Mutation and Activation of Gαs Similarly Alters Pre- and Postsynaptic Mechanisms Modulating Neurotransmission

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INTRODUCTION

A vital property of neuronal synapses is the ability to alter the efficacy of transmission with changes in usage. This plastic nature of synaptic communication is generally believed to underlie behavioral learning and memory formation (Bhalla and Iyengar 1999). A burst of high-frequency presynaptic activity generally results in a transient increase in synaptic transmission (facilitation, augmentation), and repeated bursts or sustained high-frequency activity can result in a sustained, long-lasting increase in synaptic efficacy (potentiation) at single excitatory synapses (Alberini et al. 1995). The mechanisms that underlie synaptic facilitation and potentiation have been shown to occur presynaptically, postsynaptically, or both, in different synaptic classes (Bao et al. 1997; Sheng and Kim 2002). Convergent genetic, biochemical, and pharmacological studies have revealed conserved biochemical pathways that mediate the use-dependent alterations in transmission properties underlying behavioral change (reviewed in Martin et al. 2000; Roberson et al. 1999; Silva et al. 1998; Xia and Storm 1997). Synaptic plasticity observed in both pre- and postsynaptic compartments is mediated by receptor activation of the stimulatory alpha subunit of the heterotrimeric G protein (hereafter denoted Gαs), which positively regulates the activity of adenyl cyclase (see Fig. 1A) (Abrams et al. 1991; Bourne and Nicoll 1993; Neer 1995; Quan et al. 1991). Activated adenyl cyclase (AC) synthesizes cyclic AMP (cAMP) that is degraded by a cAMP-specific phosphodiesterase (Fig. 1A). The primary target of cAMP regulation is protein kinase A (PKA), which regulates the phosphorylation state of numerous proteins with critical roles in neurotransmission on both sides of the synaptic cleft (Bao et al. 1998; Bhattacharya et al. 1999; Drain et al. 1994; Hirling and Scheller 1996; Hosaka et al. 1999; Raymond et al. 1993; Roberson et al. 1999; J. Wang et al. 1999; L. Y. Wang et al. 1999; Zhou et al. 2002).

Dissection of Gαs and cAMP synaptic signaling has been achieved by genetic and pharmacological methods in different systems, prominently including Aplysia and rodent systems. The role of the cAMP cascade has been probed using agonists of Gαs (Abrams et al. 1991; Artalejo et al. 1990; Beaumont and Zucker 2000; Bevilaqua et al. 1997; Eliot et al. 1994; Gereau and Conn 1994; Mitoma and Koniishi 1999; Morimoto and Koshland 1991; Wayman et al. 1994) as well as direct pharmacological manipulation of cAMP levels (Barad et al. 1998; Brown et al. 2000; Chen and Regehr 1997; Dixon and Atwood 1989; Kameyama et al. 1998; Makhinson et al. 1999; Salin et al. 1996; Wang et al. 1991; Yoshimura and Kato 2000). These studies reveal a role for cAMP in the dynamic regulation of synaptic vesicle fusion probability (Beaumont and Zucker 2000; Chen and Regehr 1997). Further studies also show that postsynaptic mechanisms are regulated by cAMP (Brown et al. 2000; Kameyama et al. 1998; Wang et al. 1991). Thus the temporal regulation of [cAMP] acts to tune synaptic function in response to activity and is a critical regulator of short-term plasticity in both invertebrates and vertebrates.

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FIG. 1. Synaptic manipulation of Gαs pathway with targeted transgenic expression of a constitutively active form of the protein. 

A: cartoon of the Drosophila Gαs pathway. An unidentified G-protein-coupled receptor (GPCR) binds the heterotrimeric G-protein-containing Gαs (dgs loss of function mutants). Activated Gαs binds adenylyl cyclase (AC; rut mutants) to drive synthesis of cAMP, which activates protein kinase A (PKA; DC0 mutants). Signaling is terminated by a cAMP-specific phosphodiesterase (PDE; dnc mutants). The βγ complex independently regulates AC, protein kinase C (PKC), and other targets. 

B: a point mutation engineered at Q215L in a GTP-binding site of Gαs eliminates GTP hydrolysis making it constitutively active (designated dgs*) (Connolly et al. 1996). The GAL4-UAS system allows cell-specific targeted expression of dgs* (Brand and Perrimon 1993). Specific GAL4 drivers mediated expression of UAS-dgs* either ubiquitously, only in the nervous system (GAL4-ELAV), only in the musculature (GAL4-G7), or in an acutely induced pattern driven by heat-shock. 

C: tissue-specific GAL4 lines were used to drive expression of the activated G-protein α subunit either presynaptically (GAL4-ELAV) or postsynaptically (GAL4-G7) at the 3rd instar neuromuscular junction. Targeted expression visualized by crossing GAL4 lines to UAS-GFP.
From the beginning, *Drosophila* has been a prominent system for the study of cAMP-dependent regulation of both behavior and synaptic properties. Numerous associative learning mutants have identified genes responsible for regulation of intracellular concentrations of cAMP [e.g., *dunce*, *rutabaga*; reviewed in (Davis 1996; Dubnau and Tully 1998); see Fig. 1A] and downstream proteins (PKA, CREB responsive element binding [CREB], CREB-binding protein). Electrophysiological analyses at the glutamatergic neuromuscular junction (NMJ) of these mutants have demonstrated altered basal neurotransmission properties and a disruption of short-term synaptic plasticity (Engel and Wu 1996; Griffith et al. 1994; Lee and O’Dowd 2000; Zhong and Wu 1991; reviewed in Davis 1996) attributed to changes in presynaptic efficacy. Recent work using FM1-43 dye labeling (Kurumi and Kidokoro 2000) and single-bouton recordings (Renger et al. 2000) point to a significant defect in synaptic vesicle translocation and release probability when cAMP levels are affected. Work in the embryonic NMJ (Suzuki et al. 2002; Yoshihara et al. 1999, 2000), and in cultured neurons (Alshuaib and Mathew 1998; Berke and Wu 2002; Lee and O’Dowd 2000; Yao and Wu 2001; Yao et al. 2000; Zhao and Wu 1997) verify a significant presynaptic contribution of cAMP to regulated vesicle release and facilitation. Other studies in *dnc* and *rut* suggest that the cAMP-dependent potentiation of the larval NMJ is due to increased activity in calcium-activated K⁺ channels, which bind PKA (slopoke) (Wang et al. 1999; Zhou et al. 2002), and other channel subunits (ether à go-go, hyperkineletic) (Engel and Wu 1998). L-type calcium channels have also been implicated (Bhattacharya et al. 1999). More recent studies have shown that postsynaptic expression of PKA and CREB-binding protein also affect synaptic function (Davis et al. 1998; Marek et al. 2000). Similarly, increases in cAMP activity in *dnc* mutants increases the open probability of voltage-gated potassium channels postsynaptically (Delgado et al. 1991, 1992, 1994; Zhong and Wu 1993) likely due to rapid reactivation of shaker K⁺ channels (Drain et al. 1994).

These classical “severe” learning mutants result in an ~60% loss of performance in behavioral tests for learning (Tully and Quinn 1985). However, the most dramatic block of associative learning in *Drosophila* has been achieved by the targeted expression of a constitutively active G-protein alpha subunit (*dgs*; see Fig. 1B) in mushroom bodies, centers for olfactory learning and memory (Connolly et al. 1996). Surprisingly, constitutive activation of Gos mimics ablation of the mushroom bodies to eliminate detectable olfactory learning (de Belle and Heisenberg 1994). This learning blockade is much more severe than that of any single cAMP cascade mutant (e.g., *dnc, rut, DCO*), suggesting that Gos is regulating multiple downstream targets in parallel to control the synaptic modulation underlying associative learning. The complete learning blockade also suggests the possibility that Gos may be regulating synaptic mechanisms independently of an exclusive cAMP cascade. The goal of the present study was to use the *dgs* gain-of-function transgenic mutants, coupled with Gos loss-of-function mutants, to comprehensively dissect the role of Gos in the regulation of synaptic transmission and plasticity. At the larval NMJ, *dgs* was expressed in neuronal (presynaptic) and/or muscle (postsynaptic) cells to examine the consequence on synaptic efficacy and plasticity (see Fig. 1C). Likewise, a viable hypomorphic mutation of *dgs*, which has <20% of normal cAMP production (Wolfgang et al. 2001), was used to examine the consequence of loss of Gos activity. To our enormous surprise, expression of *dgs* (either pre- or postsynaptically) and *dgs* mutants similarly displayed increased basa transmission, decreased short-term facilitation, and abolished posttetanic potentiation. A further surprise was that both loss and gain of Gos activity, on either side of the synaptic cleft, nearly eliminated detectable GluR1A subunits in the postsynaptic glutamate receptor field. In contrast, all three genotypes showed increased FM1-43 dye cycling in the presynaptic boutons, indicating a Gos-dependent enhancement of the synaptic vesicle cycle. These results demonstrate that the levels of Gos must be tightly regulated to maintain appropriate basal synaptic efficacy and to permit manifestation of functional plasticity. We conclude that both sides of the NMJ synapse respond to alteration in Gos activity and that Gos regulation involves transsynaptic communication controlling both the rate of the presynaptic vesicle cycle and the composition of the postsynaptic glutamate receptor field.

**METHODS**

*Drosophila* stocks and animal preparation

Loss of function mutants of *Drosophila* Gos (*dgs*) examined included *dgs*<sup>R60</sup>, a *dgs* null allele that reduces [cAMP] to 20–25% of wild type and is lethal at the late embryo early first instar interface (Wolfgang et al. 2001), and *dgs*<sup>R19</sup>, a *dgs* partial loss-of-function hypomorph with 40% [cAMP] relative to wild type and reduced viability (Wolfgang et al. 2001). Both mutants were maintained over TM3-GFP and CyO-GFP balancer chromosomes (CyO {ubiquitin-GFP} and TM3 {actin5c-GFP} balancers were gifts of C. Thummel, University of Utah), and genotyping of homozygous and transheterozygous *dgs* mutant larva was done based on absence of GFP fluorescence in the CNS or somatic tissues, respectively. For all transgenic studies, the UAS line used was w; UAS-*dgs*<sup>G16-8</sup>/TM3{actin5c-GFP} (Connolly et al. 1996). UAS-*dgs*<sup>G16-8</sup> encodes a constitutively active Gos transgene that is defective in GTP hydrolysis, due to the point mutation Q215L (referred to as *dgs*; see Fig. 1B) supplied as a gift from J. Connolly (Cold Spring Harbor Laboratory, NY). GAL4 drivers lines used included 1) GAL4-ELAV: w; P[w+; w;+=GawB];elav<sup>C153</sup> (Bloomington Stock No. 458), for pan-neural expression (Lin and Goodman 1994), 2) GAL4-G7: created as w;+/w; G7:G7; W+; PGAWB p-element (Brand and Perrimon 1993), for pan-muscle expression, and 3) HS-GAL4: heat-shock inducible hs70 GAL4 construct (Grotewiel et al. 1998) for acute, ubiquitous expression studies. Expression patterns for the presynaptic GAL4-ELAV and postsynaptic GAL4-G7 driving a UAS-τ-GFP reporter construct (courtesy of A. Brand, University of Cambridge, UK) at the larval NMJ is shown in Fig. 1C. Inducible expression via the HS-GAL4 construct showed strong global expression of UAS-τ-GFP at 4–6 h after 1-h heat shock at 35 °C, although leaky expression was observed prior to heat shock in the salivary glands of the larva, even if the animals were raised at 18°C. Control *Drosophila* strains included the wild-type Oregon-R (OR) and heterozygous GAL4/+ or UAS/+ lines (+ = OR) for transgenic studies.

Experimental lines assayed in this study include 1) *dgs*<sup>B19</sup>/B19 (homozygous; weak hypomorph loss-of-function), 2) *dgs*<sup>B19</sup>/B19 (transheterozygous; strong hypomorph loss-of-function), 3) w, GAL4-ELAV/+;Y; UAS-*dgs*<sup>+/+</sup> (chronic presynaptic gain-of-function), 4) w; GAL4-G7/+; UAS-*dgs*<sup>+/+</sup> (chronic postsynaptic gain-of-function), and 5) w; HS-GAL4/+;UAS-*dgs*<sup>+/+</sup> (acute ubiquitous gain-of-function; temporally inducible). Breeding flies were maintained at 25°C on apple juice agar plates, and allowed to lay eggs for 24-h periods. Larvae from the staged lays were genotyped based on absence of GFP fluorescence in the CNS or somatic tissues, respectively.
replaced on fresh apple juice agar plates at 25°C. All experiments were conducted on wandering third instar (L3) larvae. For dissection, a larva was placed on silicone-elastomer (Sylgard)-coated coverslips under recording saline (see following text) and tacked to the coverslip using Histoacryl glue (Braun Surgical, Germany). The larva was then cut longitudinally along the dorsal surface using fine iris scissors. The gut was removed to expose the ventral nerve cord (VNC), peripheral nervous system, and somatic musculature. The lateral body walls were then tacked to the coverslip using Histoacryl glue, the trachea was removed, and the segmental nerves were cut at their exit point from the VNC. The larva was then used immediately (immunocytochemistry, electrophysiology, etc.), unless otherwise noted.

**Immunohistology**

Dissected larvae were fixed for 30–45 min in 4% paraformaldehyde (αCSP), or 10–30 min in Bouin’s Fixative (αDGluRIIA, αDGluRII, αHRP) (Budnik et al. 1990; Featherstone et al. 2002). Preparations were washed in 0.1% Triton X-100 in phosphate-buffered saline [PBS-TX; 0.02M phosphate buffer, 0.1M NaCl (pH 7)] plus bovine serum albumin (0.5%; Sigma Chemicals, St. Louis) several times over a period of 1 h. Preparations were then incubated overnight at 4°C with mouse anti-cysteine string protein (αCSP, 1:50; gift from Dr. W. Zinsmaier, University of Arizona), mouse anti–DGluRIIA (α-DGlurIIA; Petersen et al. 1997), 1:50; obtained from the Iowa Hybridoma Band, University of Iowa), and/or rabbit anti–DGluRII (α-DGluRII; Petersen et al. 1997), 1:50; obtained from the Iowa Hybridoma Band, University of Iowa), and/or rabbit anti-DGluRIIA (α-DGlurIIA; Petersen et al. 1997), 1:50; obtained from the Iowa Hybridoma Band, University of Iowa), and/or rabbit anti-DGluRII (α-DGluRII; Petersen et al. 1997), 1:50; obtained from the Iowa Hybridoma Band, University of Iowa), and/or rabbit anti-DGluRIII (AS5, 1:2000; gift from Dr. C. Schuster, Max-Planck Ge sellschaft, Tubingen, Germany). Preparations were then washed in PBS-TX with 2% horse serum (Sigma) and incubated with appropriate secondary antibodies. Mouse anti-horseradish peroxidase (αHRP, 1:500), conjugated to Texas Red-labeled anti-mouse secondary was added with secondary antibodies to counterstain preparations as indicated. αCSP localization was visualized using a biotinylated anti-mouse secondary followed by development with peroxidase substrates (Vectastain ABC Elite; Burlingame, CA) (Beumer et al. 1999). αDGluRIIA and αDGluRII were visualized with Alexa 488 (green) fluorescently labeled anti-mouse or –rabbit secondary antibodies, respectively (Molecular Probes, Eugene, OR). In all cases, control and experimental preparations were processed simultaneously in the same solutions.

Confocal images were taken with a Biorad 2000 microscope, running Lasersharp 2000 software, and presented with Adobe Photoshop 6.0 software. Quantification of antibody staining was performed using National Institutes of Health Image. Intensity of staining was determined by the following protocol: single color images were converted to 8-bit grayscale, filtered using “despeckle” noise reduction, and mean signal within the area of the NMJ measured after subtracting background from each image. Area of the NMJ was determined by setting threshold above background, then measuring area of signal above threshold. All NMJ staining was normalized by comparison to OR within each experimental run.

**Electrophysiology: two-electrode voltage clamp**

Two-electrode voltage-clamp recordings were performed on muscle 6, in anterior abdominal segments A2–4 of wandering L3 larva, according to previously published methods (Rohrbough et al. 1999, 2000). Briefly, the dissected larva was placed in a Plexiglas recording chamber and viewed in transmitted light using a compound microscope (Zeiss) fitted with differential interference contrast (DIC) (Nomarski) optics and a ×40 water-immersion lens. Recordings were made at 18°C with sharp glass electrodes pulled from fiber-filled borosilicate glass (World precision Instruments, Sarasota FL) using an electrode puller (P-97 or P2000, Sutter Instruments, Novato CA) to resistances of 10–30 MΩ and filled with a solution of 3:1 mixture of 3M KAc/KCl. Stimulation of the motor nerve was achieved by brief (0.5–1 ms) positive current stimulation of a loop of motor nerve in a suction electrode, using a Grass S88 Stimulator (Grass Instruments; Warwick, RI), at frequencies of 0.5–20 Hz. Stimulation threshold was determined by varying stimulation intensity and duration until ~50% of episodes did not result in transmission failure [excitatory junction current (EJC) amplitude >1 nA). Stimulation intensity (voltage and/or duration) was then increased by roughly 20% or until no episodes resulted in transmission failure, and this stimulation intensity was used for the remainder of the experiment. Suction electrodes were made using pulled fiber filled borosilicate glass heated polished to final internal diameter of 10–12 μm and filled with bath saline. Recording bath solution was a modified standard saline, and consisted of (in mM) 128 NaCl, 2 KCl, 4 MgCl, 70 sucrose, and 5 HEPES. CaCl2 was added to pH 7.2 solutions, to bring final [Ca2+] to 0.2–1.8 mM.

Two-electrode voltage-clamp recordings were recorded using an Axoclamp 2B amplifier in two-electrode voltage-clamp (TEVC) mode (~60 mV; Axon Instruments; Foster City, CA). The signal was filtered at 0.5 kHz on-line, converted to a digital signal using a Digidata 1200 A/D interface (Axon Instruments), and stored on computer (Gateway P5–166 MHz) for later analysis. All analysis was done off-line, using pCLAMP6 program suite (Axon Instruments). Miniature EJC (mEJC) frequency and amplitude were assayed in 0.2 Ca2+ modified standard saline, plus 3 μM tetrodotoxin (TTX; Sigma Chemicals), to block endogenous activity. Current traces were filtered at 0.2 kHz, and analyzed using Mini Analysis software 5.5.1 (Synaptosoft; Leonia, NJ). All mEJC experiments recorded spontaneous activity from at least five larvae per genotype, with >125 responses per larva.

**Glutamate pressure ejection**

Glutamate receptor function was assayed directly using directed pressure ejection application of l-glutamate onto single NMJ boutons visualized with DIC optics. Brief (200 ms) pulses of 1 mM l-glutamate was delivered using an electronically controlled valve system at 4–6 psi through a pipette of 10–12 μm diam, placed adjacent to the nerve bifurcation point between muscle 6/7. Responses were monitored in TEVC recording mode for 5 s after glutamate application, filtered at 0.2 kHz on-line, and recorded on computer using pClamp software.

**FM1-43 dye imaging**

Wandering third instar larvae were dissected in 0 Ca2+ saline as described in the preceding text. The preparation was incubated in normal (1.8 mM Ca2+) saline containing 10 μM cyclosporin A for 20 min, followed by 5 min in high-concentration (90 mM) K+ saline containing 10 μM FM1-43 dye (Molecular Probes, Eugene, OR). This protocol has been reported previously to load both the endo/exo cycling and the reserve synaptic vesicle pools (Kuromi and Kidokoro 2000). The preparation was then washed for 15 min in 0 Ca2+ saline to remove nonspecific dye staining. Identified NMJs were imaged using a Biorad 2000 confocal microscope, using Lasersharp 2000 software. NMJs were stimulated by bath exchange of high-[K+] (90 mM) 0.2 mM Ca2+ saline for 5 min, followed by 15 min wash in 0 Ca2+ saline. Single NMJs were imaged using identical acquisition settings before and after dye unloading. Images were analyzed using National Institutes of Health Image. Five to 10 boutons from NMJ 6/7 were outlined individually, and mean intensity compared between the loaded and unloaded conditions after subtracting background fluorescence.

**Statistics**

All between-group comparisons were performed using Welch-corrected t-test with Graphpad 3.0a for Macintosh (Graphpad Software, San Diego, CA), unless otherwise noted. Ca2+ dependency was fit using a variable slope sigmoidal dose response curve, and the resulting slopes then were compared with OR with Welch-corrected t-test.
using Graphpad 3.0a. Significant differences from OR are indicated as follows: *P < 0.05, **P < 0.01, ***P < 0.001.

RESULTS

Gαs is required for viability and regulates coordinated behavior

Homologous null Gαs mutants (dgsR60) were 100% embryonic lethal, whereas the weak hypomorphic allele dgsB19 were viable although behaviorally sluggish (Wolfgang et al. 2001). The most severe loss of function mutation permitting viability was the heteroallelic combination dgsR60/dgsB19. In this genotype, lethality was commonly observed by the second instar, and viable animals in the third instar were extremely sluggish and responded poorly to nose-touch. In addition, dgsR60/dgsB19 larvae developed slowly and maturation to the wandering third instar stage took 10 days on average compared with 5 days in controls.

Transgenic animals with UAS-dgs* (constitutively active Gαs) driven by the GAL4-e22c promoter (ubiquitous expression from early stages of embryonic development) (Brand and Perrimon 1993) were developmentally arrested in the early postembryonic first instar stage, were extremely sluggish, and died at ~5 days after egg laying (AEL). Targeted expression of dgs* only in the nervous system under control of the GAL4-ELAV promoter (pan-neuronal expression from embryonic stage 12) permitted full viability but delayed development rate with puparium formation taking on average 10 days AEL (compared with 5 days AEL in controls). These larvae were slightly behaviorally sluggish but responded normally to nose touch and grew to normal size at the time of puparium formation. Muscle specific expression of dgs* under control of the GAL4-G7 promoter (expression in all muscles beginning in 2nd instar) resulted in much more markedly sluggish behavior, and some lethality was observed starting at the early third instar stage. Viable animals were also delayed in maturation taking on average 8–9 days to pupariation. These larvae commonly responded to nose touch by an aberrant convulsion and often showed abnormal, spastic contraction of mouthhooks and anterior tip of the body. Both neuronal and muscle transgenic dgs*-expressing lines were viable as adults and showed normal gross morphology and fertility. Larvae expressing dgs* under the control of a heat-shock inducible GAL4 (HS-GAL4) were viable at 18°C but conditionally lethal within 12 h at 25°C after 1-h heat shock at 35°C.

We conclude that Gαs is required for viability in Drosophila with null mutants dying during late embryogenesis consistent with previous studies (Wolfgang et al. 2001). Drosophila also cannot tolerate global activation of Gαs in all tissues, dying during postembryonic development, and on global induction during later larval developmental stages dying in <12 h. These results show that there is a continued requirement for tight control of Gαs activity. Drosophila can survive constitutive activation of Gαs in all neurons or all musculature with clearly compromised coordinated behavior and response to tactile stimulation. These findings contrast with previous analyses in Caenorhabditis elegans, in which constitutive activation of Gαs in the nervous system causes widespread neuronal cell death and early lethality (Berger et al. 1998; Korswagen et al. 1997, 1998).

NMJ structure normal in Gαs mutants

Given the dramatic effects of both gain- and loss-of-function Gαs mutants on viability and coordinated behavior, we first assayed possibly phenotypes in the anatomical development of the neuromusculature. In all larval viable mutants, the CNS appeared to be of normal size and structure and the muscles of normal size and patterning compared with size-matched controls (data not shown). Therefore more-detailed anatomical analyses were conducted on the NMJ. NMJs were labeled with CSP antibody, which recognizes a synaptic vesicle associated protein, to assay synaptic architecture and the number of synaptic boutons. Specifically, NMJs at muscle 6/7 of anterior segment A3 were quantitatively assayed for synaptic branching and number of boutons (Beumer et al. 1999). This NMJ is the subject for all further analyses given in the following text.

The morphology of the 6/7 NMJ in both the pre- and postsynaptic dgs*-expressing lines were similar to those seen in wild-type controls. In both OR and lines with altered Gαs activity, there were ~130 boutons shared between muscle 6/7 (in mean number of boutons ± SE: OR, 129.4 ± 7.2, n = 9; GAL4-ELAV/dgs*, 130.4 ± 6.2, n = 12; GAL4-G7/dgs*, 139.1 ± 15.3, n = 9; dgsR60B19, 113.7 ± 11.3, n = 7). Interestingly, the absence of NMJ structural defects differs from previous reports showing a NMJ structural overgrowth in cAMP metabolic mutants. Specifically, dnc (cAMP phosphodiesterase) mutants have an increase in arborization at the NMJ (Zhong et al. 1992), while rut (adenyl cyclase) mutants have a decrease in the number of synaptic varicosities (Cheung et al. 1999). A hypomorphic mutation in dCBP results in a slight (25%) increase in bouton number (Marek et al. 2000). The difference here shows that Gαs mutants are not comparable to single cAMP metabolic mutants in respect to controlling the structural differentiation of the NMJ. The absence of NMJ growth changes in both loss and gain Gαs mutants means that we can consider any alterations in synaptic function independently of the compounding variable of altered synaptic structure.

Loss and gain of Gαs activity both increase basal synaptic transmission

The first interest was to assay consequences of both removing and adding Gαs activity to the pre- and postsynaptic cells to fundamental aspects of neurotransmission. Six primary genotypes were functionally assayed: moderate hypomorphic loss of function (dgsB19/dgsB19; hereafter referred to as dgsB19), severe hypomorphic loss of function (dgsR60/null/dgsB19), constitutive postsynaptic activation (G7/dgs*), constitutive presynaptic activation (ELAV/dgs*), induced global activation (heat-shock driven dgs*), and control [including wild-type (OR) and heterozygous chromosomes]. The UAS-dgs*/+, G7, GAL4/+; and ELAV/+; genotypes did not vary significantly in any parameter from the OR control, and so for simplicity, these data are not listed separately in the figures.

Basal-evoked synaptic transmission was assayed by stimulating the segmental nerve at 0.5 Hz in low (0.2 mM) Ca2+ bath solution and recording synaptic currents in the voltage-clamped muscle (Fig. 2). Evoked responses at this stimulation frequency were reliable, nonfatigable, and consistent over many minutes of stimulation in all genotypes. All control lines
showed EJCs of similar amplitude: OR, 7.12 ± 1.44 (SD) nA; GAL4-ELAV/+/, 8.63 ± 4.71 nA; GAL4-G7/+, 7.13 ± 4.27 nA; UAS-dgs*/+, 8.45 ± 4.31 nA; HS-GAL4/dgs* without heat shock, 12.60 ± 7.73 (see also Fig. 2A). Presynaptic expression of dgs* resulted in a highly significant increase in EJC amplitude (GAL4-ELAV/UAS dgs*, 21.73 ± 13.81 nA, \( P = 0.0058 \); Fig. 2A). This degree of enhanced transmission amplitude is similar to that reported for dnc null (\( dnc^{M1} \)) mutants (Zhong and Wu 1991), suggesting that the phenotype may arise entirely from elevated presynaptic cAMP. Surprisingly, postsynaptic expression of dgs* resulted in an even more striking increase in EJC amplitude (GAL4-ELAV/UAS dgs*, 21.73 ± 13.81 nA, \( P = 0.0058 \); Fig. 2A). This result shows that Gos activity in the postsynaptic cell also plays a central role in determining basal neurotransmission level at this Ca\(^{2+}\) concentration. Also surprisingly, EJC transmission amplitude in dgs hypomorph larvae was also significantly increased (\( dgs^{R60/B19} \), 13.37 ± 8.11 nA, \( P = 0.011 \)), a very significant enhancement compared with controls. This result shows that both increasing and decreasing Gos activity results in increased basal synaptic function at low bath [Ca\(^{2+}\)]. Acute induction of Gos with HS-GAL4/dgs* was performed to determine if the effect of dgs* on synaptic transmission is acute or a consequence of aberrant development of synaptic function. Acute expression of dgs* did not cause a significant alteration of the basal EJC amplitude until 6 h after heat shock (9.72 ± 6.35 nA, \( P = 0.366 \) after 1 h; 8.30 ± 4.33 nA, \( P = 0.479 \) after 4 h; 5.16 ± 0.96 nA, \( P = 0.003 \) after 6 h). The delayed decrease in transmission is likely attributable to nonspecific loss in transmission due to excitotoxic death of the animal (see preceding text). Rise and decay times were quantitatively analyzed but were not significantly different between any of the control and experimental genotypes (data not shown). These results show that both loss and gain of Gos pre- or postsynaptically similarly elevate basal transmission amplitudes in low [Ca\(^{2+}\)] conditions.

One possibility for increased evoked transmission is an altered Ca\(^{2+}\) cooperativity of neurotransmitter release, a char-

**FIG. 2.** Gain- or loss-of-function of Gos mutants both increase basal neurotransmission amplitude and reduce the calcium dependence of neurotransmission. A: evoked synaptic current amplitudes in response to nerve stimulation at 0.5 Hz in 0.2 mM Ca\(^{2+}\) saline. Genotypes assayed include G7/dgs* (postsynaptic gain-of-function), ELAV/dgs* (presynaptic gain-of-function), Oregon-R (OR, wild-type), \( dgs^{B19/B19} \), \( dgs^{B19/B19} \) (heterozygous loss-of-function; \( \sim 40\% \) of Gos activity [Wolfgang et al. 2001]), and \( dgs^{R60/B19} \) [transheterozygote loss-of-function hypomorph; \(< 20\% \) of Gos activity; Wolfgang et al. 2001]). GAL4/+ and UAS-dgs*/+ controls were not significantly different from OR (data not shown). Inset: example excitatory junction current (EJC) traces and histogram shows mean ± SE. Significance from OR is indicated (*\( P < 0.05 \); **\( P < 0.01 \); ***\( P < 0.001 \)). B: calcium dependence is reduced when Gos activity is misregulated. Each datum is the average of 20–40 consecutive traces evoked at 0.5 Hz for the calcium range 0.15–1.8 mM. Plot shows averaged results for ≥5 larvae per genotype for each [Ca\(^{2+}\)]. EJC amplitude as a function of [Ca\(^{2+}\)] was modeled as a sigmoidal dose-response curve with variable slope, and the resulting slopes (inset) were compared.
characteristic defect of other *Drosophila* learning mutant phenotypes. Therefore calcium dependence of transmission was assayed from 0.15 to 1.8 mM \([\text{Ca}^{2+}]_{\text{bath}}\). In wild-type animals, calcium dependence has an exponential relationship in this range, with a slope \(-3.5\) (Fig. 2B), similar to values reported in other systems and close to the theoretical relationship of 4 (Heidelberger et al. 1994; Zhong and Wu 1991). All control lines showed a calcium dependence of evoked transmission indistinguishable from wild type; all calcium dependencies for controls ranged from 3.2 to 3.5. In contrast, calcium dependence for both *dgs*-expressing lines were reduced compared to wild type, all calcium dependencies for *dgs* lines showed a calcium dependence of evoked transmission (Heidelberger et al. 1994; Zhong and Wu 1991). All control genotypes showed a proportional increase in EJC transmission amplitude with the increasing frequency of the stimulus train, indicating increased postsynaptic plasticity due to high-frequency presynaptic activity. Such a short-term plasticity defect has been shown previously in a number of other *Drosophila* learning mutants at the NMJ, both where Ca\(^{2+}\) dependence and structure is similar to wild type (Rohrbough et al. 1999) and where NMJ structure and Ca\(^{2+}\) dependence are altered in parallel (Rohrbough et al. 2000; Zhong and Wu 1991). As a first assay, short-term facilitation (STF) was assayed in low (0.2 mM) \([\text{Ca}^{2+}]_{\text{bath}}\) by stimulation at frequencies from 0.5 to 20 Hz (Fig. 3). The last 10 responses of each stimulus train were averaged, and the amplitude normalized to the starting EJC amplitude. Trains of 20 stimuli were delivered at 0.5–20 Hz in 0.2 mM Ca\(^{2+}\) saline with 30-s rest between each train. The last 10 responses of each stimulus train were averaged, and the amplitude normalized to the starting EJC amplitude with the increasing frequency of the stimulus train, facilitating more than threefold at 20 Hz (OR, 3.13 \(\pm\) 0.31-fold increase, \(n = 18\); Fig. 3, A and B). In contrast, both pre- and postsynaptic expression of *dgs* caused a decrease in the proportional increase EJC transmission amplitude with increasing stimulus frequency. The loss of STF in the presence of constitutively active Gs was significant at frequencies >10 Hz (at 20 Hz: ELAV/dgs*, 2.09 \(\pm\) 0.39, \(n = 10\), \(P = 0.034\); G7/dgs*,

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**Gas misregulation reduces expression of short-term plasticity**

An increase in basal transmission could be indicative of a prefacilitated synapse, which would occlude further increases in synaptic efficacy due to high-frequency presynaptic activity. Such a short-term plasticity defect has been shown previously in a number of other *Drosophila* learning mutants at the NMJ, both where Ca\(^{2+}\) dependence and structure is similar to wild type (Rohrbough et al. 1999) and where NMJ structure and Ca\(^{2+}\) dependence are altered in parallel (Rohrbough et al. 2000; Zhong and Wu 1991). As a first assay, short-term facilitation (STF) was assayed in low (0.2 mM) \([\text{Ca}^{2+}]_{\text{bath}}\) by stimulation at frequencies from 0.5 to 20 Hz (Fig. 3). The last 10 responses of each stimulus train were averaged, and the amplitude normalized to the starting EJC amplitude.

All control genotypes showed a proportional increase in EJC amplitude with the increasing frequency of the stimulus train, facilitating more than threefold at 20 Hz (OR, 3.13 \(\pm\) 0.31-fold increase, \(n = 18\); Fig. 3, A and B). In contrast, both pre- and postsynaptic expression of *dgs* showed no change in either basal EJC amplitude or the calcium dependence of transmission over this range. However, the more severe loss of function condition, *dgs*s, resulted in a dramatically decreased calcium dependence to 1.86 (Fig. 2B). All genotypes converged at higher \([\text{Ca}^{2+}]_{\text{bath}}\) concentrations, showing that the primary transmission enhancement is manifest only at lower Ca\(^{2+}\) concentrations. These results support the *dnc* mutant phenotype, which show that an increase in \([\text{cAMP}]\) correlates with a decrease in the Ca\(^{2+}\) dependence of neurotransmitter release (Zhong and Wu 1991). *dnc* mutants show an increase in the variability of transmission at the single bouton level, specifically an increase in the number of failures (Renger et al. 2000). Therefore the coefficient of variation of EJCs (CV = SD/mean amplitude) at the lowest \([\text{Ca}^{2+}]_{\text{bath}}\) (0.15 mM, see Fig. 2B) condition were analyzed post hoc to measure the fidelity of transmission. This analysis revealed that presynaptic overexpression of *dgs* and hypomorph mutation of *dgs* decrease the CV significantly, indicating increased fidelity of evoked transmission, whereas postsynaptic overexpression of *dgs* does not alter the CV relative to wild type (OR (CV \(\pm\) SE), 0.46 \(\pm\) 0.03; ELAV/dgs*, 0.33 \(\pm\) 0.09, \(P = 0.003\); G7/dgs*, 0.0.40 \(\pm\) 0.05; \(P = 0.317\); *dgs* S60/B19, 0.28 \(\pm\) 0.05, \(P = 0.013\). This comparison between increased CAMP due to *dnc*, versus activation of Gs, points to an additional signaling role of the Gs cascade.
1.99 ± 0.28, n = 7, P = 0.043; Fig. 3). The level of activity-dependent facilitation in dgsR60/B19 was also significantly reduced, to a level similar to that in the dgs* transgenic animals (dgsR60/B19, 1.96 ± 0.37, n = 8, P = 0.044). When dgs* was acutely driven with heat shock-inducible GAL4, a significant decrease in facilitation was also seen at 20 Hz even without induction (HS-GAL4/dgs* without heat shock, 2.03 ± 0.17, n = 6, P = 0.005). This decrease in activity-dependent facilitation is consistent with the pre- and postsynaptic effect of dgs* in the preceding text and is likely due to a small constitutive background expression of dgs* (“leakage”) of the HS-GAL4 construct.

These results show that a decrease in STF is manifest at low [Ca^{2+}β], when Gos is constitutively activated in both pre- and postsynaptic cells. The presynaptic effect is consistent with the previously characterized dnc phenotype, showing that increased [cAMP] correlates with decreased STF (Zhong and Wu 1991). Surprisingly, loss of Gos activity also results in a loss of STF, but this also supports the previous finding of reduced STF in rut with decreased [cAMP] (Zhong and Wu 1991). These results together support the hypothesis that dynamic changes in [cAMP] are important for the manifestation of STF and that bidirectional misregulation of cAMP results in a similar loss of STF. A new finding here is that Gos misregulation in the postsynaptic cell also affects proper manifestation of STF.

**Posttetanic potentiation is eliminated when Gos function is altered**

During sustained moderate frequency stimulation (5 Hz, >30 s) in low [Ca^{2+}β], the *Drosophila* NMJ shows a dramatic increase in transmission, followed by a sustained increase in synaptic efficacy at basal stimulation frequency for the length of the recording (Delgado et al. 1992; Rohrbough et al. 1999, 2000; Zhong and Wu 1991). There are two components of the response: a period of augmentation during tetanus, resembling prolonged facilitation, and a sustained response after tetanus, representing posttetanic potentiation (PTP). Behavioral learning mutants, including cAMP metabolic mutants such as dnc and rut, show a decrease in augmentation and a loss of PTP (Zhong and Wu 1991). Maintained synaptic potentiation is widely believed to be a cellular correlate of learning. Both augmentation and potentiation were assayed at the NMJ during and after a 60-s tetanic stimulation at 5 Hz, in low (0.2 mM) [Ca^{2+}β] (Fig. 4).

In both wild-type and genetic control lines, augmentation to more than double the basal EJC amplitude was observed during the tetanic stimulus (OR, 2.21 ± 0.22 times basal amplitude, n = 9; Fig. 4, and data not shown). When Gos was activated presynaptically, the level of augmentation was very significantly decreased (ELAV/dgs*, 1.37 ± 0.18, n = 8, P = 0.011), similar to the previously reported dnc phenotype (Zhong and Wu 1991). However, when Gos was activated postsynaptically, augmentation amplitude was no longer significantly different from controls (G7/dgs*, 1.64 ± 0.21, n = 10, P = 0.079). When a low level of dgs* is constitutively expressed both pre- and postsynaptically, augmentation was also significantly reduced (HS-GAL4/dgs*, 1.56 ± 0.17, n = 7, P = 0.044). Conversely, when Gos activity was reduced in dgs mutants, augmentation was also very significantly decreased (dgsR60/B19, 1.35 ± 0.13, n = 7, P = 0.008), similar to the results previously reported in rut mutants (Zhong and Wu 1991). These results show that both increase and decrease of Gos activity in the presynaptic terminal depress the expression of augmentation.

In all loss and gain of function Gos mutants assayed, maintained posttetanic potential was essentially eliminated compared with robust potentiation in all controls (Fig. 4). At 5 min after induction of the stimulus train, both wild-type and genetic controls show maintained potentiation over basal amplitude (OR, 1.62 ± 0.17-fold increase, n = 9; Fig. 4, and data not shown). In both the dgs*-expressing transgenic lines, pre- and postsynaptic, potentiation was essentially eliminated with a loss of PTP caused by presynaptic Gos activation (ELAV/dgs*, 1.00 ± 0.05, n = 9, P = 0.007) and a significant reduction in PTP caused by postsynaptic Gos activation (G7/dgs*, 1.16 ± 0.06, n = 9, P = 0.020). In support of these observations, “leaky” expression of dgs* on both sides of the synapse (HS-GAL4/dgs*, no heat shock) also caused a very significant loss of PTP (1.05 ± 0.07, n = 7, P = 0.011). This loss of PTP is similar to results reported for dnc mutants.

![Figure 4](http://jn.physiology.org/content/89/3/2627/F4)

**FIG. 4.** Maintained augmentation and posttetanic potentiation (PTP) are both reduced by altered Gos activity. A: tetanic stimulation (5 Hz) was delivered for 60 s, preceded and followed by basal stimulation at 0.5 Hz. EJC amplitudes are plotted normalized to the mean pretetanus amplitude. Amplitudes of 10 consecutive EJCs were averaged for each point shown. Error bars are SE. GAL4/+ and UAS-dgs/+ controls were not significantly different from OR (data not shown). B: altered Gos function resulted in significant loss of both augmentation and synaptic potentiation. Mean fold increase in EJC amplitude relative to 0.5 Hz (baseline) are reported as mean ± SE. Augmentation was defined as the average fold increase of EJC amplitude in the last 10 s of tetanus (5 data points/genotype). Potentiation was defined as the average fold increase in EJC amplitude 5 min after the initiation of tetanus (4 data points/genotype). The significance from OR is indicated (*P < 0.05; **P < 0.01).
NMJ can be eliminated by misregulation of Gαs activity in Drosophila (Zhong and Wu 1991). Again conversely, loss of Gαs function in dgs mutants also recapitulated the rut phenotype (Zhong and Wu 1991), with a significant loss of PTP (dgsR60B19, 1.12 ± 0.05, n = 7, P = 0.021).

These results clearly show that posttetanic potentiation at the Drosophila NMJ can be eliminated by misregulation of Gαs, which occurs through both pre- and postsynaptic mechanisms. Both loss and gain of Gαs activity results in loss of potentiation, indicating that tight regulation of [cAMP] is required for potentiation properties of Gαs/H9251 fusion events.

The frequency of spontaneous fusion events was not significantly altered when controls and any of the transgenic lines which increase Gαs activity (OR, 1.60 ± 0.20 Hz, n = 12; ELAV/dgs*, 1.32 ± 0.16 Hz, n = 19, P = 0.428; G7/dgs*, 1.70 ± 0.20 Hz, n = 5, P = 0.814) or mutants with reduced Gαs activity (dgsR60B19, 1.79 ± 0.26 Hz, n = 7, P = 0.636; see Fig. 5A). This result indicates that the basal fusion properties of synaptic vesicles at the NMJ are not significantly altered by altered Gαs activity in contrast to work on the embryonic NMJ showing that effectors or antagonists of the cAMP cascade can regulate vesicle fusion properties (Yoshihara et al. 2000; Zhang et al. 1999). The mean amplitude of mEJC, acquired in the same experiment, are also roughly similar in wild type and larvae with increased presynaptic Gαs function (OR, 528 ± 14 pA, n = 12; ELAV/dgs*, 554 ± 13 pA, n = 18, P = 0.189), although there is a significant increase in the quantal size when Grs is activated postsynaptically (G7/dgs*, 587 ± 16 pA, n = 5, P = 0.022; see Fig. 5B). Conversely, when Gαs function is reduced globally, the mean mEJC amplitude is slightly but significantly reduced (dgsR60B19, 528 ± 14 pA, n = 12).

**FIG. 5.** Analysis of pre- and postsynaptic function fail to provide an explanation for the elevated synaptic transmission observed in Gαs mutants. Miniature EJC (mEJC) frequency and amplitude were assayed in 0.2 mM Ca2⁺ saline with the addition of 3 μM TTX used to block evoked activity. Present are the combined results from ≥5 larvae per genotype with >125 responses per larva (>600 mEJC events per genotype). A: mEJC frequency was not significantly affected by altered Gαs activity. B: mEJC amplitude was slightly but significantly increased by postsynaptic dgs* expression (*P < 0.05) and decreased in dgsR60B19/dgsB19 mutants. C: mEJC amplitude distribution was not detectably affected in dgs mutants as shown in a cumulative histogram of mEJC amplitudes. The difference in distributions was not significant by Kolmogorov-Smirnov test. D: 200-ms pulses of 1 mM t-glutamic acid were applied to the neuromuscular junction (NMJ) and the glutamate-gated current was monitored (see METHODS). Pressure ejection application of glutamate onto the postsynaptic field resulted in no significant differences between genotypes, although a similar trend was observed compared with the mEJC analysis. Shown is peak response amplitude ± SE for ≥13 animals per genotype.
480 ± 11 pA, n = 7, P = 0.014). It is unclear whether these slight (~10%) changes in mEJC amplitude are functionally relevant, but such changes would not appear to account for the 400% increase in evoked transmission with postsynaptic activation of Gs (7 nA in OR vs. 28 nA in G7/dgs*; Fig. 2A). Cumulative histogram presentation of individual mEJC events, shown in Fig. 5C, shows that the distribution of mEJC events are not skewed toward larger or smaller amplitudes.

It is possible that more postsynaptic glutamate receptors, or even a different pool of receptors, are accessed by evoked multivesicular release versus activation by the content of a single synaptic vesicle. In addition, mEJC quanta do not summate linearly to result in EJC amplitude and are imperfect as a measure of the size of the postsynaptic field. Therefore to assay whether the postsynaptic receptor field was somehow altered in a manner undetectable in mEJC amplitude analyses, direct pressure ejection of glutamate onto the NMJ was used to monitor postsynaptic glutamate-gated currents (Fig. 5D). There is no significant difference in the peak amplitude of postsynaptic glutamate-gated current in wild type relative to mutant with increased or decreased Gs activity (Fig. 5D), although it may be of interest to note that the results follow a similar trend to mEJC amplitude analysis. Also note that the application of exogenous glutamate is a flawed measure of postsynaptic receptor field size/density in larval NMJ due to steric constraints imposed by the subsynaptic reticulum, which closely opposes the fully enclosed presynaptic varicosity and limits access of solution-borne transmitter to the synaptic cleft (Atwood et al. 1993; Johansen et al. 1989). As a result, the time scale and amplitude of the postsynaptic response to direct application of glutamate are qualitatively different from that of endogenously released transmitter.

Taken together, mEJC analyses indicate no change in basal synaptic vesicle fusion probability correlating with altered Gs activity. Likewise, mEJC and direct application of glutamate onto the postsynaptic cell reveal only subtle changes in the postsynaptic receptor field. These results indicate that the dramatic increases observed in basal transmission must be due to alterations specific to the machinery involved in the evoked release of neurotransmitter at low [Ca2+]_bath or to a change in the postsynaptic receptor field that is invisible to mEJC amplitude analyses and glutamate application studies described above. Thus the next question was whether activity could cause detectable changes in single synapse properties.

**Single synapse properties not altered following high-frequency stimulation**

One mechanism underlying synaptic plasticity at mammalian glutamatergic synapses is the regulated postsynaptic insertion or removal of glutamate receptors, especially AMPA-type receptors, in response to presynaptic stimulation (Malinow and Malenka 2002). Such an activity-dependent mechanism might explain the absence of robust mEJC alterations in different Gs mutants (Fig. 5). Previous assays at the *Drosophila* NMJ have highlighted presynaptic plasticity mechanisms, and it has been an open question whether postsynaptic plasticity of this type may be present. Therefore to assay whether activity-dependent glutamate receptor insertion occurs at the *Drosophila* NMJ, the amplitude and frequency of endogenous excitatory junction currents was monitored 1 min before and 4 min after induction of PTP (5-Hz stimulation for 60 s, 0.2 mM [Ca2+]_bath; see Fig. 4A) in wild-type animals.

No apparent change was observed in spontaneous EJC amplitude or frequency after tetanic stimulation that produced robust PTP. In wild type, mean spontaneous current amplitude prior to the stimulus train was 585 ± 34 pA and 614 ± 38, 623 ± 42, 623 ± 38, and 594 ± 42 pA at 1-min intervals minutes after stimulation (n = 5). Spontaneous current amplitude in activated Gs transgenic lines was likewise unaltered [ELAV/dgs* 560 ± 30 pA prior to tetanus and 639 ± 54, 629 ± 20, 640 ± 15, and 627 ± 13 pA after tetanus (n = 5); G7/dgs* 633 ± 9 pA before tetanus, and 625 ± 21, 616 ± 7, 620 ± 1, and 620 ± 3 pA after tetanus (n = 4)]. Loss of Gs activity also did not affect spontaneous current amplitude after tetanic stimulation (dgs_R60B19 683 ± 18 pA prior to tetanus, and 687 ± 18, 690 ± 14, 686 ± 9, and 682 ± 13 pA after tetanus; n = 4).

This negative result indicates that rapid postsynaptic glutamate receptor trafficking is likely not a mechanism used at the *Drosophila* NMJ for short-term synaptic plasticity. Thus having exhausted the available electrophysiological assays, we next turned to other assays of the NMJ to reveal the mechanisms by which changes in Gs activity level cause dramatic changes in both basal evoked transmission and activity-dependent plasticity. Because both pre- and postsynaptic Gs activation cause similar dramatic changes in neurotransmission, quantified optical assays were used to examine the composition and function of both sides of the synaptic cleft.

**Postsynaptic GluRIIA receptor subunit is dramatically downregulated by misregulation of Gs activity**

Previous work at the *Drosophila* NMJ has shown that CAMP-dependent perturbations in synaptic function are compensated for by pre- and postsynaptic compensation mechanisms (“homeostasis”), which act to keep transmission in a normal range (Davis 1995; Davis and Goodman 1998; Davis et al. 1996, 1998; Marek et al. 2000; Paradis et al. 2001; reviewed in Davis and Goodman 1998). More recent studies have shown that specific increases in the GluRIIA subunit of the glutamate receptor increases transmission at the NMJ, whereas decreased GluRIIA decreases synaptic response to glutamate (DiAntonio et al. 1999; Petersen et al. 1997; Sigrist et al. 2002). Changes in responsiveness to glutamate are also compensated for by an increase in presynaptic vesicle release and/or an increased number of presynaptic active zones (DiAntonio et al. 1999; Petersen et al. 1997; Reiff et al. 2002). These studies raised the possibility that increased evoked transmission due to altered Gs activity may be due to changes in the number or distribution of GluRIIA receptors. We used specific antibodies to quantitatively assay the presence of GluRIIA receptor subunits at the NMJ (Petersen et al. 1997) (see also METHODS) in the spectrum of different Gs mutants (Fig. 6).

In control larvae, immunocytochemical GluRIIA staining was tightly localized to the NMJ, and present in punctate domains in the postsynaptic SS, consistent with other reports (Fig. 6A). Incredibly, GluRIIA expression at the NMJ was essentially undetectable after manipulation of Gs activity, with both loss-of-function mutants and either pre- or postsyn-
aptic Gα activation resulting in negligible signal (Fig. 6A). The internal control using an anti-HRP antibody, which detects an epitope on neuronal tissues, was present at similar levels in all control and experimental genotypes (Fig. 6A). To quantify these results, staining intensity of HRP and DGluRIIA were normalized between experimental and wild-type control in the

![Diagram showing staining intensity and area ratio](image)

**FIG. 6.** Postsynaptic GluRIIA receptors are nearly eliminated by increases or decreases in Gα activity in both presynaptic and postsynaptic cells. The NMJ was probed with 2 anti-glutamate receptor II (GluRII) antibodies: a polyclonal antibody that recognizes all 3 GluRII subunits (subunits A-C; not shown) and a monoclonal antibody specific to GluRIIA (glutamate binding pocket of the receptor; Petersen et al. 1997; shown below). Both antibodies showed similar types of changes in all genotypes. **A:** representative images of Anti-GluRIIA staining at the NMJ. GluRIIA (green channel) overlaps with anti-HRP antibody staining (HRP, red channel), which recognized a membrane epitope at synaptic boutons. The normal GluRIIA expression pattern is shown for wild type (OR). Detectable GluRIIA expression was nearly absent in ELAV/dgs*, G7/dgs*, and dgsR60/B19, whereas HRP expression was unaffected. All animals were processed identically to controls, and independent staining experiments were repeated ≥4 for each genotype. **B:** the fluorescent intensity of GluRIIA immunoreactivity was quantified, and normalized to OR (pixel value minus background) within each experiment. Shown are normalized intensities with means ± SE (***P < 0.001). **C:** postsynaptic distribution of GluRIIA was reduced by changes in Gα activity. The area of GluRIIA immunoreactivity signal (independent of intensity) was measured within the bouton defined by HRP staining, and the ratio of GluRIIA to total bouton surface area was determined. In OR, about one-fifth of the presynaptic bouton area is apposed to GluRIIA receptor subunits. The significance from OR is indicated (**P < 0.01; ***P < 0.001).
same experimental trial (GluRIIA, 1.0 ± 0.02; HRP 1.0 ± 0.02; n = 19). An extremely significant loss of GluRIIA expression was observed when Gos activity was increased presynaptically (ELAV/dgs* 25.5 ± 3.8% of control, P < 0.0001, n = 7), increased postsynaptically (G7/dgs* 26.7 ± 2.8%, P < 0.0001, n = 9), or decreased globally (dgs R60/B19, 43.2 ± 5.8%, P < 0.0001, n = 8; see Fig. 6B). In the same animals, the area ratio of postsynaptic (GluRIIA-containing) receptor field to the presynaptic terminal (HRP-positive staining) was also analyzed, independent of GluRIIA signal intensity (Fig. 6C, see also METHODS). In wild-type controls, approximately one-fifth of the presynaptic terminal is directly apposed to GluRIIA-containing postsynaptic receptor domains (area GluRIIA/HRP = 0.21 ± 0.01). This ratio was dramatically decreased in synapses with either an increase or decrease in Gos activity (ELAV/dgs*, 0.02 ± 0.005, P < 0.0001; G7/dgs*, 0.09 ± 0.027, P = 0.002; dgs R60/B19, 0.07 ± 0.027, P = 0.001). Surprisingly, the loss of receptor s was present after both pre- and postsynaptic Gos activation and was actually significantly more severe in the presynaptic Gos gain-of-function synapses (ELAV/dgs* vs. G7/dgs*, P = 0.034).

To verify these results and to assay whether there was a compensatory increase in other glutamate subunits, we used a second polyclonal antibody directed against the ligand binding site, which recognizes multiple GluRII subunits (AS5) (Saitoe et al. 2001). Extrasynaptic staining was observed with this antibody, which likely represents a real population of extrasynaptic GluRII receptors (DiAntonio et al. 1999). This extrasynaptic pattern was not observed with the GluRIIA-specific antibody (Fig. 6A), suggesting that extrasynaptic receptors do not contain DGluRIIA in any appreciable amount. Using the global GluRII antibody, immunocytochemical assays indicated a similar, but less severe, reduction in both the intensity and distribution of DGluRIII receptors. When the signal was normalized to control (GluRII 1.0 ± 0.02, HRP 1.0 ± 0.01, n = 14), mutants with altered Gos function again displayed significantly reduced GluRII expression (ELAV/dgs*, 61.8 ± 10%, P = 0.005, n = 11; G7/dgs*, 64.4 ± 13.8%, P = 0.003, n = 8; dgs R60/B19, 50.2 ± 10.1%, P = 0.001, n = 10). The area ratio of GluRII (AS5) to HRP was compared as in the preceding text and a similar trend observed. Both Gos gain- and loss-of-function mutants showed reduced GluRII/HRP synaptic areas (OR, GluRII/HRP, 0.71 ± 0.04; ELAV/dgs*, 0.56 ± 0.06, P = 0.071; G7/dgs*, 0.44 ± 0.09, P = 0.025; dgs R60/B19, 0.48 ± 0.07, P = 0.009).

In conclusion, in two independent assays of GluRII receptors at the NMJ, we saw a significant decrease after all manipulations of Gos activity (increase, decrease, presynaptic, and postsynaptic). The GluRIIA subunit expression was largely eliminated in these mutants (Fig. 6), and the overall abundance of GluRII signal was significantly reduced. Based on the preceding functional studies, and previous reports, the loss of GluRIIA was a surprising phenotype and one not predicted by current models. At a minimum, this result indicates that activation of Gos leads to a direct alteration of the glutamate receptor field, clearly requiring trans synaptic signaling in the case of presynaptic Gos activation. The significance of the striking change in the receptor field to the similarly remarkable enhancement in synaptic transmission in currently unclear.

Synaptic vesicle cycling rate is increased when Gos misregulated

The activity of the presynaptic vesicle pool is clearly regulated by cAMP-dependent changes at multiple points. The size of the readily releasable vesicle pool is regulated by the levels of cAMP, acting through a PKA-mediated pathway (Davis et al. 1998; Kuromi and Kidokoro 2000; Suzuki et al. 2002; Yoshihara et al. 2000). Increased cAMP or PKA activity results in a larger number of vesicles being released due to depolarization and due to increased translocation from the reserve pool of vesicles (Kuromi and Kidokoro 2000). Such an increase in the size of the endo/exo pool (ECP) may not necessarily be revealed by mEJC analysis but is a good estimator of the size of the vesicle pool released per EJC on depolarization (Kuromi and Kidokoro 1999). Therefore we next quantified the size of the ECP in mutants with altered Gos activity in an attempt to correlate increased EJC amplitude with an increase in the number of vesicles being released presynaptically, using an independent measure of evoked presynaptic vesicle fusion. Using an established protocol, vesicles in NMJ terminals were loaded with FM 1-43 dye under conditions of normal (1.8 mM [Ca 2+ ] bath, high (90 mM) [K + ] bath, and in the presence of cyclopiazonic A, a calcineurin inhibitor (Kuromi and Kidokoro 2000; Kuromi et al. 1997). Inhibiting calcineurin blocks endocytosis and acts to load the reserve pool of vesicles under conditions that normally only access the ECP (Kuromi et al. 1997). Once the presynaptic terminal was loaded and the fluorescence intensity measured, the ECP was unloaded using low (0.2 mM) Ca 2+ and high [K+] stimulation (see METHODS; Fig. 7).

The amount of FM 1-43 dye loading was similar in control and all Gos mutants, indicating that endocytosis was not detectably perturbed by altered Gos activity at the Drosophila NMJ (Fig. 7, A and B). The intensity of the loaded FM 1-43 fluorescent signal, based on a pixel intensity range of 0–256, was indistinguishable in all genotypes (OR, 104.02 ± 5.97, n = 6; ELAV/dgs*, 102.87 ± 3.37, P = 0.871, n = 6; G7/dgs*, 108.4 ± 3.74, P = 0.550, n = 7; dgs R60/B19, 102.01 ± 8.84, P = 0.856, n = 5). Specific conditions were determined to stimulate dye release (see METHODS), which allowed evaluation of bidirectional changes in ECP size. In wild-type synapses, a loss of staining in the periphery of the NMJ bouton was observed, whereas staining in the central region of the bouton was largely unaffected (Fig. 7A) with the result of an ~50% loss of fluorescence intensity (53.06 ± 4.94; Fig. 7 B and C). The rate of dye unloading was significantly increased in transgenic animals with increased Gos activity pre- or postsynaptically (ELAV/dgs*, 31.68 ± 3.52, P = 0.0065; G7/dgs*, 36.38 ± 5.31, 0.044). Loss of Gos activity at the NMJ resulted in a loss of dye similar to that of the control (dgs R60/B19 47.91 ± 4.11, P = 0.446). When the amount of dye unloading was normalized to the loaded intensity, the gain-of-function Gos mutants also displayed a greater amount of dye loss during stimulation than controls (OR, 48.93 ± 4.01% unloading; ELAV/dgs*, 68.93 ± 3.65%, P = 0.005; G7/dgs*, 97.07 ± 4.08%, P = 0.010; dgs R60/B19, 52.29 ± 4.11, P = 0.573). These results indicate that both pre- or postsynaptic Gos activation increase vesicle mobility/fusion rate at low [Ca 2+ ], consistent with previous studies of the effect of pre- or postsynaptic activation of the cAMP-PKA cascade on
quantal content (Davis et al. 1998; Kuromi and Kidokoro 2000). Decreasing Gos activity did not show increased vesicle fusion/mobility, consistent with previous FM dye experiments, which show that inhibitors of the cAMP-PKA cascade reduced vesicle mobility from the reserve pool only, leaving the ECP unaffected (Kuromi and Kidokoro 2000).

**Discussion**

The objective of this study was to determine the role of the Gos pathway in the regulation of synaptic transmission and functional plasticity and especially to assay synaptic correlates of the striking loss of behavioral learning observed following the constitutive activation of Gos in the *Drosophila* brain (Connolly et al. 1996). As with previous studies of plasticity mechanisms in *Drosophila*, this investigation made use of the larval, glutamatergic NMJ as the synaptic system for all assays. Surprisingly, both loss of Gos (dgs mutants) (Wolfgang et al. 2001) and activation of Gos (dgs* transgene; Fig. 1) in either pre- or postsynaptic cells similarly resulted in a dramatic increase in evoked synaptic efficacy and concomitant loss of functional plasticity (facilitation, augmentation, and potentiation) in reduced \( [Ca^{2+}]_{\text{bath}} \) conditions. These results indicate that synaptic plasticity is dependent on proper Gos-mediated signaling on both sides of the synapse. The behavioral learning deficit after dgs* expression is far worse than defects in cAMP metabolic mutants (dnc, rut, DCO; Fig. 1) (Connolly et al. 1996), suggesting that cAMP-independent mechanisms are being misregulated via Gos manipulation (Blackmer et al. 2001; Herlitze et al. 2001). Similarly, the NMJ transmission defects following dgs* expression are also more severe than those of cAMP metabolic mutants (Zhong and Wu 1991). We conclude that the total scope of Gos synaptic signaling, through cAMP and other pathways, is responsible for keeping the synapse within specific functional parameters to allow for rapid modulation of transmission strength. Functionally, misregulation of Gos activity affects the presynaptic vesicle cycle to change evoked pathway-specific changes in vesicle fusion probability. In addition, increased or decreased Gos activity strikingly alters the composition of the postsynaptic glutamate receptor field, although the functional significance of this regulation is presently unclear. Finally, the level of Gos activity is communicated transsynaptically to bidirectionally control both pre- and postsynaptic mechanisms of neurotransmission.

**Gos- and cAMP-dependent regulation of synaptic transmission at the Drosophila NMJ**

The exaggerated EJC basal neurotransmission and reduced STF in reduced \( [Ca^{2+}]_{\text{bath}} \) conditions caused by Gos activation is consistent with other conditions in which the cAMP-signaling pathway is activated. For example, the classic *Drosophila* plasticity mutant *dnc* (PDE; elevated cAMP; Fig. 1) displays a twofold increase in basal EJC amplitude and reduced STF in 0.2 mM \( [Ca^{2+}] \) (Delgado et al. 1992; Zhong and Wu 1991). Similarly, the increased transmission caused by activated Gos correlates with reduced \( Ca^{2+} \) dependence of transmission, similar to the reduction of \( Ca^{2+} \) dependence in *dnc* mutants (Zhong and Wu 1991). The observation that increased transmission caused by expression of dgs* is greater than that observed in *dnc* null mutants could point to opposing or compensatory roles of Gos signaling, through cAMP or other intracellular cascades, in pre- versus postsynaptic compartments. Alternatively, the difference could be due to the simple fact that cAMP levels are more elevated in the dgs* GAL4-UAS manipulations than in the *dnc* mutants. Direct measure-
ments of [cAMP] have been performed in both dnc mutants and in cells expressing the dgs* construct used here. In the null dnc alleles used in plasticity studies, cAMP was increased five- to sixfold over controls (Davis and Kiger 1981), and dnc hypomorphs showed a twofold increase in [cAMP] (Byers et al. 1981). dgs*-expressing cells show a much larger increase in basal [cAMP], reportedly 60-fold over controls (Quan et al. 1991). dnc* and dgs* alleles used in plasticity studies, cAMP was increased nearly twofold (Davis et al. 1998). Thus a change in quantal fusion rate (number of fusion sites or probability of fusion sites) but rather correlate with changes specific to evoked fusion (e.g., gating of ion channels, response to Ca\(^{2+}\) fusion trigger). Mutants defective in the cAMP pathway (dnc, rut) both result in a twofold decrease in mEJC frequency when measured at the single bouton level (Renger et al. 2000) and a dramatic (3- to 4-fold) increase in mEJC frequency when measured in culture or in central neurons (Lee and O’Dowd 2000; Rohrbough and Brodie 2002); however, mEJC frequency at the whole NMJ level has not been measured for these animals. The level of intracelluar cAMP directly affects opening probability of shaker-type K\(^+\) channels, with dnc mutants showing a greater open probability (Delgado et al. 1991), resulting in hyperexcitability (Salkoff and Wyman 1981; Wu and Haugland 1985) and presumably altered mEJC frequency.

Similarly, mEJC amplitude was largely unaffected in Gos mutants, suggesting little change in the density or conductance of postsynaptic glutamate receptors downstream of Gos activity. After postsynaptic activation of Gos, a modest increase in quantal size (approximately 10%) was observed, but this increase is insufficient to explain the 400% increase in EJC amplitude (assuming that the relationship between spontaneous and evoked responses is linear). In dnc and rut mutants, mEJC amplitude is not altered (Renger et al. 2000; Zhong and Wu 1991). Thus an increase in basal glutamate receptor density or conductance properties does not appear to contribute significantly to the Gos mutant phenotype.

It is important to note that similar enhancement in synaptic transmission amplitude and loss of functional plasticity, without any change in mEJC properties, has been reported for a number of other learning mutants in Drosophila (Rohrbough et al. 1999, 2000). These findings suggest that alteration to evoked synaptic transmission mechanisms is the primary mechanism for regulating synaptic plasticity at the Drosophila NMJ. Numerous potential targets for this presynaptic regulation have been identified (see following text). In addition, activity-dependent changes in postsynaptic responsiveness are also possible. Fast glutamate receptor insertion and removal from postsynaptic membranes has been widely discussed recently as a mechanism for postsynaptic plasticity with the observation that AMPA receptors are quickly inserted into the postsynaptic membrane after tetanus (Shi et al. 1999). This insertion can be driven by CamKII activity, or PKC (Hayashi et al. 2000; Malinow et al. 1989). Our assays to monitor spontaneous activity after tetanic stimulation indicate that this mechanism is unlikely to act at the Drosophila NMJ, at least in the 5-min time frame monitored here. Thus the results to date are consistent with an evoked pathway-specific alteration of presynaptic efficacy as the primary mechanism of plasticity downstream of both Gos and cAMP.

**Possible pre- and postsynaptic mechanisms underlying plasticity at the NMJ**

The results presented here are consistent with a persistent state of presynaptic potentiation resulting when Gos activity is either increased or decreased. FM 1-43-dye-labeling assays demonstrate that the rate of synaptic vesicle cycling through the exo-endo pool is significantly increased in low [Ca\(^{2+}\)] submaximal conditions when Gos is activated. Loss of function Gos mutants show no significant difference in the size of the exo-endo pool. This is consistent with prior work, which has shown that...
the translocation from the reserve pool was selectively affected by PKA inhibitors (Kuromi and Kidokoro 2000). The more modest increase in transmission in these mutants (200%) must be restricted to altered evoked release of vesicles in the exo-endo pool only. Previous work in Drosophila has shown that cAMP-dependent regulation of Shaker K+ channels is likely to be a primary mechanism explaining elevated vesicle cycling. PKA phosphorylates K+ channels (Drain et al. 1994), and dnc and rut mutants interact with K+ channel mutants (Delgado et al. 1991, 1992; Zhong et al. 1992) and differentially affect K+ channel conductance (Alshuaib and Mathew 1998; Delgado et al. 1991, 1998; Yu et al. 1999; Zhong and Wu 1993). A second likely mechanism involves Gs-dependent increased Ca2+ influx. In mammalian neuronal cultures, cAMP has been shown to increase N- and L-type Ca2+ channel currents, in a PKA-dependent manner (Artalejo et al. 1990; Gross et al. 1990), and these same Ca2+ currents are altered proportionally by dnc, rut, or effectors or inhibitors of the cAMP pathway (Bhattacharya et al. 1999). Such a mechanism would explain the altered Ca2+ dependence of neurotransmitter release observed in Gs mutants. A third possible mechanism is directly increased vesicle mobilization, resulting in an activity-dependent alteration in the distribution of vesicles (Hirling and Scheller 1996; Kuromi and Kidokoro 2000; reviewed in Rodesch and Brodie 2000). Increased [cAMP] directly affects the mobility of the readily releasable synaptic vesicle pool at the Drosophila NMJ, making it more accessible to stimulated release (Kuromi and Kidokoro 2000). One or more of these mechanisms would explain the heightened low-Ca2+-evoked neurotransmission in Gs-gain-of-function mutants in the absence of any change in mEJC characteristics.

This study provides no evidence that postsynaptic function is regulated by the level of Gs activity or that alterations in the postsynaptic glutamate receptor field play any role in short-term plasticity at the Drosophila NMJ. In both gain- and loss-of-function Gs mutants, there is no substantial change in glutamate receptor conductance, density, or distribution based on mEJC amplitude analyses and direct assay of glutamate-gated currents in the muscle. This finding is extremely surprising in light of the dramatic alteration of the molecular character of the postsynaptic glutamate receptor field in both loss and gain of function Gs mutants. Two different antibodies were used to assay the GluR fields: a polyclonal antibody against all GluRII subunits (Saitoe et al. 2001) showed a significant reduction of signal in all Gs mutants and a monoclonal antibody specific to GluRIIA (Petersen et al. 1997) showed a nearly complete loss of signal in all Gs mutants. Immunoreactivity against DGlurIA in the embryo appeared normal in dgsR60 homozygous mutants, indicating a postembryonic modification of DGlurIA expression under the control of dgs (D. E. Featherstone, personal communication). At a minimum, these analyses reveal a striking molecular alteration of the Glur field downstream of Gs, possibly to the extent of nearly eliminating GlurIA subunits.

Complete loss of GlurIA has been shown to cause significantly decreased mEJC amplitudes (DiAntonio et al. 1999; Petersen et al. 1997), whereas we report here a nearly complete loss of GlurIA immunoreactivity, using two antibodies, without a similar change in mEJC amplitudes. One way to rationalize this apparent contradiction is to postulate that the reduced presence of GlurIA after Gs manipulation is not sufficient to alter significantly mEJC kinetics or amplitudes. The present report shows a 75% reduction of receptor abundance, whereas Petersen et al. (1997) examined GlurIA genetic nulls. More recently, the effect of graded expression levels of GlurIA was examined by DiAntonio et al. (1999), revealing that local expression levels of GlurIA, in the absence of GlurIB, results in an overcompensation of presynaptic transmitter release, doubling the amplitude of glutamatergic transmission at the NMJ. At higher levels of GlurIA expression, this phenotype was eliminated. If the levels of DGlurIB were also downregulated (or eliminated) by altered Gs signaling, our findings would be in agreement with those of DiAntonio et al. A second possibility is that the loss of GlurIA immunoreactivity caused by Gs manipulation may represent epitope masking rather than loss of GlurIA subunits. Extracellular binding of an auxiliary protein to glutamate receptors has recently been reported in C. elegans (Zheng 2001), and an essential auxiliary subunit of mammalian AMPA receptors (stargazin) has recently been found (Letts et al. 1998). Stargazin is essential for proper insertion and localization of receptors with the postsynaptic density and is modulated by PKA phosphorylation, thereby controlling receptor number (Chen et al. 2000; Choi et al. 2002; Schnell et al. 2002). Interaction with such proteins, or other changes in the accessibility/confirma-

Gas-independent signaling by the βγ complex

Work on Gs-mediated plasticity has focused almost entirely on the cAMP cascade regulated by the α subunit. However, activation of Gs results in the dissociation of the heterotrimeric complex into free α subunits and βγ complexes, and each mediates distinctive intracellular signaling (Clapham and Neer 1993; Sternweis 1994; Tang and Gilman 1992). Relatively little work has been published specifically on the effects of βγ signaling due to Gs-coupled receptor activation, but it is believed that the βγ subunits are not very selective and can be shared between all G proteins (Dascal et al. 1993; Iniguez-Lluhi et al. 1992). Recent work has shown that the addition of βγ increases L-type channel Ca2+ influx via a PKC-mediated pathway (Zhong et al. 1999) and directly mediates activation of K+ channels current due to receptor activation of Gs (Sorota et al. 1999). If these two results are extrapolated to the Drosophila NMJ, transgenic manipulation of Gs signaling could give rise to alterations in Ca2+ and K+ currents independent of Gs activity. βγ subunits have been shown to decrease P/Q and N-type Ca2+ channels by binding them directly at the I–II intracellular loop (Herlitze et al. 1996; Ikeda 1996). Classical second-messenger pathways are also modulated by βγ signaling: type-specific inhibition or activation of adenylyl cyclase (AC1and AC2, respectively) (Tang and Gilman 1991; Taussig et al. 1993) as well as activation of phospholipase Cβ, re-
viewed in Sternweis and Smrcka (1992). In addition, recent work has shown that other receptor kinases, such as ras/MAP kinase, can be activated specifically by βγ (Crespo et al. 1994; Daaka et al. 1997).

It is not clear how free βγ subunits are regulated by the cell, although there is evidence that they are physically sequestered by tethering proteins such as phoshducin (Lee et al. 1992; Schroder et al. 1997). It is possible that excess βγ complexes are present when Gαs is constitutively activated and that they could effect a wide variety of intracellular signaling cascades, leading in part to the novel physiological phenotypes seen here. However, there are few examples where endogenous βγ signaling predominates the modulation of intracellular signaling.

Transynaptic signaling downstream of the Gαs pathway

Numerous lines of evidence have demonstrated the existence of both anterograde and retrograde transsynaptic signals at the Drosophila NMJ (reviewed in Broadie and Richmond 2002; Davis and Goodman 1998; Featherstone and Broaddie 2000). Such signals are involved in induction of postsynaptic receptor fields (Broadie and Bate 1993), pruning of postsynaptic receptor fields (Featherstone et al. 2000, 2002), and compensatory regulation of presynaptic quantal content (Davis et al. 1998; Petersen et al. 1997). The present study shows that increasing Gαs function either pre- or postsynaptically results in nearly identical phenotypes, and independent assays of presynaptic and postsynaptic function indicate similar mechanisms. Specifically, presynaptic Gαs activation modifies the postsynaptic GluRIIA receptor field, and postsynaptic Gαs activation heightens presynaptic vesicle cycling. Moreover, global loss-of-function Gαs mutants also modify the postsynaptic GluRIIA field. Are these paired pre- and postsynaptic alterations a form of compensation or are they independent, Gαs-dependent mechanisms? What signals are used to communicate the level of Gαs activity in both directions across the synaptic cleft?

The identity of the messenger(s) is still unclear, but there are a few likely suspects. Glutamate itself has been shown to act as a retrograde messenger (Silber et al. 2003; Staubli et al. 1998). Integrins are known to signal through a common downstream pathway (Bahr et al. 1997; Davis and Goodman 1998; Featherstone and Broadie 2000). Activation of facilitatory calcium channels in chromaffin cells by cAMP-dependent protein kinase and transmitter.

REFERENCES


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