGGCCAGCAGACAACCACGTTTACTGGACACTGGAGATGTCATGAGCCTGTCTCTTGCTCTGACACCAGACTG  
W D I E T G Q Q T T T F T G H T G D V M S L S L A P D T R L 198

631 TTTGTCTCTGGTCTTGGCATGCTTCGGTCAAGCTCTGGGATGTCCGAGAAGGGATGTGCGGCAGACCTTTACAGGACACGAGTCTGAC  
F V S G A C D A S V K L W D V R E G M C R Q T F T G H E S D 228

721 ATCAATGCCATATGTTTCTTTCCCAATGGCAATGCCTTTGCCACTGGCTCAGACGATGCTACATGCAGGCTGTTTGACCTTCGTGCAGAC  
I N A I C F F P N G N A F A T G S D D A T C R L F D L R A D 258

811 CAGGAGCTCATGACCTACTCCATGACAACATTATCTGTGGTATCACATCTGTCCCTTCTCCAAGAGTGGCCGCCTCTCTCGGTGGC  
Q E L M T Y S H D N I I C G I T S V P F S K S G R L L L G G 288

901 TATGACGACTTCAACTGTAATGTCTGGGATGCACTCAATGCTGACAGAGCAGGTTCCCTAGCTGGACATGACAACCCAGTCTGAGTCTGCT  
Y D D F N C N V W D A L N A D R A G V L A G H D N R V S C L 318

991 GGGGTGACTGATGATGGCATGGCTGTGGCAACAGGATCCTGGGACAGCTTCTTCAAGATCTGGAACTAAGGCCACAGCAGGTGGATGGAT  
G V T D D G M A V A T G S W D S F L K I W N \* 340

1081 GCCATGGCGACTGGAAGACCATTCCAACCTTGAAGCGTTACTGCGATAGCATATCCTATCCAACCATACTAACGTGGACACCCACACCTC  
1171 CCCTCAGAAGTTCAAAGGGCAAGATCTTTTCTTCACTTATTGCTGAAACCAAGAGCACAACCTCCCATTAAGAGAAGGATCTCTGTG  
1261 CTGTAAACTAAAACAAATTGTGCATTTTTCTGGGCCATTGTCTTTGGTTTATTTGTTTGTTTTGTTTTGTTTTGTTTTGTCTTGG  
1351 GCAAATTTTAAAGGAAATACTACAATAAAAATGTTAACAGAAGGTAACCTGAGTATGATTGTGAGAGACACACCTTCTTACTAGT  
1441 TCATTGCAATTTAAATCAGTGATCTGTGGCAATCATGAAAAACAAGTTGAAGACTTTGTTTATCTGGAATGTTTCTAAATATA

**Figure 2.** Nucleotide and predicted amino acid sequences of the rG $\beta_1$  cDNA. The rG $\beta_1$  cDNA nucleotide sequences are numbered on the left, and amino acids of a predicted open reading frame are numbered on the right. Underlining designates the nucleotide sequences used for RNase protection and Northern analyses. Sequences complementary to those of the anti-rG $\beta_1$  oligonucleotides are boxed.

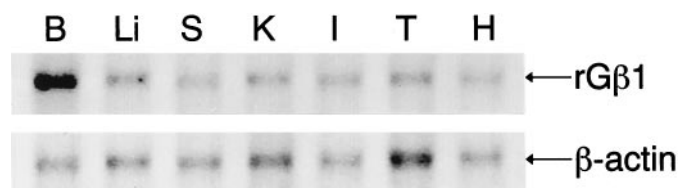
sequence, an open reading frame of 1020 bp, and 2453 bp of a 3' untranslated sequence (Fig. 2). The 340 amino acids predicted from the open reading frame would yield an unmodified molecular mass of  $\sim 37.5$  kDa. This cDNA displayed 84% identity to the bovine and human G-protein  $\beta_1$  subunits, 63–78% identity to  $\beta_2$ – $\beta_5$  (Fong et al., 1987; Levine et al., 1987; Watson et al., 1994), and only 72% identity to the rat sequence previously reported as G $\beta$  (GenBank accession no. L29090). We thus termed this cDNA sequence as encoding rat G-protein  $\beta_1$  subunit (rG $\beta_1$ ).

#### Differential expression of rG $\beta_1$

Northern analyses revealed a single 3.6 kb mRNA that hybridized with rG $\beta_1$  hybridization probes. This mRNA was expressed more

abundantly in rat brain than in several peripheral tissues examined (Fig. 3) and was expressed unevenly in different brain regions. RNase protection studies using a cRNA transcribed from SDD 1 using T7 polymerase documented high rG $\beta_1$  expression in cerebral cortex and striatum + accumbens, intermediate levels in hippocampus, and low levels in thalamus. Immunohistochemical studies revealed rG $\beta_1$ -like immunoreactivity in neuronal patterns in several brain regions, including nucleus accumbens (see Fig. 5), a brain region heavily implicated in psychostimulant actions (Koob, 1992).

Specimens from striatum + accumbens dissected from rats treated with *d*-amphetamine (7.5 mg/kg, i.p., 4 hr before sacrifice)



**Figure 3.** Expression of rG $\beta_1$  mRNA. RNase protection assessments of the relative levels of rG $\beta_1$  (top) and  $\beta$ -actin expression (bottom) in rat tissues. B, Brain; Li, liver; S, spleen; K, kidney; I, intestine; T, testis; H, heart.

revealed more than twofold enhancement of rG $\beta_1$  expression, as assessed by RNase protection (Table 1). This upregulation was noted 2 hr after drug injection and persisted for at least 48 hr (Fig. 4). Cocaine treatment (50 mg/kg, i.p., 4 hr) also stimulated up to 2.5-fold enhancement of rG $\beta_1$  mRNA expression in striatum + accumbens specimens (Table 1). Western analyses showed that protein expression was also enhanced in striatum + accumbens dissected from amphetamine-treated (131%  $\pm$  37, mean percentage of saline control  $\pm$  SEM;  $n$  = 4) or cocaine-treated (138%  $\pm$  29, mean percentage of saline control  $\pm$  SEM;  $n$  = 4) rats. Immunohistochemical studies revealed that cocaine-induced increases in rG $\beta_1$  immunoreactivity were most remarkable in a cluster of nucleus accumbens shell region neurons lying close to the lateral ventricle (Fig. 5). Altered expression in other brain regions displayed more variability; 7.5 mg/kg amphetamine increased rG $\beta_1$  mRNA in hippocampus of rats killed 4 hr after injections but decreased rG $\beta_1$  mRNA in cerebral cortex and thalamus. Cocaine treatments induced little change in rG $\beta_1$  expression in either the hippocampus or thalamus, whereas they enhanced rG $\beta_1$  mRNA in cerebral cortex (Table 1).

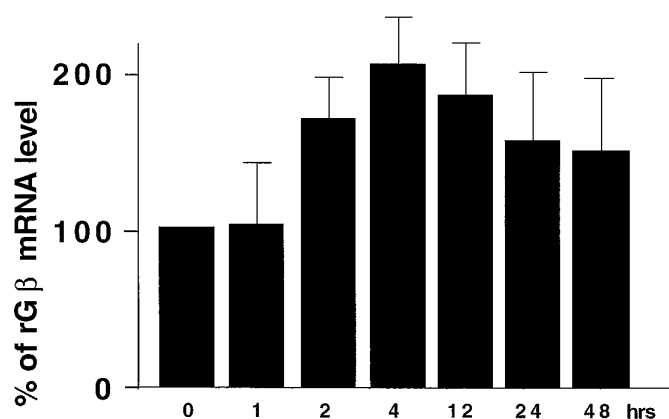
### Effects of anti-rG $\beta_1$ on sensitization

To seek evidence for possible involvement of rG $\beta_1$  expression in long-term adaptations to psychostimulants, we examined the effects of transient attenuation of rG $\beta_1$  expression on psychomotor stimulant-induced sensitization, one of the most robust behavioral adaptations to repeated stimulant administration readily demonstrable in animal models. Three alternate-day intracerebroventricular administrations of an anti-rG $\beta_1$  oligonucleotide complementary to sequences flanking the ATG start codon of the rG $\beta_1$  cDNA (Figs. 2, 7A) reduced levels of rG $\beta_1$  immunoreactivity in striatum + accumbens, whereas a missense oligonucleotide infused on the same schedule had no effect (Fig. 6). The animals' appearance, gross behavioral activity, body weight, and magnitude of locomotor responses to acute administration of amphetamine or cocaine was not altered by these treatments.

**Table 1. Psychostimulant-induced rG $\beta_1$  mRNA changes in different brain regions**

Drugs	Striatum + accumbens	Prefrontal cortex	Thalamus + hypothalamus	Hippocampus
Cocaine (50 mg/kg, i.p.)	246 $\pm$ 48 ( $n$ = 9)	145 $\pm$ 45 ( $n$ = 6)	96 $\pm$ 30 ( $n$ = 6)	108 $\pm$ 47 ( $n$ = 6)
<i>d</i> -Amphetamine (7.5 mg/kg, i.p.)	218 $\pm$ 63 ( $n$ = 9)	72 $\pm$ 31 ( $n$ = 6)	81 $\pm$ 26 ( $n$ = 6)	337 $\pm$ 79 ( $n$ = 5)

Values represent mean percentage of control  $\pm$  SEM; rG $\beta_1$  mRNA levels were normalized to levels of expression of  $\beta$ -actin.



**Figure 4.** Time course for amphetamine-induced alterations in rG $\beta_1$  expression in striatum + accumbens of rats. Rats receiving single *d*-amphetamine injections (7.5 mg/kg, i.p.) were killed at various times after injection, total RNA was extracted from striatum + accumbens, and expression of rG $\beta_1$  mRNA was analyzed by an RNase protection assay. Variation in recoveries between samples was normalized by assessments of hybridization to  $\beta$ -actin mRNA.

Animals whose rG $\beta_1$  expression was attenuated by anti-rG $\beta_1$  oligonucleotide displayed enhanced locomotor responses to an initial injection of 20 mg/kg cocaine that were indistinguishable from those in control animals injected with missense control oligonucleotide (Fig. 7). In animals injected with missense oligonucleotide, marked behavioral sensitization was noted after the five daily cocaine administrations. Locomotor scores in these animals doubled those induced by the cocaine dose that preceded sensitization. In striking contrast, however, animals treated with anti-rG $\beta_1$  oligonucleotide revealed no evidence of behavioral sensitization in any of three replicate experiments (Fig. 7C). The effects of antisense oligonucleotide treatments were reversible. Anti-rG $\beta_1$  pretreated rats in each of the three groups displayed full recovery of cocaine-induced sensitization when new sensitizing treatments were begun 3 weeks after cessation of antisense treatments (Fig. 7B). When antisense treatments were administered after establishment of sensitization, they failed to alter the robust sensitization displayed by presensitized rats (Fig. 8). These results strongly implicate rG $\beta_1$ , or a closely related protein, as being required for the establishment of cocaine-induced behavioral sensitization.

### DISCUSSION

The current results document region-specific psychostimulant upregulation of neuronal expression of a gene whose expression or upregulation seems to play a necessary role in the establishment of psychostimulant-induced sensitization. These data add a specific example to a growing body of literature that collectively and increasingly implicates contributions of regulated expression of specific genes of neurotransmission to some of the long-term effects of abused substances in the brain.

### Implications of identification of rG $\beta_1$ regulation by drugs

The G-protein identified here has such high homology to previously described G-protein  $\beta$  subunits that it is very unlikely to have any other function. Its designation as G $\beta_1$  is also based on sequence homologies, although this provisional designation should be confirmed by results from functional studies.

Our identification of rG $\beta_1$  cDNA was unlikely to have been

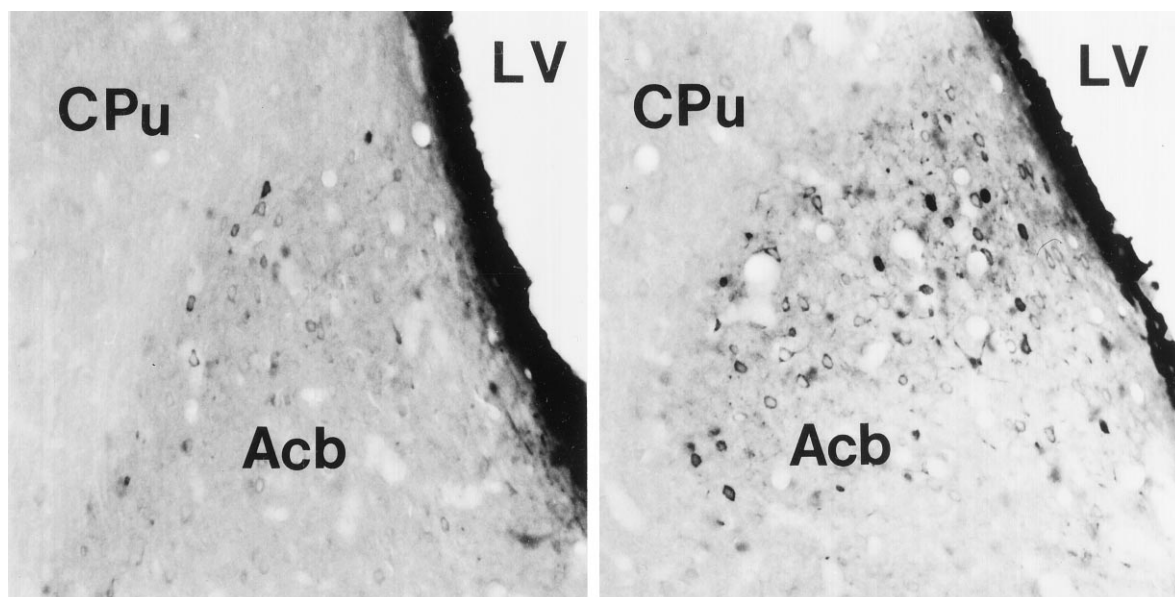


Figure 5. Cocaine-induced alterations in  $rG\beta_1$  expression in striatum + accumbens. Expression of  $rG\beta_1$  immunoreactivity in nucleus accumbens neurons in rats killed 4 hr after intraperitoneal treatment with saline (left panel) or 50 mg/kg cocaine (right panel). LV, Lateral ventricle; Acb, nucleus accumbens; CPu, caudate putamen.

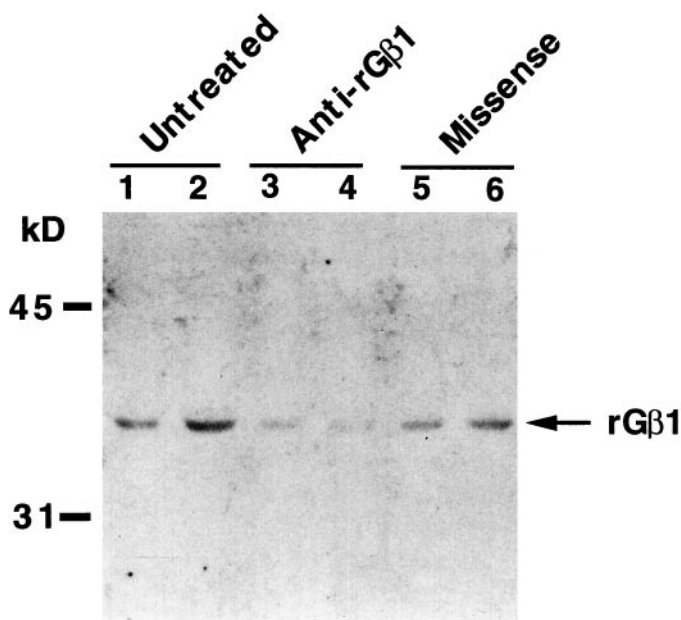


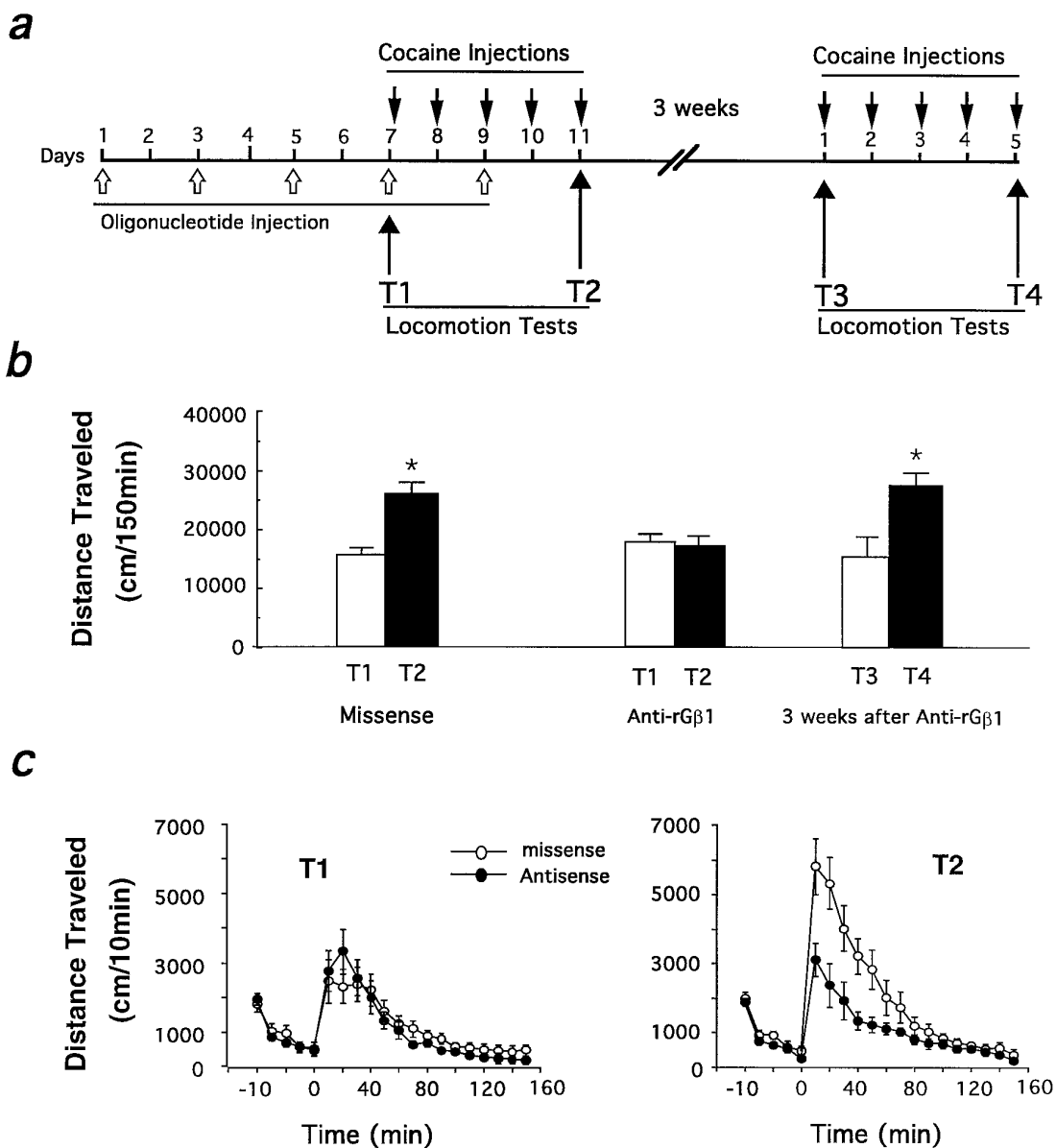
Figure 6. Inhibition of  $rG\beta_1$  translation by anti- $rG\beta_1$  oligonucleotide treatments. Expression of  $rG\beta_1$  gene product in Western blot of 10  $\mu$ g protein samples from striata dissected from each of two rats that were untreated (lanes 1 and 2), treated with anti- $rG\beta_1$  oligonucleotide (lanes 3 and 4), or treated with missense oligonucleotide (lanes 5 and 6). Molecular size standards migrated as displayed at the left of the figure; the  $rG\beta_1$  immunoreactivity migrates at an  $M_r$  of 37 kDa.

random. Its detection could have been influenced by the extent of psychostimulant regulation of  $rG\beta_1$  gene expression, the abundance of  $rG\beta_1$  mRNA, the complementarity of  $rG\beta_1$  cDNA sequences to the oligonucleotide used in the differential display, and other factors. Although the PCR-DD techniques used do not necessarily provide a representative sample of drug-regulated genes, 2 of 10 initially evaluated SDD products did encode  $rG\beta_1$ , whereas several other cDNAs encode molecules involved in in-

tracellular signal transduction, such as the calcium-regulated neuronal protein phosphatase calcineurin. These observations are thus consistent with the emerging idea that many of the genes regulated by abused drugs may be implicated in the biochemical and/or structural bases of inter- and intracellular brain signaling pathways, as has also been suggested by studies of transcription factor and neuropeptide gene regulation by abused drugs (for review, see Persico and Uhl, 1997). Amplification of additional differentially expressed cDNAs from drug-treated brains using different oligonucleotides will allow further tests of this idea.

#### Patterns of expression of $rG\beta_1$ in normal and drug-treated brains and possible functional significance

The observed pattern of expression of  $rG\beta_1$  in normal rats was distinct from that described for the  $G\beta$  subunit family members termed 1 through 4 in humans. Although each of the other  $G\beta$ s are expressed ubiquitously (Fong et al., 1987; Levine et al., 1987; Weizsacker et al., 1992; Neer, 1995),  $rG\beta_1$  is expressed much more prominently in brain, and in a markedly region-specific fashion. This pattern of regional expression of  $rG\beta_1$  in brain is reminiscent of that of the murine  $G\beta_5$  gene, which displays selectively enhanced expression of two transcripts in brain in markedly different regional distribution patterns (Watson et al., 1994). The high level expression of  $rG\beta_1$  in brain is consistent with possibly important functions in CNS signal transduction. Although the intracellular pathways triggered by  $rG\beta_1$  activation have not been elucidated clearly, there is increasing evidence to suggest that activated  $G\beta\gamma$  dimers can directly influence several signal transduction pathways (for review, see Clapham and Neer, 1993). These include regulation of the potassium channels opened by muscarinic cholinergic receptors (Reuveny et al., 1994; Wickman et al., 1994), selected isoforms of adenylylase, phospholipase  $C\beta$ , cytosolic IP3 kinase (Blank et al., 1991; Tang and Gilman, 1991; Boyer et al., 1992; Katz et al., 1992; Watson et al., 1994), and the ras-dependent MAP kinase pathway (Crespo et al., 1994; Faure et al., 1994). G-protein  $\beta\gamma$  mechanisms have also

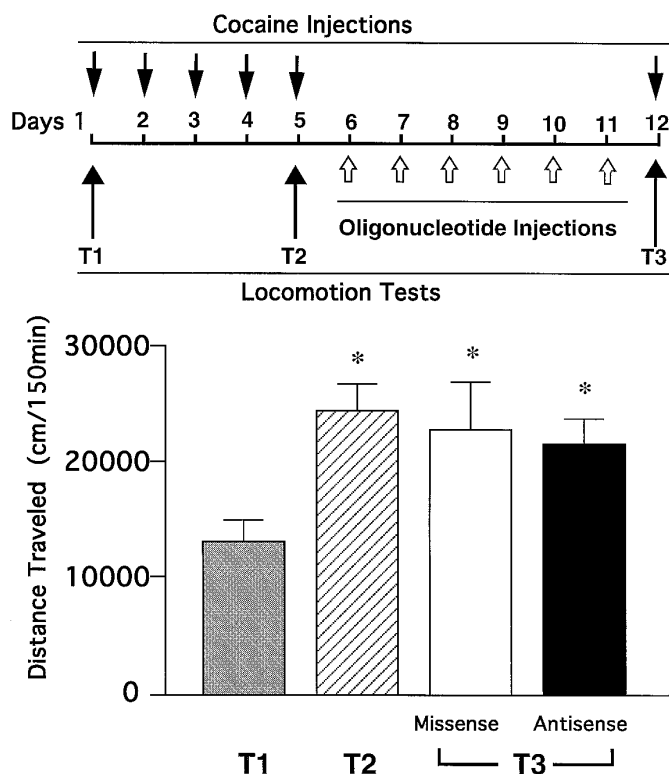


**Figure 7.** Anti-rG $\beta_1$  oligonucleotide treatments block cocaine-induced sensitization. *a*, Schedule of oligonucleotide treatments, cocaine administrations, and behavioral testing. *b*, Locomotor responses to cocaine administration in rats tested at the beginning (*T1*) and end (*T2*) of a 5 d series of cocaine administrations resulting in sensitization of control rats. Data at *T3* mark testing 3 weeks after cessation of cocaine injection. Bars reflect total motor activity scores from time 0 to 150 min after cocaine injection. Significant differences between locomotor responses to cocaine at *T2* versus *T1* are found in the missense treatment group but not in the antisense treatment group (anti-rG $\beta_1$ ;  $p = 0.123$ ). Difference at *T4* versus *T3* are significant for the antisense treatment group ( $*p < 0.05$ ). *c*, rG $\beta_1$  missense (open circles) and antisense (filled circles) oligonucleotide treatments precede the establishment of cocaine sensitization. Lines display the time course in minutes relative to the time of cocaine injection. Thirty rats were tested in each group.

been strongly implicated in modulation of voltage-gated calcium channels (Herlitze et al., 1996; Ikeda, 1996).

Patterns of rG $\beta_1$  upregulation after psychostimulants also reveal regional specificity, evidence for drug specificity, and possibly persistent time courses. Prominent upregulation of protein immunoreactivity in Western analyses and mRNA in Northern studies was found in striatum + accumbens specimens after amphetamine and cocaine treatments. The neuronal localization of this upregulation was documented after cocaine administration and after amphetamine treatment in work in progress. Upregulation of mRNA in hippocampus is the other most prominent change noted after amphetamine

treatments. Other brain regions, including the thalamus and cerebral cortex, fail to display such alterations. Interestingly, initial data from samples of the ventral tegmental area, a brain region also implicated in psychostimulant-induced sensitization (Kalivas and Weber, 1988; Perugini and Vezina, 1994; Sorg and Ulibarri, 1995), fail to display substantial upregulation of rG $\beta_1$  mRNA levels (X.-B. Wang and G. Uhl, unpublished observations). These upregulatory events can persist. Initial data suggest that rG $\beta_1$  upregulation persists for the course of 10–14 d of amphetamine or cocaine treatments, and that the upregulation persists for at least 48 hr after the last of these chronic doses (our unpublished observations).



**Figure 8.** Effects of intracerebroventricular oligonucleotide infusions on locomotion activity of rats presensitized with cocaine. *A*, Schedule of oligonucleotide treatments, cocaine administrations, and behavioral testing. *B*, rG $\beta_1$  missense (open bars) and antisense (filled bars) oligonucleotide treatments follow the establishment of cocaine sensitization. T3 represents an additional test of cocaine effects on locomotor responses after the oligonucleotide treatments. Sensitization is demonstrated at T2 and at T3 for both missense and antisense-treated rats (\* $p < 0.05$  compared with T1 for each). Twenty rats were tested in each group.

### Comparisons with drug-induced alterations in G $\alpha$ expression

Activation of various G $\alpha$  subunits by activated G-linked neurotransmitter receptors can also alter activities of G-linked channels conducting ions such as potassium and calcium and of second messenger systems, including adenylyl cyclase, guanylyl cyclase, and phospholipase C (Casey and Gilman, 1988). Modest alterations in levels of expression of G $\alpha$  subunit protein have been reported in studies of brain regions dissected from rats treated with psychostimulants or opiates (Nestler et al., 1989; Steketee et al., 1991; Striplin and Kalivas, 1992, 1993; Przewlocka et al., 1994; Self et al., 1994). A chronic cocaine and morphine regimen led to ~15% reductions in G $_i$ /G $_o$   $\alpha$  subunit activity in samples from ventral tegmental area, nucleus accumbens, and locus ceruleus, whereas chronic opiate treatment resulted in modest G $_i$ /G $_o$   $\alpha$  subunit upregulation in locus ceruleus and downregulation in nucleus accumbens (Nestler et al., 1989; Przewlocka et al., 1994). Interestingly, acute opiate treatments induced downregulation of G $_i$ /G $_o$   $\alpha$  subunit expression in locus ceruleus samples (Self et al., 1994). Although these alterations are modest in magnitude, they might conceivably work synergistically with the more robust alterations in G $\beta_1$  subunits described in this report. The changes identified may be more prominent for G $\beta_1$  than for other G $\beta$  subunits; immunoreactivity corresponding to a common G $\beta$  epitope was not altered by psychostimulants in a study using antibodies raised against a peptide sequence displaying >90% identity across G $\beta_{1-4}$  se-

quences (X.-B. Wang and G. R. Uhl, unpublished observations). Conceivably, the alterations in brain G $\beta_1$  activities larger than those reported previously for measures of some G $\alpha$  subunit activities could reflect involvement of alterations in other  $\alpha$  subunits that have resisted identification by the ADP-ribosylation or Western blotting techniques used to date.

### Dopaminergic and nondopaminergic interactions with rG $\beta_1$ regulation

rG $\beta_1$  regulation by cocaine and amphetamine in dopamine-rich striatal and accumbens regions would be consistent with roles of dopamine D $_1$  and D $_2$  receptors in psychostimulant-induced locomotion and reward (Henry and White, 1991; Asin et al., 1994; Hooks et al., 1994; Silvia et al., 1994; Xu et al., 1994). Conceivably, these drug-induced rG $\beta_1$  expression changes could change the molecular balances between  $\beta$ ,  $\gamma$ , and  $\alpha$  G-protein subunits and/or alter quantitative features of the ratios between G-linked receptors and G-proteins and change signal transduction pathways mediated by  $\alpha$  and/or  $\beta\gamma$  subunits, including those activated by D $_1$  and D $_2$  family receptors. Such mechanisms could contribute to the neuroplastic changes likely to underlie behavioral alterations noted after chronic psychostimulant administration.

Results obtained in other brain regions also suggest that non-dopaminergic mechanisms triggered by treatment with only one of the psychostimulants examined could also play roles in rG $\beta_1$  regulation. The hippocampus, where rG $\beta_1$  upregulation is triggered only after amphetamine, and the cortex, where it is triggered only by cocaine, are thus unlikely to alter rG $\beta_1$  expression only on the basis of the dopaminergic mechanisms that both psychostimulants share. These differences may have functional consequences: both of these regions are differentially implicated in associative learning processes such as those likely to accompany establishment of addiction. The alterations in these regions could thus have substantial importance in long-term neuroadaptive responses to psychostimulants even though they might not be as directly dependent on dopaminergic circuits.

### rG $\beta_1$ antisense treatments and sensitization

The effects of transient attenuation of rG $\beta_1$  expression on sensitization provide evidence for involvement of rG $\beta_1$  expression in one robust model for behavioral adaptations to psychostimulants. Antisense treatments reduced rG $\beta_1$  immunoreactivity and mRNA profoundly in striatum + accumbens. Other regions including prefrontal cortex, thalamus, and hippocampus displayed more modest alterations. Conceivably, the close localization of the striatum and accumbens to the sites of intracerebroventricular oligonucleotide injections could provide high local concentrations of anti-rG $\beta_1$  oligonucleotides and contribute to this specificity of biochemical effect. No antisense experiment can exclude participation of some nonspecific influences. Nevertheless, substantial evidence for specificity of results includes the failure of missense oligonucleotide to block production of sensitization, the reductions in rG $\beta_1$  immunoreactivity in striatum + accumbens tissue samples after treatment with antisense but not missense oligonucleotides, the times during which antisense treatments were successful in blocking the development of sensitization, the failure of antisense infusions to produce alterations in animals' appearances, gross behavioral activities, body weights, magnitudes of locomotor responses to acute administration of amphetamine or cocaine, and the animals' recovery of susceptibility to cocaine-induced sensitization several weeks after the end of oligonucleotide treatments. Each of these features adds to confidence that

normal patterns of expression and/or regulation of expression of  $rG\beta_1$ , or a very closely related molecule, are necessary for establishment of cocaine sensitization. Although the data do not allow separation of these two possibilities, it is interesting to note that reductions to ~60% of normal levels of  $rG\beta_1$  expression are compatible with the sorts of locomotor responses to cocaine found in nonsensitized animals but not with the higher levels found in sensitized animals.

The current results add to growing evidence that genes encoding important cellular regulators could play significant roles in biochemical "adaptive" brain processes after drug administration. Definition of the likely participation of  $rG\beta_1$  in psychostimulant-induced sensitization also makes it a prominent candidate mechanism for participation in other long-term consequences of drug use, including tolerance, craving, and relapse. Identifying each of the genes whose expression is regulated with abused drugs in acute and more chronic fashions and defining the behavioral effects that can be elicited by altering this regulation provide a powerful tool for defining biochemical bases of addiction.

## REFERENCES

- Asin KE, Nikkel AL, Wirtshafte D (1994) Repeated D1 receptor agonist treatment blocks cocaine-induced locomotor activity and c-fos expression. *Brain Res* 637:342-344.
- Blank JL, Brattain KA, Exton JH (1991) Activation of cytosolic phosphoinositide phospholipase C by G-protein  $\beta\gamma$  subunits. *J Biol Chem* 267:23069-23075.
- Boyer JL, Waldo GL, Harden TK (1992)  $\beta\gamma$ -Subunit activation of G-protein-regulated phospholipase C. *J Biol Chem* 267:25451-25456.
- Casey PJ, Gilman AG (1988) G protein involvement in receptor-effector coupling. *J Biol Chem* 263:2577-2580.
- Clapham DE, Neer EJ (1993) New role for G-protein  $\beta\gamma$ -dimers in transmembrane signalling. *Nature* 365:403-406.
- Crespo P, Xu N, Simonds WF, Gutkind JS (1994) Ras-dependent activation of MAP kinase pathway mediated by G-protein  $\beta\gamma$  subunits. *Nature* 369:418-420.
- Ellinwood S, Cohen S (1971) Amphetamine abuse. *Science* 171:420-421.
- Faure M, Voyno-Yasenetskaya A, Bouren HR (1994) cAMP and  $\beta\gamma$  subunits of heterotrimeric G proteins stimulate the mitogen-activated cyclic AMP response. *J Biol Chem* 269:7851-7854.
- Fong HKW, Amatrua TT, Birren BW, Simon MI (1987) Distinct forms of the  $\beta$  subunit of GTP-binding regulatory proteins identified by molecular cloning. *Proc Natl Acad Sci USA* 84:3792-3796.
- Henry DJ, White FJ (1991) Repeated cocaine administration causes persistent enhancement of D1 dopamine receptor sensitivity within the rat nucleus accumbens. *J Pharmacol Exp Ther* 258:882-890.
- Herlitze S, Garcia DE, Mackle K, Hille B, Scheuer T, Catterall WA (1996) Modulation of  $Ca^{2+}$  channels by G-protein  $\beta\gamma$  subunits. *Nature* 380:258-262.
- Hooks MS, Juncos JL, Justice Jr JB, Meiergerd SM, Povlock SL, Schenk JO, Kalivas PW (1994) Individual locomotor response to novelty predicts selective alterations in  $D_1$  and  $D_2$  receptors and mRNAs. *J Neurosci* 14:6144-6152.
- Hyman SE (1996) Addiction to cocaine and amphetamine. *Neuron* 16:901-904.
- Ikeda SR (1996) Voltage-dependent modulation of N-type calcium channels by G-protein  $\beta\gamma$  subunit. *Nature* 380:255-258.
- Kalivas PW, Weber B (1988) Amphetamine injection into the ventral mesencephalon sensitizes rats to peripheral amphetamine and cocaine. *JPET* 245:1095-1102.
- Karler R, Finnegan KT, Calder LD (1993) Blockade of behavioral sensitization to cocaine and amphetamine by inhibitors of protein synthesis. *Brain Res* 603:19-24.
- Katz A, Wu D, Simon MI (1992) Subunits  $\beta\gamma$  of heterotrimeric G protein activate  $\beta$  2 isoform of phospholipase C. *Nature* 360:686-689.
- Koob GF (1992) Drugs of abuse: anatomy, pharmacology and function of reward pathways. *Trends Pharmacol Sci* 13:177-184.
- Levine MA, Smallwood PM, Moen Jr PT, Helman LJ, Ahn TG (1987) Molecular cloning of  $\beta_3$  subunit, a third form of the G protein  $\beta$ -subunit polypeptide. *Proc Natl Acad Sci USA* 87:2329-2333.
- Liang P, Pardee AB (1992) Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science* 257:967-970.
- Neer EJ (1995) Heterotrimeric G proteins: organizers of transmembrane signals. *Cell* 80:249-257.
- Nestler EJ (1992) Molecular mechanisms of drug addiction. *J Neurosci* 12:2439-2450.
- Nestler EJ, Erdos JJ, Terwilliger R, Duman RS, Tallman JF (1989) Regulation of G proteins by chronic morphine in the rat locus ceruleus. *Brain Res* 476:230-239.
- Nestler EJ, Hope BT, Widnell KL (1993) Drug addiction: a model for the molecular basis of neural plasticity. *Neuron* 11:995-1006.
- Persico AM, Uhl GR (1997) Transcription factors: potential roles in drug-induced plasticity. *Rev Neurosci* 7:233-275.
- Perugini M, Veznia P (1994) Amphetamine administered to the ventral tegmental area sensitizes rats to the locomotor effects of nucleus accumbens amphetamine. *JPET* 270:690-696.
- Post RM, Rose H (1976) Increasing effects of repetitive cocaine administration in the rat. *Nature* 260:731-732.
- Przewlocka B, Lason W, Przewlocki R (1994) The effect of chronic morphine and cocaine administration on the  $G_s$  and  $G_o$  protein messenger RNA level in the rat hippocampus. *Neuroscience* 63:1111-1116.
- Reuveny E, Slesinger PA, Inglesse J, Morales JM, Iniguez-Lluhi LA, Lefkowitz RJ, Bourne HR, Jan LY (1994) Activation of the cloned muscarinic potassium channel by G protein  $\beta\gamma$  subunits. *Nature* 370:143-146.
- Self DW, Terwilliger RZ, Nestler EJ, Stein L (1994) Inactivation of  $G_i$  and  $G_o$  proteins in nucleus accumbens reduces both cocaine and heroin reinforcement. *J Neurosci* 14:6239-6247.
- Silvia CP, King GR, Lee TH, Xue Z-Y, Caron MG, Ellinwood EH (1994) Intranigral administration of D2 dopamine receptor antisense oligodeoxynucleotides establishes a role for nigrostriatal D2 autoreceptors in the motor actions of cocaine. *Mol Pharmacol* 46:51-57.
- Sorg BA, Ulibarri C (1995) Application of a protein synthesis inhibitor into the ventral tegmental area, but not the nucleus accumbens, prevents behavioral sensitization to cocaine. *Synapse* 20:217-224.
- Steketee JD, Striplin CD, Murray TF, Kalivas PW (1991) Possible role for G-proteins in behavioral sensitization to cocaine. *Brain Res* 545:287-291.
- Striplin CD, Kalivas PW (1992) Correlation between behavioral sensitization to cocaine and G protein ADP-ribosylation in the ventral tegmental area. *Brain Res* 579:181-186.
- Striplin CD, Kalivas PW (1993) Robustness of G protein changes in cocaine sensitization shown with immunoblotting. *Synapse* 14:10-15.
- Tang WJ, Gilman AG (1991) Type-specific regulation of adenylyl cyclase by G protein beta gamma subunits. *Science* 254:1500-1503.
- Watson AJ, Katz A, Simon MI (1994) A fifth member of the mammalian G-protein  $\beta$ -subunit family. *J Biol Chem* 269:22150-22156.
- Weizsacker E, Strathmann MP, Simon MI (1992) Diversity among the beta subunits of heterotrimeric GTP-binding proteins: characterization of a novel  $\beta$  subunit cDNA. *Biochemistry* 183:350-356.
- Wickman KD, Iniguez-Lluhi JA, Davenport PA, Taussig R, Krapivinsky GB, Linder ME, Gilman AG, Clapham DE (1994) Recombinant G-protein  $\beta\gamma$  subunits activate the muscarinic gated atrial potassium channel. *Nature* 368:255-257.
- Xu M, Hu X-T, Cooper DC, Moratalla R, Graybiel AM, White FJ, Tonegawa S (1994) Elimination of cocaine-induced hyperactivity and dopamine-mediated neurophysiological effects in dopamine D1 receptor mutant mice. *Cell* 16:923-926.