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Activators of G-protein Signaling 3: A drug addiction molecular gateway

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Abstract

Drug addiction is marked by continued drug-seeking behavior despite deleterious consequences and a heightened propensity to relapse notwithstanding long, drug-free periods. The enduring nature of addiction has been hypothesized to arise from perturbations in intracellular signaling, gene expression, and brain circuitry induced by substance abuse. Ameliorating some of these aberrations should abate behavioral and neurochemical markers associated with an “addiction phenotype”. This review summarizes data showing that protein expression and signaling through the non-receptor Activator of heterotrimeric G-protein Signaling 3 (AGS3) is altered by commonly abused substances in rat and in vitro addiction models. AGS3 structure and function are unrelated to the more broadly studied Regulator of G-protein Signaling (RGS) family. Thus, the unique role of AGS3 is the focus of this review. Intriguingly, AGS3 protein changes persist into drug abstinence. Accordingly, studies probing the role of AGS3 in the neurochemistry of drug-seeking behavior and relapse are reviewed in detail. To illuminate this work, AGS3 structure, cellular localization, and function are covered so that an idealized AGS3-targeted pharmacotherapy can be proposed.

Keywords

Drug addiction; Cocaine; Heroin; Alcoholism; AGS3; Gpsm1; G-protein; Second messenger systems; Drug design

Introduction

Addiction is becoming increasingly understood as a neuropathological disorder comprised of chronic and compulsive drug relapse episodes during which the drive to seek and use drug often cannot be controlled (O'Brien 1996; Heyman 2009). The enduring nature of addiction and a high relapse liability even after extended abstinence has led to the hypothesis that drugs of abuse displace normal molecular machinery of reward-related associative learning (Koob et al. 1998; Hyman and Malenka 2001; Jones and Bonci 2005; Hyman et al. 2006; Kalivas and O'Brien 2008). Intriguingly, the mechanisms underlying relapse can be dissociated from those involved in drug reinforcement and dependence (Shaham et al. 1996; Cornish and Kalivas 2000; Grimm and See 2000). Thus, pharmacotherapeutics that promote abstinence without interference with non-drug derived reinforcement may arise from a through understanding of drug-mediated perturbations in second messenger systems.

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Modeling Drug Relapse

Much work examining the behavioral and molecular responses to commonly abused substances has been accomplished in protocols where drugs are administered to laboratory animals by the experimenter. Two of these paradigms, behavioral sensitization and conditioned place preference, have contributed much to our knowledge (Le et al. 2001; Robinson and Berridge 2008; Vezina and Leyton 2009). Nonetheless, a growing literature has highlighted significantly different outcomes following experimenter-administered or subject-administered, operant self-administration paradigms (Dworkin et al. 1995; Hemby et al. 1997; Markou et al. 1999; Jacobs et al. 2002, 2003, 2004, 2005; Hemby et al. 2005; Chen et al. 2008) and it is postulated that the molecular mechanisms of drug relapse can be probed with various manipulations of operant drug self-administration paradigms (Davis and Smith 1976; de Wit and Stewart 1981; Le and Shaham 2002; Shaham et al. 2003; Epstein et al. 2006). In operant self-administration addiction models, the animal manipulates some operandum, such as a lever or nosepoke, resulting in response-contingent drug delivery. Operant paradigms have been developed for nearly all substances abused by humans and these models can be used to quantify the motivation to seek drug (Richardson and Roberts 1996; Salamone et al. 2009) as well as to examine the choice structure of drug pursuit (Whitelaw et al. 1996; Ping and Kruzich 2008).

Addiction and the Mesocorticolimbic system: general concepts

All drugs of abuse, albeit via different mechanisms, increase extracellular levels of the neuromodulator dopamine (Nestler 2005). Once released and before being sequestered into the pre-synaptic terminal, dopamine can activate both pre-synaptic and post-synaptic heterotrimeric G-protein-coupled dopamine receptors. Increased signaling through dopamine receptors has been shown to facilitate learning and/or incentive salience (Berridge and Robinson 1998; Berke and Hyman 2000; Jay 2003) and can also change the gene expression and function (Berke and Hyman 2000; Canales 2005; Nestler 2005) of circuitry that normally processes motivationally relevant environmental stimuli and adaptive goal-directed responding (Koob et al. 1998; Kelley 2004). The intricate modulation of motivated behavior and learning by mesocorticolimbic dopamine receptors has been previously reviewed (Berke and Hyman 2000; Nicola et al. 2000; El-Ghundi et al. 2007; Dalley and Everitt 2009) as has the role of other heterotrimeric G-protein coupled receptors in the enduring nature of drug and alcohol-induced plasticity (Hack and Christie 2003; Waldhoer et al. 2004; Gravanis and Margioris 2005; Ong and Kerr 2005; Cowen and Lawrence 2006; Ferre et al. 2008; Abbracchio et al. 2009; Dalley and Everitt 2009; Olive 2009). Thus dopamine action within the mesocorticolimbic system or “motive circuit” (Fig. 1) (Mogenson et al. 1993) has been an active area of investigation.

Within the mesocorticolimbic system, the addiction literature has primarily focused on the ventral tegmental area and substantia nigra, dorsal and ventral striatum, amygdala, and frontal cortical regions that are referred to as the rat prefrontal cortex (PFC) or human anterior cingulate cortex (Ongur and Price 2000; Goldstein and Volkow 2002). Important reviews have covered the complex circuitry recruited during the different behavioral phases of and situations leading to drug relapse (Leshner and Koob 1999; See et al. 2003; Kalivas and Volkow 2005; Rebec and Sun 2005; Kalivas et al. 2006). Included among these is the glutamatergic projection from the PFC into the NAc that appears essential for cue or drug-prime to precipitate relapse to cocaine or heroin seeking (Park et al. 2002; McFarland et al. 2003; McFarland et al. 2004; LaLumiere and Kalivas 2008) and, as will be discussed below, may be under control of the intracellular non-receptor Activator of G-protein Signaling 3 (AGS3) as well as some members of the Regulators of G-protein Signaling (RGS) family. Since the role of the RGS family in addiction has been previously reviewed (Neubig 2002; Burchett 2005; Ron and Jurd 2005; Traynor and Neubig 2005; Garzon et al. 2008; Hooks et al. 2008; Lomazzi et al. 2008; Traynor 2010), the focus here is on reviewing data implicating the apparent role of AGS3 in the medial

ventral striatum (NAcore) and the PFC in shaping addiction- and alcoholism-associated aberrant signaling through G-protein coupled receptors, neurochemistry, and behavior.

G-protein coupled receptors

Appreciation of the intracellular signaling cascades that mediate biological effects of receptor activation largely began with description of the heterotrimeric guanine nucleotide binding proteins (G-proteins) as a necessary interface between metabotropic cell surface receptors, such as dopamine, and enzymes, such as adenylyl cyclase, that generate intracellular signaling molecules (Gilman 1987; Ross 1989). Thus, heterotrimeric G-proteins are now known to transmit and amplify signal while serving as a primary level of integration and signal sorting (Gilman 1987; Bourne and Nicoll 1993; Neer 1995). Figure 2a illustrates canonical G-protein signal transduction across the cell membrane. However, the precise molecular events of the G-protein cycle have not been fully elucidated (Oesterhelt and Stoeckenius 1971; Stickle and Barber 1993; Tian and Deth 1993; Bourne 1997; Hamm 2001). For example, intrinsic GTP hydrolytic rate constants (Breitwieser and Szabo 1988) of reconstituted $G\alpha$ are slower than that of crude membrane preparations (Brandt and Ross 1985; Brandt and Ross 1986; Sato et al. 1995). This discrepancy is only partially explained should diffusion kinetics alter subcellular compartmentalization (Lamb and Pugh 1992). Thus, the search for G-protein accessory proteins, such as AGS3 and the RGS family, began.

G-protein Relevance to Addiction

Disruptions in G-protein kinetics or subcellular stoichiometry has been described in several disease states (Manji 1992; Ostrom et al. 2000), including alcohol (Waltman et al. 1993; Pattiselanno et al. 1994; Ferguson and Goldberg 1997; Nestby et al. 1999), opiate (Griffin et al. 1985; De Vries et al. 1991; Suzuki et al. 1991; Terwilliger et al. 1991; Van Vliet et al. 1991,¹⁹⁹³; Guitart et al. 1993; Ronken et al. 1994; Self et al. 1994; Schoffelmeer et al. 1995,¹⁹⁹⁷; Nestby et al. 1997; Selley et al. 1997; Kaplan et al. 1998; McLeman et al. 2000), methamphetamine (Iwasa et al. 1996; Ujike et al. 1996; McLeman et al. 2000; Nishio et al. 2002; Schmauss et al. 2002; Kitanaka et al. 2003; Tong et al. 2003; Yang et al. 2008), and cocaine (Kleven et al. 1990; Nestler et al. 1990; Terwilliger et al. 1991; Striplin and Kalivas 1992; Self et al. 1994; Henry and White 1995; Volkow et al. 1999; Morgan et al. 2002; Xi et al. 2002; Kobayashi et al. 2007) addiction models. Thus, since the recent discovery of the AGS and RGS heterotrimeric G-protein modulators, the potential involvement of these molecules in addiction has been an active area of research.

G-protein modulators: AGS3 and the RGS Family

Yeast-based functional assays for receptor-independent activation of heterotrimeric G-protein signaling using mammalian cDNAs revealed four structurally and functionally unique “non-receptor Activators of G-protein Signaling” proteins (Cismowski et al. 1999; Takesono et al. 1999; Cao et al. 2004). Given the stark structural and functional dissimilarities between the AGS proteins, new nomenclature has been assigned (Siderovski and Willard 2005). Nonetheless, within the addiction literature, the older nomenclature predominates. Of the AGS proteins, only AGS3 has been evaluated in addiction models. AGS3 is a highly conserved G-protein dissociation inhibitor (GDI, Fig. 2b) (Takesono et al. 1999; De Vries et al. 2000a; Bernard et al. 2001; Peterson et al. 2002). In brief, a GDI prevents activation of the G-protein $G\alpha$ subunit. AGS3 was also among the first GDI described that simultaneously stimulates signaling through $G\beta\gamma$ while inhibiting $G\alpha$ activation and association of G-protein with receptor. Moreover, while AGS3 was first described in a receptor-independent functional screen, AGS3 is most likely to inhibit $G\alpha$ and stimulate $G\beta\gamma$ subsequent to receptor activation (Webb et al. 2005).

Of the 30+ members of the Regulators of G-protein Signaling (RGS) family, all share a RH homology domain (RGS box) that imparts to all currently known RGS proteins GTPase-accelerating (GAP) activity on $G\alpha_i/o$ and $G\alpha_q$ (Berman and Gilman 1998; De Vries et al. 2000b; Ross and Wilkie 2000; Hollinger and Hepler 2002). A GAP promotes G-protein deactivation and can thereby limit and/or focus signaling through G-protein-coupled receptors. However, other proteins that do not function like a RGS; e.g., RhoGEF, GRK, and Conductin, also contain the RGS box. Work describing the role of the RGS family in addiction (predominantly the psychostimulants and opiates) has been the topic of several reviews (Neubig 2002; Burchett 2005; Ron and Jurd 2005; Traynor and Neubig 2005; Garzon et al. 2008; Hooks et al. 2008; Lomazzi et al. 2008; Traynor 2010). In brief, most studies on RGS and addiction have focused on mRNA levels rather than protein. Depending on the substance studied and the paradigm applied, RGS mRNA is often elevated or decreased in brain structures of relevance to addiction and pain (Traynor and Neubig 2005) and, via increased GAP activity modulating metabotropic receptor signaling, may regulate the behavioral impact of addictive drugs (Kenny and Markou 2004). Intriguingly, while RGS4 mRNA upregulation in pre-clinical addiction studies is not as dramatic as some other RGS family mRNA (Traynor 2010), RGS4 protein was found to be acutely upregulated in the rat locus coeruleus following chronic morphine (Gold et al. 2003) and inducible RGS4 knockouts display a profound morphine phenotype (Han et al. 2010). A similar time-dependent protein expression profile has been reported for RGS9-2 and RGS7 following acute morphine (Traynor 2010). However, only a small rise in RGS9-2 protein is observed following chronic morphine and this upregulation is often disconnected from mRNA expression (Traynor 2010). Thus, studies of the RGS family have revealed a sophisticated repertoire in ameliorating as well as facilitating psychiatric conditions, including response to addictive drugs. However, due to space limitations, the interested reader is referred to the primary literature discussed in the many reviews cited above.

AGS3: Structure and Function

Higher organisms express one AGS3 homolog, LGN/GPSM2, that along with AGS3 is found in several rat tissues including neurons, astroglia, and microglia (Blumer et al. 2002; McCudden et al. 2005). Full length AGS3 is enriched in brain and testes and primary cortical culture suggests that AGS3 may be predominantly neuronal (Blumer et al. 2002).

Rat AGS3 is 650 residues and contains tandem repeats in both the N- and C- terminus. The amino terminus encompasses seven tetratricopeptide repeats (TPR) that form a coiled-coil domain of antiparallel helices that can modulate protein-protein and protein-membrane interactions (Das et al. 1998; Blatch and Lassel 1999). Intriguingly, in protein phosphatase 5, the N-terminal TPR domains regulate the catalytic carboxyl terminus (Kang et al. 2001). However, no regulatory function of AGS3 TPR has been observed. Instead, AGS3 TPR is thought to mediate compartmentalization or scaffolding, as TPR deletion results in a homogenous, cytoplasmic distribution (Pizzinat et al. 2001).

The N-terminal AGS3 TPR repeats are followed by a ~100 amino acid linker and four, conserved ~25 amino acid C-terminal G-protein regulatory motifs known as GPR or GoLoco (Siderovski et al. 1999; Takesono et al. 1999; De Vries et al. 2000a; Natochin et al. 2000; Adhikari and Sprang 2003; Siderovski and Willard 2005). The GPR motif is also found in other proteins including RGS14 (Kimple et al. 2001; Natochin et al. 2001; Peterson et al. 2002; Kimple et al. 2004). Intriguingly, although all four AGS3 GPR motifs differ in primary sequence and affinity (De Vries et al. 2000a; Natochin et al. 2000; Adhikari and Sprang 2003) and surrounding residues appear to determine both affinity and GDI efficacy (Luo and Denker 1999; Ponting 1999; Siderovski et al. 1999; Kimple et al. 2001; Kimple et al. 2002; Adhikari and Sprang 2003), the C-terminal GPR domains of AGS3 are both necessary and sufficient for GDI activity on $G\alpha_i$ (Takesono et al. 1999; De Vries et al. 2000a; Pizzinat et al.

2001). Moreover, each of the GPR domains can function independently and also perhaps cooperatively as a GDI on $G\alpha_{i1}$ (Adhikari and Sprang 2003). Together, these data suggest that each GPR repeat can bind $G\alpha$ independently and/or cooperatively (Adhikari and Sprang 2003).

Together with site-directed mutagenesis (Natochin et al. 2002; Willard et al. 2008) and simulated molecular dynamics (Khafizov 2009) studies, crystal structures of the GPR motif bound to chimeric $G\alpha$ (Kimple et al. 2002) have been solved and subsequently revealed an elegant mechanism of AGS3 GDI involving both stabilization of inactive conformations and steric hindrance. Thus, AGS3 binding $G\alpha$ is thought to regulate guanine nucleotide exchange and may also physically occlude the GDP exit route of $G\alpha$ (Kimple et al. 2002; Natochin et al. 2002; Willard et al. 2008; Khafizov 2009). Together, these data suggest that AGS3 is an effective GDI preventing $G\alpha$ activation by both stabilizing inactive $G\alpha$ and physically preventing $G\alpha$ activation (Fig. 2b).

Importantly, AGS3 binding is not selective for $G\alpha_i$, as $G\alpha_t$ and weak $G\alpha_o$ and $G\alpha_q$ binding has also been observed, but AGS3 only exhibits GDI activity at $G\alpha_i$ and $G\alpha_t$ (Takesono et al. 1999; De Vries et al. 2000a; Bernard et al. 2001; Natochin et al. 2001; McCudden et al. 2005). $G\alpha_t$ is enriched in retina while brain is enriched with $G\alpha_{i/o}$. Thus, via TPR and GPR repeats, brain AGS3 may scaffold a complex $G\alpha$ mixture at the membrane to increase signaling efficiency through G-protein coupled receptors (Siderovski et al. 1999; Bernard et al. 2001; Pizzinat et al. 2001; Natochin et al. 2002) and perhaps facilitate second messenger crosstalk (Fan et al. 2009). This action is contrasted with the RGS family that appears to preferentially accelerate deactivation of $G\alpha_{i/o}$, $G\alpha_q$, as well as the cognate $G\beta\gamma$.

In summary, AGS3 preferentially binds to, and stabilizes, all three $G\alpha_i$ isoforms in the GDP bound inactive state (Fig. 2) through GPR domains that inhibit guanine nucleotide exchange. Guanine nucleotide exchange (GDP for GTP) is necessary for heterotrimeric G-protein activation. In this manner, AGS3 inhibits subsequent $G\alpha_i$ activation while simultaneously facilitating signaling through the now displaced $G\beta\gamma$ complex (Fig. 2) (Takesono et al. 1999; De Vries et al. 2000a; Webb et al. 2005). Thus, AGS3, the first G-protein dissociation inhibitor (GDI) described for heterotrimeric G-proteins can dramatically shape signaling through $G\alpha_i$ -coupled receptors.

Relevance of AGS3 to Addiction

The following sections summarize data collected over the past decade beginning with expression profiling and moving on to functional studies that assay both neurochemistry and behavior.

AGS3: Expression Profile in Addiction Models

In rat models of alcoholism and cocaine addiction, AGS3 expression has been examined in a limited number of mesocorticolimbic regions. A ~60% upregulation was detected in the NAc and PFC 3 or more weeks after either repeated cocaine injections or 3 weeks extinction from operant cocaine self-administration. AGS3 expression did not change in several other mesocorticolimbic regions at these time points. Intriguingly, following cocaine, AGS3 upregulation appears to require both repeated cocaine exposure and a protracted abstinence period (Bowers et al. 2004). Intriguingly, mRNA encoding AGS3 remained unchanged in both the NAc and PFC 3 weeks after repeated cocaine injections (Bowers, M.S., unpublished observations), suggesting that AGS3 expression is not transcriptionally regulated. In fact it was recently shown that AGS3 expression can be regulated by a ubiquitin specific protease (USP9x) in vitro and that AGS3 and USP9x can be co-regulated in the rat PFC 3 weeks after repeated cocaine injections (Xu et al. 2010).

The dynamics of AGS3 protein expression in rat NAc and PFC is not equivalent. For example, the ~60% AGS3 protein upregulation observed in the PFC 3 weeks after repeated cocaine injections fell to ~30% upregulation in a separate cohort 8 weeks after repeated cocaine injections (Bowers et al. 2004). Thus, although still significantly elevated, rat PFC AGS3 content declined between the 3 and 8 week cocaine abstinence time points. In contrast, no decline was observed in the rat NAc between the 3 week and 8 week cocaine abstinence time points. This regional distinction was also apparent during alcohol abstinence (Bowers et al. 2008). For example, AGS3 protein was upregulated ~25% in the NAc 3 weeks after operant ethanol self-administration. No AGS3 expression change was observed in PFC or several other mesocorticolimbic regions following ethanol self-administration. Further, NAc AGS3 expression returned to baseline after 6 additional weeks. Intriguingly, AGS3 expression changes may be dose-responsive to alcohol (a dose-dependent upregulation was observed 3 weeks after repeated ethanol injections: Bowers, M.S., unpublished observations and Bowers et al. 2008, supplemental).

Taken together, these data suggest that AGS3 expression is not altered by repeated exposure to commonly abused substances until after protracted abstinence. Thus, initial drug sensitivity or mechanisms that support on-going drug taking are unlikely mediated via changes in AGS3 expression. In partial support of this hypothesis, brief (10 min) opioid receptor activation on primary striatal neurons in vitro facilitated AGS3 binding to inactive $G\alpha$ subunits (Yao et al. 2005). However, AGS3 protein upregulation and the subsequent superactivation of downstream signaling occurred only as a function of morphine washout (Fan et al. 2009). The upregulation of AGS3 in brain regions that are implicated in motivation, reinforcement, and reactivity to drug-associated cues during abstinence from commonly abused substances led to the hypothesis that AGS3 modulates critical aspects of drug and alcohol relapse. Data testing this hypothesis are described next.

Neurobiology of Cocaine and Cocaine Relapse: Role of AGS3

Repeated cocaine injections normally augment the motoric and neurochemical response to a subsequent cocaine challenge (Post and Rose 1976). This sensitization effect is typically more pronounced after abstinence (Izenwasser and French 2002), contains elements of conditioning, and has been associated with the reinstatement of drug-seeking behavior (De Vries et al. 1998). Intriguingly, pertussis toxin injection (that prevents $G\alpha_{i/o}$ dissociation and activation) into the NAc (Hummel and Unterwald 2003) or ventral tegmental area (Steketee and Kalivas 1991) increased behavioral sensitization to cocaine, and expression of sensitization is decreased by activation of a $G\alpha_{i/o}$ -coupled receptor (Beyer and Steketee 2002; Xie and Steketee 2009).

During abstinence from repeated cocaine injections, AGS3 knockdown prevented uncoupling of $G\alpha_i$ from receptor (Bowers et al. 2004). Further, AGS3 knockdown restored $G\alpha_i$ receptor coupling to drug-naïve levels and prevented expression of cocaine-mediated behavioral sensitization. Importantly, following antisense washout, AGS3 expression returned and behavioral sensitization to cocaine challenge expressed (Bowers et al. 2004). Thus, during cocaine abstinence, AGS3 expression increased, signaling through $G\alpha_i$ -coupled receptors decreased, and behavioral sensitization was expressed. However, if PFC AGS3 is knocked down, signaling through $G\alpha_i$ -coupled receptors is increased to drug-naïve levels and expression of behavioral sensitization is blocked. Thus, AGS3 expression appears to gate cocaine-mediated sensitization. Importantly, AGS3 knockdown does not change the locomotor response to a novel environment, injection stress, or acute cocaine.

Complimentary experiments to this reversible knockdown were performed with a cell-permeable AGS3-like mimetic peptide (Bowers et al. 2004) containing a single GPR consensus motif derived from the C-terminal AGS3 GPR repeats (Peterson et al. 2002). This peptide,

similar to AGS3 upregulation, binds inactive $G\alpha_i$ and uncouples the cognate receptor (Bowers et al. 2004). This is not observed with a similar peptide containing a point mutation. Injection of the AGS3 mimetic peptide into the drug-naïve PFC, followed by acute, systemic cocaine induced a sensitized-like behavioral and neurochemical response similar to that observed after abstinence from repeated cocaine exposures (Fig. 3) (Bowers et al. 2004). Neither the sensitized behavioral nor neurochemical response was observed following acute systemic cocaine if the mutant peptide was injected into the PFC instead of the AGS3 mimetic peptide. Neither AGS3 mimetic nor mutant peptide altered the locomotor or neurochemical response to systemic saline. Taking these data together with the knockdown data described above leads to the interpretation that AGS3 expression levels gate some behavioral and neurochemical plasticity that is commonly observed during abstinence from repeated cocaine exposure.

Given the intriguing role of AGS3 to modulate some of the neurochemistry thought to be involved in cue- cocaine-, or heroin primed relapse (Park et al. 2002; McFarland et al. 2003, 2004; LaLumiere and Kalivas 2008), the propensity to relapse into cocaine-seeking behavior was assayed following reversible AGS3 knockdown or treatment with the AGS3-like mimetic peptide (Bowers et al. 2004). Figure 4a illustrates that animals were trained to press a lever in order to receive a cocaine infusion or food reinforcement. Once this responding was stable, lever pressing was extinguished by removing the reinforcer. During this drug-free period, AGS3 expression increased (data not shown in Fig. 4). AGS3 knockdown did not effect food-primed reinstatement of food-seeking behavior (Fig. 4b), yet cocaine-primed seeking of cocaine was blocked (Fig. 4c). Further, a cocaine prime failed to elicit drug seeking in rats where the PFC was treated with scrambled oligonucleotide (Fig. 4c,d). Nonetheless, following antisense washout and return of AGS3 protein, cocaine-prime reliably precipitated relapse in the same rats that failed to reinstate drug seeking under AGS3 knockdown (Fig. 4d). Perhaps most intriguingly, injection of the AGS3-like mimetic peptide into the PFC shifted the cocaine-dose response for drug-primed relapse leftward (Fig. 4e). In summary, AGS3 expression levels appear to gate several cocaine addiction-associated neurochemical and behavioral phenomena including sensitivity to cocaine-primed relapse, expression of behavioral and neurochemical sensitization, and uncoupling of at least 2 receptors that signaling through $G\alpha_i$.

Despite the apparent congruity of these data, the precise mechanism of AGS3 protein induction and the primary class of affected receptors remains unknown. For example, it was speculated earlier that AGS3 protein accumulation was not regulated by transcriptional processes and it has been recently shown that a ubiquitin specific protease (USP9x) can regulate AGS3 expression (Xu et al. 2010), yet the transduction pathways impinging upon this promising mechanism have not been elucidated. Moreover, while it was shown that AGS3 knockdown after multiple cocaine injections can restore the diminished coupling of the D2-like dopamine receptor to drug naïve levels and AGS3 knockdown prevents cocaine-precipitated relapse, it remains exceptionally unlikely that highly efficacious (Gal and Gyertyan 2006; Karila and Reynaud 2009) D2-like dopamine agonists would serve as anti-relapse medications (De Vries et al. 2002; Edwards et al. 2007; Bachtell et al. 2008) until pharmacogenetics (Blum et al. 2009) or promiscuous ligands (Pilla et al. 1999; Khroyan et al. 2000; Preti 2000; Gasior et al. 2004; Feltenstein et al. 2007) are more widely adopted.

Heroin Relapse: Role of AGS3

The reinforcing effects of heroin are mediated through μ -opiate receptors (Matthes et al. 1996) that couple through $G\alpha_i$ to acutely inhibit adenylyl cyclase (AC) activity. During continued opiate receptor stimulation (Sharma et al. 1975; De Vries et al. 1991) as well as during opiate blockade, washout, or withdrawal (Avidor-Reiss et al. 1995; Schoffelmeer et al. 1995; Watts 2002; Fan et al. 2009) the increase in AC activity has been associated with opiate

tolerance and dependence (Sharma et al. 1975; Van Vliet et al. 1992, 1993; Chao and Nestler 2004). Precisely how AC activity increases during opiate withdrawal remains unknown.

Thus, it is intriguing that the AGS3 stabilization of GDP-bound $G\alpha_i$ liberates $G\beta\gamma$ (see Fig. 2), which can subsequently stimulate signaling through $G\beta\gamma$ -mediated effectors (Takesono et al. 1999; Natochin et al. 2000; Bernard et al. 2001; Webb et al. 2005; Ferre et al. 2008) including some AC isozymes (Yao et al. 2005). Importantly, AGS3 protein expression increased during morphine withdrawal, and AGS3 acted as a central molecular mediator of signaling pathway crosstalk that culminated in increased AC activity (Fan et al. 2009). That AGS3 upregulation during morphine withdrawal can stimulate AC type 5 (Fan et al. 2009) is especially intriguing because striatal regions, including the NAc, are enriched with AC5 (Defer et al. 2000) and AC5 has been implicated in opiate tolerance and withdrawal (Avidor-Reiss et al. 1997). Accordingly, knockdown of rat NAc AGS3 during heroin abstinence blocked heroin-primed relapse (Yao et al. 2005). Taken together, AGS3 appears to represent a critical molecular integrator leading to AC heterologous sensitization following prolonged μ -opiate receptor activation or withdrawal, and AGS3 expression gates the propensity of heroin to precipitate relapse.

Alcohol Relapse: Role of AGS3

The role that AGS3 may play in cocaine and heroin addiction was probed following small doses of experimenter-administered drug used to precipitate relapse. Compared to these drugs, alcohol only weakly primes relapse in laboratory rodents (Stewart 2000; Le and Shaham 2002). However, by manipulating conditions governing alcohol availability (Bowers et al. 2008) and by presenting compound stimuli previously associated with alcohol (Katner and Weiss 1999; Katner et al. 1999; Bowers et al. 2008), robust relapse can be obtained with standard, outbred rats, and alcohol-seeking motivation quantified (Bowers et al. 2008).

Abstinent alcoholics are prone to relapse (Larimer et al. 1999) due to a heightened motivation to seek alcohol (Sanchis-Segura and Spanagel 2006). Increased alcohol-seeking during abstinence is expressed by humans (Burish et al. 1981), non-human primates (Weerts et al. 2006), and rodents (Sinclair and Senter 1968; Sanchis-Segura and Spanagel 2006). Intriguingly, during prolonged abstinence, this motivation can be cue-rather than pharmacologically-driven and regulated by NAc AGS3 protein expression (Bowers et al. 2008). In other words, the heightened motivation to seek alcohol following abstinence can be primed and maintained by cues previously associated with alcohol alone, and rat NAc AGS3 knockdown significantly interferes with expression of this motivation to seek alcohol. More specifically, following prolonged alcohol abstinence, AGS3 knockdown reversed both the fast and persistent response ultrastructure exhibited during alcohol seeking without impairing motoric capacity or cue-primed sucrose seeking during abstinence from operant sucrose self-administration (Fig. 5).

At least part of the AGS3 contribution to heightened, cue-precipitated alcohol seeking during prolonged abstinence is mediated via $G\beta\gamma$, which AGS3 liberates upon binding GDP- $G\alpha_i$ (see Fig. 2), as $G\beta\gamma$ sequestration partially blocked alcohol-seeking behavior (Bowers et al. 2008). However, AGS3 knockdown does not block the motivation of sucrose-abstinent rats to seek sucrose (Fig. 5f, g). Thus, the increased motivation to seek alcohol and reactivity to alcohol-related cues that develop during prolonged abstinence, and that drive relapse, may be modulated through AGS3. Intriguingly, AGS3 is not upregulated following brief, 24 hour, abstinence and AGS3 knockdown does not impact motivation to seek alcohol following short-term abstinence.

Is AGS3 Upregulation Behaviorally Relevant?

While the AGS3 addiction-related data described above appear compelling, studies probing the role of AGS3 in cocaine (Bowers et al. 2004) and heroin (Yao et al. 2005) relapse models only investigated the efficacy of a drug-prime to precipitate drug-seeking behavior. Thus, the study evaluating the capacity of AGS3 knockdown and G β γ sequestration to normalize the cue-primed motivation to seek alcohol during abstinence (Bowers et al. 2008) represents an important advance to our understanding of the AGS3 repertoire. Specifically, in this new alcohol “relapse” model, the motivation to seek alcohol is not only robust and reproducible, but cue driven, and also independent of alcohol reinforcement. In other words, no environmental renewal is observed during alcohol abstinence, as alcohol seeking was not primed upon omission of previously alcohol-associated cues. However, after abstinence, the cue-primed navigation of the progressive ratio schedule by alcohol-reinforced rats is strikingly similar to that expressed by alcohol-abstinent rats responding for cues previously associated with alcohol alone (not alcohol reinforced). In these cue-only experiments, a clear plexiglass barrier prevented access to the alcohol-containing cup. Thus, these alcohol-abstinent rats experienced all cues except those specifically related to alcohol ingestion, yet the breakpoints obtained, cumulative responding, run rate, and inter-response interval profile overlaid with alcohol reinforced rats.

Thus, the motivation to seek alcohol after abstinence in this paradigm may be cue driven and also potentially linked to NAc core excitability (Hopf et al. 2010). However, the affect of AGS3 knockdown on alcohol-seeking behavior was only evaluated in cued and alcohol reinforced rats (Bowers et al. 2008). While the efficacy of AGS3 knockdown under these “real world” conditions is promising, the capacity of AGS3 to modulate response to drug-associated cues has not been explicitly evaluated in any addiction model to date. Nonetheless, increased AGS3 protein expression during alcohol abstinence was significantly correlated to both the expression of this enhanced cue-primed motivation to seek alcohol during abstinence as well as to prior operant alcohol self-administration history.

Thus, upregulated AGS3 may decrease drug efficacy at eliciting drug-seeking behavior or sensitization. However, the contribution of “low dose” drug-mediated interoceptive cues in priming AGS3-modulated drug-seeking behavior and sensitization, versus the purely pharmacological effects of drug prime interacting with AGS3, has not been fully elucidated either. Although, it was shown that AGS mimetic peptides shift the dose response curve of cocaine-primed relapse leftward (Bowers et al. 2004). Thus, it is intriguing that AGS3 upregulation appears to require *abstinence* from multiple drug exposures. Nonetheless, AGS3 may also modulate drug-associated cue reactivity, as AGS3 knockdown not only reduced breakpoint during cue-primed, reinforced alcohol seeking to pre-abstinence levels; time to first response, run rate, and inter-response intervals were also returned to pre-abstinence levels (Bowers et al. 2008).

Intriguingly, AGS3 knockdown during cocaine abstinence blocked the expression of behavioral sensitization to cocaine, but AGS3 knockdown *prior* to cocaine exposure failed to block behavioral sensitization to cocaine (Bowers et al. 2004). AGS3 knockdown also failed to impede the motoric response to novelty, mild restraint stress, or acute cocaine injection. Moreover, AGS3 knockdown also failed to block food-primed reinstatement of food seeking or cue-primed reinstatement of liquid sucrose seeking (both during abstinence from operant sessions), suggesting that changes in AGS3 expression may be relatively specific for drug-related stimuli. This is in contrast to alcoholism pharmacotherapies like naltrexone that also reduce concurrent alcohol and sucrose-seeking behaviors (Bienkowski et al. 1999; Steensland et al. 2007).

Together, these data suggest an AGS3 upregulation may only be pathogenic following abstinence from repeated drug exposures and, that when upregulated, AGS3 may modulate either or both the response to drug-related pharmacology or previously associated cues.

AGS3 Insights Informing a Therapeutic Future

Data summarized above suggest that AGS3 could represent an etiological molecular gateway of cocaine, heroin, and alcohol addiction. Importantly, AGS3 knockdown during cocaine abstinence restored diminished signaling through $G\alpha_i$ -coupled receptors to drug-naïve levels (Bowers et al. 2004). Decreased $G\alpha_i$ -coupled receptor signaling capacity is seen in rat strains with increased responsiveness to repeated cocaine (Haile et al. 2001) or morphine (Guitart et al. 1993), as well as in non-human primates self-administering cocaine (Morgan et al. 2002) and human addicts (Volkow et al. 1999; Heinz et al. 2005; Martinez et al. 2005; Watanabe et al. 2008). These data suggest that decreased $G\alpha_i$ signaling may support addiction pathogenesis. Importantly, AGS3 knockdown, unlike current addiction pharmacotherapies (e.g., naltrexone or acamprosate), does not impair seeking of non-addictive substances (Fig. 4b and Fig. 5f,g) or motoric capacity (Fig. 4b and Fig. 5e-g), including under conditions of drug-seeking as well as the drug-free response to mild stress or novelty (Bowers et al. 2004, 2008).

Thus, increased AGS3 expression appears to be a deleterious gatekeeper on the path towards addiction. Unfortunately, clinical knockdown strategies face significant barriers and receptor-mediated approaches are limited by a potential ubiquitous promoter or enhancer upstream of AGS3 (Fan et al. 2009). Nonetheless, the robust structural and functional information summarized in Section 3, above, can inform rational design of AGS3-targeted antagonists. For example, the necessary residues of AGS3 binding (Peterson et al. 2002; Willard et al. 2008) and action (Kimple et al. 2002, 2004) on $G\alpha_i$ have been identified and confirmed in crystal structures of an AGS3-like GPR motif bound to chimeric $G\alpha$ (Kimple et al. 2002). Together these data can inform design of small, peptide mimetics that can selectively disrupts the Janus-like nature of AGS3; i.e., binding or GDI activity. Selectively disrupting binding would simultaneously augment signaling through $G\alpha_i$ -coupled receptors and focus signaling through $G\beta\gamma$ -mediated effectors. Leaving binding intact, but disrupting GDI activity would leave signaling through $G\alpha_i$ -coupled receptors dampened while perhaps further augmenting signaling through $G\beta\gamma$. Thus, the potential exists to tailor treatment to the individual's current experience with the drug, abstinence status, or motivational level (Yao et al. 2005; Bowers et al. 2008; Fan et al. 2009).

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Figure 1. Some “motive” circuit nuclei

mesocorticolimbic circuit parses reward valence and motivational drives to execute both novel as well as habitual responding (Mogenson et al. 1993). Two of these regions are the focus of this review; the nucleus accumbens core (NAcore) and the prefrontal cortex (PFC). Both the NAcore and PFC receive dopaminergic afferents from the ventral tegmental area (VTA). Importantly, glutamatergic projections from the PFC into the NAcore (open arrow) are necessary for the reinstatement of seeking behavior in several models of drug relapse (Park et al. 2002; McFarland et al. 2003, 2004; LaLumiere and Kalivas 2008).

Figure 2. The heterotrimeric G-protein cycle

(a) Intracellular heterotrimeric G-proteins consist of an α subunit and a tightly associated $\beta\gamma$ complex. The G-protein heterotrimer is inactive and usually receptor-associated when the α subunit ($G\alpha$) is bound to guanosine diphosphate (GDP) and $G\beta\gamma$ ($G\alpha\text{GDP}\beta\gamma$). step 1) Receptor activation facilitates mass-action exchange of guanosine triphosphate (GTP) for GDP, which lowers affinity of the GTP-bound G-protein ($G\alpha\text{GTP}\beta\gamma$) for receptor. Additionally, the activated $G\alpha$ ($G\alpha$ bound to GTP, $G\alpha\text{GTP}$) dissociates from the $G\beta\gamma$ complex (Gilman 1987; Bourne 1997). step 2) Both $G\alpha\text{GTP}$ and $G\beta\gamma$ can modulate diverse second messenger effectors (Ross 1989). step 3) $G\alpha$ terminates its own signaling by hydrolyzing GTP into GDP, releasing inorganic phosphate. step 4) $G\alpha\text{GDP}$ exhibits higher $G\beta\gamma$ affinity. step 5) Reassembled G-protein heterotrimer ($G\alpha\text{GDP}\beta\gamma$) can re-associate with receptor, which increases receptor affinity for ligand (Lorenzen et al. 2000). (b) AGS3 catches and stabilizes inactive $G\alpha_i$ (open arrow), which augments signaling through $G\beta\gamma$ -mediated effectors (closed arrow), but inhibits signaling through $G\alpha_i$ -coupled receptor onto effector, while also prohibiting G-protein heterotrimer reformation and receptor re-association (open arrow) (Takesono et al. 1999; De Vries et al. 2000a; Natochin et al. 2001; Webb et al. 2005)

Figure 3. AGS3-mimetic yields a cocaine-experienced phenotype

Cocaine (15 mg/kg, i.p.)-induced increase in extracellular glutamate in the accumbens core was augmented by pretreatment of the PFC with Tat-GPR. Data points depict mean $\pm$ SEM pmol/dialysis sample, $n = 8$. Tat-peptide, either Tat-GPR or Tat-mGPR. Challenge, either cocaine (15 mg/kg, i.p.) or saline (1 ml/kg, i.p.). * $p < 0.05$, using a two-way ANOVA with repeated measures over time, and a least significant difference test for post-hoc comparisons of Tat-GPR to TAT-mGPR. Reprinted from Bowers, M.S. et al. (2004), *Activator of G Protein Signaling 3: A Gatekeeper of Cocaine Sensitization and Drug Seeking*, *Neuron*, 42, 269-81, with permission from Elsevier.

Figure 4. Increased AGS3 facilitates cocaine, but not food seeking

(a) Time-line outlining the experimental protocol. (b) Bilaterally reducing AGS3 did not effect reinstatement of food seeking. However, (c) AGS3 knockdown blocked the reinstatement of cocaine-seeking behavior, while infusion with scrambled oligonucleotide had no effect. (d) Reinstatement of drug seeking was initiated by a cocaine injection 2 weeks after discontinuing oligonucleotide infusion in all groups. (e) Microinjection of Tat-GPR into the PFC augmented the reinstatement of drug seeking (active lever pressing) by a cocaine priming injection (5 mg/kg, i.p.). Two reinstatement trials were conducted in animals extinguished to criterion (Ext I and Ext II), and Tat-GPR or Tat-mGPR were administered 30 min prior to the cocaine priming injection in random order. Data points depict mean active lever presses + SEM, $n = 8-9$. Ext, total active lever presses made during the extinction trial the day prior to the cocaine-induced reinstatement trial; Test, total active lever presses made during cocaine reinstatement; Retest,

total active lever presses following a second extinction period and a second cocaine reinstatement; Sc, scrambled oligonucleotide. * $p < 0.05$, using two-way ANOVA with repeated measures over time, and a least significant difference test for post-hoc comparisons. Reprinted from Bowers, M.S. et al. Activator of G Protein Signaling 3: A Gatekeeper of Cocaine Sensitization and Drug Seeking, *Neuron*, 42, 269–81, (2004), with permission from Elsevier.

Figure 5. AGS3 knockdown reduced the enhanced alcohol seeking during abstinence

(a) Antisense knocked down AGS3 in the NAc core of 3-wk alcohol abstinent rats (SC-AGS3: $n = 7$; AS-AGS3: $n = 7$) and in 24 h abstinent rats (SC-AGS3: $n = 6$; AS-AGS3: $n = 6$). Representative AGS3 and calnexin control blots at 3 wks are shown (a'). (b) AGS3 knockdown (AS-AGS3) reduced alcohol seeking in 3 wk abstinent rats to 24h-abstinent levels ($n = 12$), with no effect in 24h-abstinent rats ($n = 12$), whereas a scrambled control construct (SC-AGS3) had no effect on motivation (breakpoint) at 3 wk abstinence ($n = 15$). (c and d) Reduced motivation to seek alcohol in 3 wk-abstinent rats was also evident throughout the progressive ratio testing period in terms of reduced cumulative responding (c) and in decreased number of fast and slow interresponse intervals (d). (e) No difference in motoric response capacity. (f and g) AGS3 knockdown failed to reduce motivation (breakpoint) or responding for 5% sucrose, a highly reinforcing substance not commonly thought to be addictive (AS-AGS3, sucrose: $n = 17$; SC-AGS3, sucrose: $n = 17$). The open arrowhead in a' indicates AGS3 at ~72 kDa; the filled arrowhead in a' at ~90 kDa indicates calnexin. Data represent mean $\pm$ SEM. s, scrambled; a, antisense. *, $P < 0.05$; **, $P < 0.01$ using ANOVA with Scheffé post hoc comparisons. Reprinted with permission, Proc Natl Acad Sci U.S.A. (2008) 105, 12533–12538 National Academy of Sciences, U.S.A.