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The Adenosine A_{2A} Receptor Agonist, CGS-21680, Blocks Excessive Rearing, Acquisition of Wheel Running, and Increases Nucleus Accumbens CREB Phosphorylation in Chronically Food-Restricted Rats

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Abstract

Adenosine A_{2A} receptors are preferentially expressed in rat striatum, where they are concentrated in dendritic spines of striatopallidal medium spiny neurons and exist in a heteromeric complex with D₂ dopamine (DA) receptors. Behavioral and biochemical studies indicate an antagonistic relationship between A_{2A} and D₂ receptors. Previous studies have demonstrated that food-restricted (FR) rats display behavioral and striatal cellular hypersensitivity to D₁ and D₂ DA receptor stimulation. These alterations may underlie adaptive, as well as maladaptive, behaviors characteristic of the FR rat. The present study examined whether FR rats are hypersensitive to the A_{2A} receptor agonist, CGS-21680. In Experiment 1, spontaneous horizontal motor activity did not differ between FR and ad libitum fed (AL) rats, while vertical activity was greater in the former. Intracerebroventricular (i.c.v.) administration of CGS-21680 (0.25 and 1.0 nmol) decreased both types of motor activity in FR rats, and returned vertical activity levels to those observed in AL rats. In Experiment 2, FR rats given access to a running wheel for a brief period outside of the home cage rapidly acquired wheel running while AL rats did not. Pretreatment with CGS-21680 (1.0 nmol) blocked the acquisition of wheel running. When administered to FR subjects that had previously acquired wheel running, CGS-21680 suppressed the behavior. In Experiment 3, CGS-21680 (1.0 nmol) activated both ERK 1/2 and CREB in caudate-putamen with no difference between feeding groups. However, in nucleus accumbens (NAc), CGS-21680 failed to activate ERK 1/2 and selectively activated CREB in FR rats. These results indicate that FR subjects are hypersensitive to several effects of an adenosine A_{2A} agonist, and suggest the involvement of an upregulated A_{2A} receptor-linked signaling pathway in NAc. Medications targeting the A_{2A} receptor may have utility in the treatment of maladaptive behaviors associated with FR, including substance abuse and compulsive exercise.

Keywords

food restriction; wheel running; A_{2A} receptor; nucleus accumbens; CREB; CGS-21680

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1. Introduction

In clinical populations, a number of maladaptive, compulsive, behaviors appear to coexist with and/or be predisposed by food restriction (FR), including binge eating, substance abuse, and obligatory exercise (Polivy and Herman, 1985; Krahn et al., 1992; Wilson, 1993; Davis et al., 1999; Klein et al., 2004). All of these behavioral phenomena have been modeled in preclinical studies. For example, FR increases stress-induced binge-eating of palatable foods (Hagan et al., 2002), enhances acquisition and maintenance of drug self-administration (Carroll and Meisch, 1984), and increases wheel running behavior (Routtenberg and Kuznesov, 1967). Wheel running has been shown to reinforce instrumental responding (Iversen, 1993) and place preference conditioning (Lett et al., 2000), and individual differences in expression of this behavior predict acquisition and reinstatement of cocaine self-administration (Larson and Carroll, 2005). Consequently, there may be significant overlap of neural substrates that mediate reinforcing effects of food, drugs of abuse, and at least this form of exercise. In human patients with anorexia nervosa, compulsive exercise can exacerbate self-starvation and weight loss, and contribute to fatality (Burden et al., 1993). While neuroadaptations in brain motivational/reward circuitry that develop in response to chronic FR are likely to mediate adaptive behavioral changes that facilitate food-seeking, acquisition, and ingestion, under some circumstances they may be subverted to support pathological patterns of eating behavior, drug abuse, and compulsive activity.

Studies of neuroadaptations accompanying FR that may underlie changes in motivation and reward function have, for the most part, centered on the dopamine (DA) system. For example, palatable food and acute psychostimulant drug challenge have been shown to produce higher extracellular concentrations of DA in the nucleus accumbens (NAc) of FR relative to ad libitum fed (AL) rats (Rouge-Pont et al., 1995; Bassareo et al., 1999; Cadoni et al., 2003). A potentially related finding is that FR decreases function (V_{max}) of the striatal DA transporter (Patterson et al., 1998; Zhen et al., 2006). Investigations of DA and NMDA receptor-mediated cell signaling, activation of transcription factors, and downstream gene expression have suggested a coherent pattern of postsynaptic changes in NAc. These include an upregulation of D_1 DA receptor-mediated phosphorylation of the NMDA receptor NR_1 subunit, and increased D_1 /NMDA receptor-dependent ERK and CaMK II signaling (Haberny et al., 2004; Haberny and Carr, 2005a). The increased ERK signaling is necessary for increased activation of the nuclear transcription factor CREB, which is likely to account for the downstream increase in c-fos, preprodynorphin and preprotachykinin gene expression observed in FR rats challenged with a D_1 DA receptor agonist (Carr et al., 2003; Haberny and Carr, 2005b).

There is little coexpression of D_1 and D_2 receptors in striatal neurons (Gerfen et al., 1990). Evidence of increased D_2 DA receptor function in FR subjects has been obtained in studies of D_2 agonist-induced behavior (Carr et al., 2001), pallidal Fos-immunostaining (Carr et al., 2003), and striatal functional coupling between D_2 receptors and G_i protein (Carr, 2002). Interestingly, adenosine A_{2A} receptors, which are primarily expressed in striatum (DeMet and DeMet, 2002), exist in a heteromeric complex with D_2 receptors (Hillion et al., 2002; Fuxe et al., 2003), and are localized in dendritic spines of striatopallidal medium spiny neurons (Ferre et al., 2004). Moreover, A_{2A} and D_2 receptors are coupled to $G_{s/olf}$ and $G_{i/o}$, respectively, and mediate opponent effects on activation of adenylyl cyclase, PKA, DARPP-32 (thr 34), ERK 1/2, CREB, and c-fos (Svenningsson et al., 1998; Diaz-Cabiale et al., 2001). A_{2A} agonist administration not only activates these signaling proteins and downstream transcription factors, but decreases the ligand affinity and G-protein coupling of D_2 DA receptors (Diaz-Cabiale et al., 2001; Ferre et al., 1993). Consistent with the opponent actions of A_{2A} and D_2 receptors at the cellular level, A_{2A} agonist administration decreases a number of DA-mediated behavioral responses. For example, although the A_{2A} agonist, CGS-21680, has no effect on basal locomotor activity in well-habituated rats (Filip et al., 2006), it decreases locomotor activity

in novel environments (Rimondini et al., 1998; Karcz-Kubicha et al., 2003) and attenuates the hyperlocomotion induced by cocaine and amphetamine (Turgeon et al., 1996; Filip et al., 2006). Further, while CGS-21680 appears to lack intrinsic rewarding or aversive properties (Brockwell and Beninger, 1986), it slows the initiation of responding for intravenous cocaine infusion (Knapp et al., 2001), and elevates threshold for electrical brain stimulation reward (Baldo et al., 1999). Of particular interest are demonstrations that the enduring behavioral sensitization produced by a brief regimen of daily passive injection of cocaine or amphetamine is attenuated if CGS-21680 is co-administered with each “sensitizing” dose (Shimazoe et al., 2000; Filip et al., 2006).

Considering the evidence of heteromerization, cotrafficking, and opponent cellular and behavioral effects of striatal A_{2A} and D₂ receptors, the present study was conducted to test the hypothesis that chronically FR rats are hypersensitive to cellular and behavioral effects of CGS-21680.

2. Results

2.1 Experiment 1: Effects of CGS-21680 on Horizontal and Vertical Motor Activity in Ad Libitum Fed and Food-Restricted Rats

Horizontal Activity—Figure 1 displays group mean horizontal (top panel) activity for AL and FR rats following administration of vehicle and two doses of CGS-21680. The ANOVA revealed a significant main effect of drug dose ($F_{2,44} = 12.53$, $p < .0001$), no main effect of feeding condition ($F_{1,22} = 0.8$) and a significant interaction between feeding condition and drug dose ($F_{2,44} = 4.84$, $p < .013$). Comparisons between AL and FR rats yielded a significant difference only at the 0.25 nmol dose ($t_{22} = 2.906$, $p < .017$; $t_{22} = -1.041$ and 1.307 for the vehicle and 1.0 nmol dose, respectively). The within-subjects single-degree polynomial contrasts indicated strong linear ($F_{1,22} = 7.677$, $p < .011$) and quadratic ($F_{1,22} = 19.202$, $p < .0001$) changes across drug doses. However, separate one-way ANOVA's for each feeding group showed that the dose-related changes were only significant for the FR group ($F_{2,22} = 16.228$, $p < .0001$; AL group $F_{2,22} = 1.209$). The FR group showed a significant decrease in activity from vehicle to both the 0.25 and 1.0 nmol doses ($F_{1,11} = 24.339$ and $F_{1,11} = 17.095$, $p < .002$, respectively).

Vertical Activity—Figure 1 (bottom panel) displays group mean vertical activity for AL and FR rats. The ANOVA revealed a significant main effect of drug dose ($F_{2,44} = 16.17$, $p < .0001$), no main effect of feeding condition ($F_{1,22} = 0.57$) and a significant interaction between feeding condition and drug dose ($F_{2,44} = 5.45$, $p < .008$). Between groups comparisons showed that the FR group displayed higher vertical activity than the AL group following vehicle injection ($t_{22} = -2.886$, $p < .017$) but the between group differences disappeared following drug injections ($t_{22} = 1.014$ and -0.928 for the 0.25 and 1.0 nmol dose, respectively). The within-subjects results paralleled those for horizontal activity (above) showing the linear ($F_{1,22} = 10.497$, $p < .004$) and quadratic ($F_{1,22} = 20.307$, $p < .0001$) changes across drug doses to be significant only in the FR group ($F_{2,22} = 14.371$, $p < .0001$; AL group $F_{2,22} = 2.971$, $p < .08$). The FR group displayed a significant decrease in vertical activity from vehicle to both the 0.25 nmol and 1.0 nmol doses ($F_{1,11} = 9.340$, $p < .011$).

2.2 Experiment 2: Effects of CGS-21680 on Wheel Running in Food-Restricted Rats

(a) Acquisition of wheel running in AL and FR rats—FR rats rapidly acquired wheel running and maintained a high rate across successive test sessions while AL rats did not (Figure 2). The ANOVA revealed a significant main effect of feeding condition ($F_{1,10} = 17.14$, $p < .002$), session ($F_{8,80} = 4.95$, $p < .0001$), and interaction between feeding condition and session ($F_{8,80} = 4.91$, $p < .013$). The polynomial contrasts indicated that most of the session and interaction effect could be accounted for by a linear trend over sessions ($F_{1,10} = 13.31$, $p < .004$).

and $F_{1,10}=14.80$, $p<.003$). Figure 2 clearly shows that the linear trend reflects an increase in wheel-revolutions for the FR group over sessions.

(b) Effects of CGS-21680—During the initial treatment phase, subjects injected with vehicle displayed a relatively high rate of wheel running, while subjects treated with CGS-21680 did not acquire the wheel running response (Figure 3). With the cessation of the CGS-21680 treatment, this latter group began to display wheel running, but rates were substantially lower than initially displayed by vehicle-treated subjects (main effect of treatment $F_{1,12}=30.44$, $p<.0001$; main effect of sessions $F_{1,12}=4.56$, $p<.055$; no interaction). When group treatments were reversed in the second phase of the experiment, CGS-21680 eliminated wheel running in the group that had acquired a high running rate (main effect of time $F_{2,24}=9.78$, $p<.001$). There was no main effect of treatment but a crossover interaction was found ($F_{2,24}=12.13$, $p<.0001$). The within-group ANOVAs revealed that for the group of rats which had initially acquired high rates of wheel of running (129 revolutions/30 min), administration of CGS-21680 suppressed the behavior (12 revolutions/30 min; $F_{3,15}=14.33$, $p<.0001$). However, with the termination of CGS-21680 treatment, wheel running substantially recovered, although not to the previous level (86 revolutions/30 min; contrasts between adjacent means: $F_{1,5}=16.44$, $p<.01$ and $F_{1,5}=21.30$, $p<.006$, respectively). In contrast, rats initially treated with CGS-21680 displayed a relatively low rate of wheel running following cessation of treatment with CGS-21680 (49 revolutions/30 min; $F_{3,21}=4.38$, $p<.015$) that did not increase further during the six CGS-free sessions of the second phase of testing (no difference between adjacent means).

2.3 Experiment 3: Effects of CGS-21680 on Striatal ERK 1/2 and CREB Phosphorylation in Ad Libitum Fed and Food-Restricted Rats

ERK 1/2—In CPu, the ANOVA revealed a main effect of drug treatment ($F_{1,18}=4.75$, $p<.05$), with no main effect of diet ($F_{1,18}=2.4$, $p>.10$), and no interaction between factors ($F_{1,18}=0.04$). Both diet groups displayed an approximate doubling of the optical density of pERK 1/2 bands (data not shown). In NAc, there was no main effect of drug treatment ($F_{1,18}=1.2$), no main effect of diet ($F_{1,18}=0.0$), and no interaction ($F_{1,18}=2.2$, $p>.10$).

CREB—In CPu, the ANOVA revealed a main effect of drug treatment ($F_{1,18}=13.8$, $p<.01$), with no main effect of diet ($F_{1,18}=0.02$), and no interaction between factors ($F_{1,18}=0.19$). Both diet groups displayed a 4–5-fold increase in the optical density of bands corresponding to pCREB (Figure 4). In NAc, there was no main effect of drug treatment ($F_{1,18}=0.5$), no main effect of diet ($F_{1,18}=1.5$), but a significant interaction between factors ($F_{1,18}=7.8$, $p=.01$). Pair-wise comparisons revealed that CREB phosphorylation in the FR group treated with CGS-21680 was greater than in the FR group treated with vehicle ($t(18)=3.0$, $p<.01$), the AL group treated with CGS-21680 ($t(18)=3.2$, $p<.01$), and the AL group treated with vehicle ($t(18)=2.5$, $p<.025$).

3. Discussion

CGS-21680 is a selective adenosine A_{2A} receptor agonist which has been used in many of the cornerstone studies establishing the striatal cellular and behavioral functions regulated by this adenosine receptor type and its opponent relationship with the D_2 DA receptor (Moreau and Huber, 1999; Fuxe et al., 2003). To evaluate A_{2A} receptor sensitivity in the FR rat, CGS-21680 was injected into the brain ventricular system. The i.c.v. route of administration was chosen in consideration of the variety of physiological changes that are known to alter pharmacokinetics and bioavailability of systemically administered drugs in FR subjects (Angel, 1969; Gugler et al., 1974; Ma et al., 1980). This approach was used previously to demonstrate that enhanced rewarding and motor-activating effects of abused drugs in FR rats can be attributed to an

increase in CNS sensitivity (Cabeza de Vaca and Carr, 1998). Results of Experiment 1 indicate that although well-habituated AL and FR rats do not differ with regard to basal horizontal motor activity, FR rats are nevertheless uniquely sensitive to the activity-decreasing effect of CGS-21680. Basal vertical activity, on the other hand, was significantly greater in FR than AL subjects. Increased vertical activity is also characteristic of animals placed in an environment where they have previously received cocaine (Beninger and Herz, 1986) and is selectively increased by food deprivation in amphetamine-treated rats (Cole, 1980). Vertical activity can be induced, concomitant with increased feeding, by infusing DA into the NAc shell (Swanson et al., 1997). CGS-21680 selectively decreased vertical activity in FR subjects, restoring this behavior to the level observed in AL subjects (whether injected with vehicle or CGS-21680). These results indicate that excessive rearing –a possible indicator of increased appetitive arousal– is inhibited by A_{2A} receptor stimulation in FR subjects and suggest that striatal neuroadaptations in FR subjects may extend beyond DA receptors to the opponent A_{2A} receptors.

Previous studies have indicated that CGS-21680 can diminish behavioral responsiveness to the psychostimulants, cocaine and amphetamine (Turgeon et al., 1996; Knapp et al., 2001; Filip et al., 2006). In Experiment 2, we therefore evaluated whether the A_{2A} receptor agonist diminishes a spontaneous behavior that is preferentially or uniquely expressed by FR rats and is inherently reinforcing, considered addictive, and of potential clinical importance with regard to compulsive exercise in anorexia nervosa. Most prior studies of rodent wheel running behavior, including its potentiation by FR, have involved 24 hour access to a running wheel in the home cage (e.g., Afonso and Eikelboom, 2003; Kanarek et al., 1995; Mueller et al., 1997). In such studies, free-feeding animals will run, with running being typically observed during the dark photoperiod (Edmonds and Adler, 1977; Sherwin, 1998). When meals are scheduled, running typically precedes initiation or scheduled delivery of a meal, suggesting a mediating or modulating role of appetitive arousal (Morse et al., 1995). Results of Experiment 2a demonstrate that when provided with a limited 60-min period of wheel access on any given day, outside of the home cage, FR rats reliably and uniquely acquire wheel running behavior, which escalates over several sessions to a stable high rate. In this protocol AL subjects consistently fail to acquire the behavior. This variant of the wheel running protocol brings into sharp contrast the behavioral differences between AL and FR subjects and is likely to reflect the sensitization of brain appetitive motivational/reward circuitry that also expresses, behaviorally, as increased drug reward magnitude (Carroll and Meisch, 1984; Carr, 2006). As would have been predicted based on the inhibition of excessive rearing in Experiment 1 and the inhibitory effects on psychostimulant-induced behavioral responses, CGS-21680 blocked acquisition of wheel running in FR subjects that received i.c.v. injections prior to each of their first three test sessions. Interestingly, though these subjects were tested CGS-free in nine subsequent sessions over a three week period, they never acquired a level of wheel running as high as a matched group of subjects that were pretreated with saline vehicle prior to their first three acquisition sessions. This enduring inhibition of wheel running may represent an enduring effect on underlying CNS circuitry. Alternatively, it may reflect the necessity of novelty during initial acquisition sessions in order for high levels of this behavior to develop. These are among the alternative explanations that can be explored in future studies. However, in the second group of FR rats that had already acquired a high rate of wheel running, the behavior was suppressed by CGS-21680 but displayed good recovery in the subsequent drug-free sessions. This suggests that the A_{2A} agonist may not produce enduring changes in the underlying neural circuitry, unless neuroadaptations are dependent upon an interaction between drug and behavioral history of the subject. In any event, it is noteworthy that a dose of CGS-21680 that decreases horizontal and vertical motor activity of FR rats to levels that are not appreciably different from the CGS-free activity levels of AL rats, profoundly inhibits the acquisition and expression of wheel running behavior. These behavioral findings suggest that not only are DA mechanisms that promote exploration, incentive motivation, and reward-related learning

adaptively upregulated in FR subjects (Carr, 2006), but so is an opponent system that has the potential to dampen the former. A potential implication of clinical importance is that pathological DA-mediated or -modulated behaviors in FR subjects (e.g. compulsive exercise, drug abuse) may be subject to moderation by targeting the A_{2A} receptor. The potential value of targeting this primarily striatal receptor type to treat other DA-related disorders, including schizophrenia, Parkinson's disease, and drug addiction, has been advocated (Moreau and Huber, 1999; Ferre et al., 2004).

A wide range of abused drugs activate striatal ERK 1/2 upon acute administration (Valjent et al., 2004). I.c.v. injection of the D-1 DA agonist, SKF-82958, produced enhanced activation of striatal ERK 1/2 but not adenylyl cyclase in FR relative to AL rats (Carr et al., 2003; Haberny et al., 2004). In agreement with results obtained in primary striatal cultures and neuroblastoma cells (Canals et al., 2005), i.c.v. injection of CGS-21680 in Experiment 3, activated ERK 1/2 in CPu. However, no difference was observed between feeding groups. In NAc, this dose of CGS-21680, which had selectively decreased vertical activity and acquisition of wheel running in FR rats in Experiments 1 and 2, respectively, was without effect on ERK 1/2 signaling. These results indicate that changes in ERK 1/2 signaling neither mediate nor correlate with the behavioral responsiveness of FR rats to CGS-21680. In these same subjects, CGS-21680 strongly activated CREB in CPu, but again, no difference was observed between feeding groups. However, in NAc, CGS-21680 selectively activated CREB in FR rats. This result is therefore of particular interest in so far as it is unique to FR rats, applies to the selective inhibition of vertical activity and wheel running, and is localized to the NAc in which CGS-21680 microinjection inhibits locomotor activity (Hauber and Munkle, 1997) and electrical brain stimulation reward (Baldo et al., 1999).

CREB is a downstream target of multiple signaling pathways including cAMP, MAPK and CaMK II (Dash et al., 1991; Choe and McGinty, 2001). Aside from the likely exclusion of ERK 1/2, the present results do not allow identification of the upstream signaling pathway that is activated by CGS-21680 in NAc of FR rats. However, the well documented coupling between the striatal A_{2A} receptor and the cAMP-PKA-CREB cascade (Ferre et al., 1997; Svenningsson et al., 1998) suggests that this is the pathway that is more strongly activated in FR subjects. While the behavioral and pCREB results seem to suggest that A_{2A} receptors in NAc may be hypersensitive in FR subjects, it is also possible that their increased responsiveness is an indirect result of changes in local DA transmission. It has been shown that in normal rats, striatal A_{2A} receptor stimulation only weakly activates cAMP, c-fos, and preproenkephalin gene expression due to the tonic inhibitory effect of DA at the D-2 receptor. However, these responses can be significantly increased if extracellular DA concentrations are decreased by pharmacological treatment (Karcz-Kubicha et al., 2006). Interestingly, measurements of basal extracellular DA concentrations (Pothos et al., 1995) and DOPA accumulation following administration of an aromatic amino acid decarboxylase inhibitor (Pan et al., 2006) suggest that basal DA transmission may be subnormal in NAc of FR rats. If so, this could support a DA-based mechanism for disinhibition of A_{2A} receptor responsiveness. pCREB increases the electrical excitability of NAc neurons and, in doing so, is believed to decrease behavioral responsiveness to psychostimulants (Dong et al., 2006). This mechanism could therefore play a role in the unique behavioral response of FR subjects to CGS-21680. However, it is also the case that CREB couples the A_{2A} receptor to transcription of c-fos and preproenkephalin (Agnati et al., 2003). Transcriptional responses to CGS-21680 in FR rats may mediate behavioral changes that emerge later or are more enduring. The continued low wheel running behavior of FR rats treated in their first three test sessions with CGS-21680 does raise the possibility of induced neuroadaptations that exert a long term effect on wheel running behavior. Whether the enhanced activation of CREB and correspondingly enhanced transcription of preproenkephalin play a role in this enduring behavioral change is amenable to investigation in future studies. Interestingly, striatal preproenkephalin gene expression is decreased in rats

that have had repeated access to highly palatable solution or cocaine (Dunais et al., 1997; Kelley et al., 2003) – treatments that can produce behavioral sensitization and cross-sensitization (Avena and Hoebel, 2003; Gosnell, 2005). An upregulation of preproenkephalin gene expression in NAc, stimulated by A_{2A} receptor activation, but enabled by a decrease in basal DA transmission, may represent a mechanism for dampening the otherwise enhanced incentive-motivating/positive reinforcing effects of food, drugs, and exercise in FR subjects.

4. Experimental Procedures

4.1 Subjects and surgical procedures

All subjects were mature male Sprague-Dawley rats (Taconic Farms, Germantown, NY) initially weighing 375–425 g. Food (pelleted Purina rat chow) and water were available ad libitum (AL) except when FR conditions applied. Animals were individually housed in clear plastic cages with bedding under a 12 h light:dark photoperiod with lights on at 0700 h. Each animal (with the exception of those in Experiment 2a) was deeply anesthetized with ketamine (100 mg/kg; i.p.) and xylazine (10 mg/kg; i.p.) and stereotactically implanted with a 26-gauge guide cannula (inner diameter: 0.24 mm; Plastics One, Roanoke, VA, USA) in the right lateral ventricle using the following coordinates with bregma and lamda suture landmarks in the same horizontal plane: 1.0 mm posterior to bregma, 1.5 mm lateral to the mid-sagittal suture, and 3.5 mm ventral to skull surface. The cannula was permanently affixed to the skull by flowing dental acrylic around it and four surrounding mounting screws. Patency of the guide cannula was maintained with an occlusion stylet. Five to seven days after surgery, intraventricular cannula placement was verified by a short latency, sustained drinking response to microinjection of angiotensin II (50 ng in 5 µl).

4.2 Food restriction and habituation

The day following angiotensin testing, half the subjects were switched to a restricted feeding regimen in which a single 10 g meal was delivered at approximately 1700 h each day. Rats continued to have AL access to water. Once body weight had declined by 20% (approximately 14 days) daily food allotments were titrated to maintain this target body weight for one additional week. During this approximately 3 week period all rats were habituated to the handling and microinjection (mock) procedures to be employed periodically during Experiments 1 and 2b and on the terminal day of Experiment 3. Rats to be used in Experiment 1 (spontaneous locomotor activity) were habituated on six occasions, for 30-min periods, to the test chamber in which testing was to take place. Rats to be used in Experiment 2 (wheel running) were similarly habituated to the test chamber but with the removable running wheel absent.

4.3 Drug treatment

In Experiment 1, repeated measurements were taken on all subjects, which received intracerebroventricular (i.c.v.) injections of the highly selective A_{2A} receptor agonist CGS-21680 (Jarvis et al., 1989; Sigma-Aldrich, St. Louis, MO, USA) at doses of 0.0, 0.25 and 1.0 nmol in 5 µl 0.9% sterile saline five minutes before the initiation of behavioral testing. The 1.0 nmol dose was selected as the highest to be administered based on the report that a 2.0 nmol dose produced a moderate decrease in spontaneous locomotor activity of rats in a novel environment (Janusz and Berman, 1992). In Experiment 2b, repeated measurements were taken on all subjects, which received i.c.v. infusions of CGS-21680 (0.0, 1.0 nmol in 5 µl saline) five minutes before the initiation of behavioral testing. In Experiment 3, half the subjects in each feeding condition received i.c.v. injection of CGS-21680 (1.0 nmol in 5 µl saline) and half received i.c.v. injection of saline vehicle, 20 min prior to sacrifice. For i.c.v. injection, solutions were loaded into a 30 cm length of PE-50 tubing attached at one end to a 25-µl Hamilton syringe filled with distilled water and at the other end to a 33-gauge injector cannula (inner diameter:

0.10 mm) which extended 1.0 mm beyond the implanted guide. The 5.0 μ l injection volume was delivered over a period of 95 s. One minute following injection, injector needles were removed, stylets replaced, and animals were returned to home cages.

4.4 Measurement of horizontal and vertical motor activity

Apparatus—Motor activity was monitored in four identical Lucite cages ($42 \times 42 \times 30$ cm) via a grid of 16×16 infrared light beams that traverse each animal cage front to back and left to right. An additional 16-beam sensors monitor vertical activity. Information about beam status, scanned at a rate of 100 times per second, was stored to disk and later transformed into a complete record of each animal's activity (VersaMax System, Accuscan, Columbus, OH). The two main variables, horizontal and vertical activity, were expressed as the number of beam interruptions as a function of time in a session.

Test Sessions—Each test session was preceded by a within-session habituation period whereby each rat first received a mock i.c.v. injection (i.e. held gently in a foam cushion for 3 min while stylets were removed and then returned to guide cannulas) followed by placement in the activity chamber for a period of 30 min. This was immediately followed by brief removal of the subject, an actual i.c.v. microinjection (see above) and, 5-min after completion of the microinjection, return to the activity chamber for a 30 min period of monitored activity. In the first and final test sessions, all subjects received i.c.v. injections of saline vehicle. In the two intervening test sessions they received 0.25 and 1.0 nmol of CGS-21680 with half the rats in each feeding group receiving the lower dose first and the remaining half receiving the higher dose first. Each of the two feeding groups (i.e. AL and FR) contained twelve subjects. Test sessions were run between 1000 h and 1500 h and 3–4 days apart.

Data Analysis—For each rat and session, horizontal and vertical activity counts following i.c.v. injection were recorded and analyzed separately. Results of the two saline-vehicle test sessions were averaged to produce one control value for each measure for each subject. To evaluate the effects of feeding condition and drug dose, scores were subject to a 2-way (2×3) mixed analysis of variance (ANOVA) with repeated measures on the drug dose factor. A significant interaction was followed by planned comparisons between groups (AL vs FR) using t-tests with the pooled error term from the ANOVA in the denominator and the significance level adjusted for multiple comparisons ($p < .017$, $\alpha/3$). Significant linear trends on the drug dose factor were followed by separate one-way repeated measures ANOVA and planned comparison between CGS-21680 doses (vehicle vs 0.25 nmol and vehicle vs 1.0 nmol) where appropriate.

4.5 Measurement of wheel running

Wheel Running Test Sessions—The wheel running test system was the VersaMax system used to test locomotor activity (see above), with the addition of a running wheel (26 cm diameter, Accuscan, Columbus, OH) suspended from the right interior wall of each activity chamber. Once the wheel was in position, the Versamax software automatically recorded wheel turns, as well as the beam interruptions described above.

Experiment 2a: spontaneous wheel running in ad libitum fed and food-restricted rats—Twelve naïve rats, none of which had been subject to stereotaxic surgery, were randomly assigned to AL ($n = 6$) and FR ($n = 6$) feeding conditions. Once the habituation training was completed and target body weights were stabilized, the running wheels were introduced and behavioral testing initiated. Subjects were placed in the test chamber for 60-min test periods on nine occasions separated by 2–3 days, and the total number of wheel rotations per session were automatically recorded.

Data Analysis—To evaluate the effects of feeding condition on wheel running, wheel running totals for each rat in each session were subject to a 2-way (feeding x session) mixed ANOVA with repeated measures on the session factor. A significant interaction between factors was followed by pair-wise comparisons using a t-statistic, using the pooled error term from the ANOVA in the denominator.

Experiment 2b: Effects of CGS-21680 on wheel running in food-restricted rats

—Based on the observation in Experiment 2a that only FR rats acquired wheel running behavior, all subjects in Experiment 2b (n=14), were placed on the FR regimen following recovery from stereotaxic implantation of the lateral ventricular cannula and verification of the drinking response to angiotensin II injection. Once the habituation training was completed and target body weights were stabilized, the running wheels were introduced and behavioral testing was initiated. Subjects were placed in the test chamber for 30-min periods and the total number of wheel rotations per session was automatically recorded. During the initial phase of the experiment, consisting of six test sessions, half of the rats were injected (i.c.v.) with saline-vehicle while the other half received i.c.v. injections of CGS-21680 (1.0 nmol in 5 μ l) prior to each of the first three test sessions. For the next three sessions, rats did not receive i.c.v. injections but were placed directly in the test chambers. In the second, “reversal”, phase of the experiment, also consisting of six test sessions, i.c.v. treatments were reversed such that for the next three sessions, rats previously exposed to saline vehicle received CGS-21680 while rats previously treated with CGS-21680 received saline vehicle. Three additional sessions with no i.c.v treatment followed.

Data Analysis—Wheel running totals for each rat were averaged over each block of three consecutive sessions yielding one score per rat per block. The effects of CGS-21680 on acquisition of wheel running were evaluated during the initial phase with a mixed ANOVA with repeated measures on sessions. A separate treatment x sessions mixed ANOVA evaluated CGS-21680 effects during the reversal phase.

4.4 Measurement of striatal pERK and pCREB

Treatment—Following recovery from surgery, habituation training, and stabilization of target body weights in FR subjects, the ten AL and eleven FR subjects in this experiment received their terminal treatment. Approximately half of the subjects in each feeding group received i.c.v. injection of CGS-21680 (1.0 nmol in 5 μ l) and half received i.c.v. injection of saline vehicle.

Lysate preparation—All rats were sacrificed 20 minutes after injection by brief exposure to CO₂ followed by decapitation. Brains were rapidly removed and immediately frozen. 500- μ m sections were cut using an IEC Minotome cryostat, and CPu and NAc were micropunched, under an Olympus dissecting microscope, from a series of 8 consecutive frozen sections. The tissue was then homogenized in 10 volumes of 50 mM Tris-HCl, pH 7.4, 300 mM NaCl, 1% Nonidet P-40, 10% glycerol, 1 mM EDTA, and manufacturer recommended concentrations of Phosphatase Inhibitor Cocktails I and II and Mammalian Protease Inhibitor Cocktail (all from Sigma). The homogenates were centrifuged at 30,000 $\times$ g for 20 min at 4°C, and the non-solubilized fraction discarded. Lysates were then mixed with 4x SDS-PAGE Sample Buffer containing 20 μ M DTT, boiled for 5 minutes, and unused denatured lysate was stored in sample buffer in aliquots at -80°C. The protein content was determined by the Bradford assay using bovine serum albumin as standard.

Western Blotting—Protein (10–30 μ g per lane) was separated by electrophoresis on precast 10% polyacrylamide gels (Cambrex, East Rutherford, NJ, USA). Precision Plus protein standard molecular weight markers (Bio-Rad, Hercules, CA, USA) were also loaded to assure

complete electrophoretic transfer and to estimate the size of bands of interest. The gels were transferred to nitrocellulose membrane (Osmonics) for 2 h, with a constant voltage of 100 volts. Membranes were blocked for 1 hr at room temperature with blocking buffer, 5% non fat dry milk in 50 mM Tris-HCl, pH 7.5 containing 150 mM NaCl and 0.1 % Tween 20 (TBS-T), then probed overnight at 4°C using primary monoclonal antibodies for phospho-(Thr202/Tyr204)-p44/42 ERK1/2 (mouse monoclonal, 1:2000; Cell Signaling, Beverly, MA, USA), or phospho (Ser 133) CREB (rabbit polyclonal, 1:2000; Upstate Biotechnology, Lake Placid, NY, USA). Total levels of ERK1/2 and CREB were detected on the same blots using anti-rabbit p42/44 ERK1/2 antibody 1:2000, (Cell Signaling) or anti-rabbit CREB antibody (1:2000 dilution, Calbiochem). After detection of phosphorylated ERK1/2 and CREB blots were stripped with 25 mM Glycine, pH 2.0 containing 1% SDS for 30 min at room temperature, washed six times in TBS-T buffer, blocked in blocking buffer for 1 h and then incubated overnight at 4°C in total ERK1/2 or CREB antibody. After probing with primary antibodies and washing with TBS-T buffer (3×5 min), membranes were incubated with 1:2000 dilution horseradish peroxidase conjugated anti-mouse or 1: 2000 dilution anti-rabbit IgG (Cell Signaling). Proteins were visualized using a chemiluminescence ECL kit (Pierce). Densitometric analysis of the bands was performed using NIH image software. Phospho-p42/44 MAPK and phospho CREB values were normalized to total p42/44 MAPK and CREB values, respectively. In addition, tubulin levels were analyzed in several representative gels, and no differences were observed between treatment groups.

Data Analysis—For each Western blot, film exposure time was set as needed to visualize distinct bands in the control samples (AL subjects injected i.c.v. with saline vehicle) of each experiment. Results were expressed by comparison to the normalized control. Differences between treatment conditions were analyzed by two-way ANOVA (feeding condition x injection treatment). In the case of significant interaction between factors, a t-statistic, using the error term from the ANOVA in the denominator, was used to compare cell means of interest.

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Figure 1.

Effects of intracerebroventricular (i.c.v.) injection of the adenosine A_{2A} receptor agonist, CGS-21680, on horizontal (top) and vertical (bottom) motor activity in ad libitum fed (filled circles) and chronically food-restricted (open circles) rats. Repeated measurements were taken on twenty four subjects (total), with each receiving the 0.0, 0.25 and 1.0 nmol doses in sessions spaced 3–4 days apart; dose order was counterbalanced across subjects and matched between feeding groups. Motor activity is expressed as total number of photobeam interruptions per 30-min test session (mean and SEM). CGS-21680 selectively decreased both measures of spontaneous motor activity in food-restricted subjects, including a reversal of the enhanced vertical activity (rearing) otherwise observed in these subjects. See text for data analysis.

Note: * = significant difference between groups (AL vs FR); # = significant difference from vehicle condition.

Figure 2.

Six ad libitum fed (filled circles) and six chronically food-restricted (open circles) rats were placed in an activity chamber with a running wheel suspended from an interior wall on nine occasions spaced 2–3 days apart. The total number of wheel revolutions (mean $\pm$ SEM) per 60-min acquisition session were recorded. Food-restricted rats rapidly acquired wheel running behavior while ad libitum fed rats did not. See text for data analysis.

CGS-21680 and WHEEL RUNNING

Figure 3.

Acquisition and maintenance of wheel running behavior in two groups of food-restricted rats ($n=7$ each) tested in a series of twelve 30-min test sessions spaced 2–3 days apart with results collapsed according to experimental phase and treatment as follows: During the initial phase, rats were injected 5 min prior to each of the first three test sessions with saline vehicle (5 μ l, i.c.v.) or CGS-21680 (1.0 nmol in 5 μ l, i.c.v.). For the next three test sessions, rats were tested without i.c.v. pretreatment. During the reversal phase, treatments were reversed for the first three test sessions, followed by three additional test sessions without i.c.v. pretreatments. Data are group means ($\pm$ SEM) for each block of three test sessions. Symbols indicate the i.c.v. pretreatment (i.e. VEH or CGS) each group received during the initial and reversal phases of the experiment. For each group, the filled symbol marks the block of sessions in which CGS-21680 was administered. Initial treatment with CGS-21680 blocked acquisition of wheel running and depressed acquisition during the subsequent nine drug-free sessions. In previously vehicle-treated subjects that had acquired a high rate of wheel running, CGS-21680 suppressed the behavior, which substantially recovered in drug-free sessions that followed. See text for data analysis.

Figure 4.

Ad libitum fed and food-restricted rats received i.c.v. injections of CGS-21680 (1.0 nmol in 5 μ l; black bars) or saline vehicle (gray bars) 20 min prior to sacrifice. Lysates prepared from nucleus accumbens (top) and caudate-putamen (bottom) were immunoblotted with anti-phospho-CREB or anti-CREB antibodies. Following densitometry, intensities of bands corresponding to phosphorylated proteins were divided by intensities of the corresponding total protein bands to correct for small differences in protein loading. Results (mean $\pm$ S.E.M.) are expressed in comparison to the normalized control, which was defined as the ad libitum fed group injected with vehicle. Graphed results are displayed with representative immunoblots ("43kda" indicates the band corresponding to pCREB). See text for data analysis.

Note: * = significant difference between groups (vehicle vs CGS-21680)