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Agmatine attenuates hyperactivity and weight loss associated with activity-based anorexia in female rats

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ABSTRACT

Anorexia nervosa is a debilitating eating disorder characterized by hypophagia, body weight loss, amenorrhea and intense fear of weight gain. In present study, the effect of subchronic agmatine treatment on development of activity based anorexia (ABA) in female rats has been investigated. Animals were injected with saline or agmatine (10–40 mg/kg, ip) just before the onset of dark phase and shifted to experimental cage with wheel for ABA test for 10 days. A pre-weighed quantity of food pellets (10 g) was placed daily for a restricted period of only 2 h (1700–1900 h) and food intake was monitored (g) manually by weighing the leftover food. Rats restricted to ABA paradigm, showed greater wheel running, suppressed food consumption, disrupted estrous cycle and weight loss. On the other hand, subchronic agmatine (10–40 mg/kg, ip, for 10 days) treatment decreased wheel running activity, pronounced increased in food intake and restored body weights as compared to saline treated animals. Further, agmatine treatment decreased corticosterone levels in ABA rats, thereby stabilizing HPA axis in ABA rats. Subchronic agmatine treatment also prevented the disruptions of estrous cycle. Considering the common resistance of anorexia nervosa to current pharmacotherapy, the preliminary data on reduction of physical activity by agmatine, may have potential therapeutic importance. Thus, the role of agmatine in feeding behavior is likely to provide insight into the circumstances that facilitate treatment in eating disorders like anorexia nervosa.

1. Introduction

Anorexia nervosa (AN) is a debilitating eating disorder characterized by hypophagia, body weight loss, amenorrhea and intense fear of weight gain. This eating disorder is particularly prevalent in young women than men. It is associated with high rates of depression, perfectionism, obsessive behavior and has the highest mortality rates among all psychiatric disorders (Bulik et al., 2007). In other words AN is characterized by self-imposed starvation and obsessive fear of obesity (Becker et al., 2009; Kaye et al., 2009; Klein et al., 2004). Thus, patients are motivated to restrict their eating, particularly the consumption of highly palatable, high energy density foods and continue to avoid till they get severely underweight (Klein et al., 2004). In addition to eating restraint, hyperactivity is featured in up to 75% of AN patients (Hebebrand et al., 2003). Indeed, excessive exercise has been reported to precede, follow, or coincide with the onset of strict dieting/food restriction (Davis and Kaptein, 2006). In this sense, hyperactivity not only promotes the progression, but also likely impedes the successful treatment and recovery of AN (Carter et al., 2004).

Although abnormalities of serotonergic system have been implicated in the development and persistence of AN in women, the treatment with SSRIs proved unsuccessful (Kaye et al., 1998). In contrast, dysregulation of reward and mood related systems have been identified in AN patients (Kaye et al., 2009). The dopaminergic (DA) system that regulates reward processing, movement, and feeding behavior has been reported to alter in AN patients. These patients exhibit reductions in homovanillic acid, a major metabolite of DA (Kaye et al., 1999) and increased DA D2 and DA D3 receptor binding sites (Frank et al., 2005). Furthermore, polymorphisms in DA D2 receptor are associated with AN (Bulik et al., 2005; Burden et al., 1993; Monteleone and Maj, 2008). Recent findings suggest that drugs targeting at DA receptors may be effective in treating AN. Several open label studies have reported that the treatment with atypical antipsychotics increases body weight and reduces hyperactivity and anxiety about eating and body shape in AN patients (Barbarich et al., 2004; Dennis et al., 2006; Leggero et al., 2010). However, such treatments reduced obsession about weight gain while increasing the rate of weight gain and rate of relapse in AN.

Neuroendocrinological studies in AN patients have found normal homeostatic physiological responses to starvation like elevated levels of orexigenic peptide, NPY and reduced levels of anorexigenic, CART and leptin in their CSF (Misra and Klibanski, 2010). Although no medication have been approved by FDA for treatment of AN, standard treatment for AN consist of nutritional rehabilitation, psychotherapy and adjunctive pharmacotherapy. However, eating disorders require comprehensive therapy with drugs having multidimensional activity.

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Recently, research has focused on the potential therapeutic actions of agmatine as it promotes orexigenic signals, induces antistress activity, reduces hyperactivity induced by psychostimulants and improves psychiatric disorders (Prasad and Prasad, 1996; Zhu et al., 2008; Iyo et al., 2006; Wang et al., 2006; Taksande et al., 2011; Kotagale et al., 2010). Agmatine has broad receptor bindings profile and implicated in several pharmacological actions and physiological process (Auguet et al., 1995; Reis and Regunathan, 2000; Halaris and Plietz, 2007; Yang and Reis, 1999). Thus, in view of its important role in feeding behavior and energy homeostasis, the present study may open new areas for therapeutic intervention of AN.

Activity based anorexia (ABA) is an animal model of AN in which rats are allowed free access to running wheels (RW) but restricted to 2 h food access per day. Rats exposed to this way display symptoms similar to those seen in anorexic women. These include paradoxical hyperactivity, hypophagia, extreme weight loss and estrous cycle disruption. Conversely, rodents given either restricted food access or running wheels maintain their body weight (Epling and Stefan, 1983; Routtenberg and Kuznesof, 1967). In present study, the effect of subchronic agmatine treatment on development of ABA in female rats has been investigated.

2. Materials and methods

2.1. Subjects

Sprague–Dawley female rats (age: 60 days and baseline body weight: 254.3 ± 4.5 g) were obtained from the National institute of Nutrition, Hyderabad, India. Animals were housed in acrylic cages (24 × 17 × 12 cm) under a constant room temperature (25 ± 2 °C), relative humidity (50 ± 5%), and maintained under a controlled 12:12 h light–dark cycle (light on at 0700 h). All the experimental procedures were approved and carried out under strict compliance with the Institutional Animal Ethics Committee, constituted for the purpose of control and supervision of experimental animals by the Ministry of Environment and Forests, Government of India, New Delhi, India.

2.2. Drug injections

Agmatine (Sigma-Aldrich, USA) was dissolved in saline and injected intraperitoneally (ip) (10, 20 and 40 mg/kg) daily for 10 days to rats subjected to activity based anorexia protocol.

2.3. Apparatus

A running wheel cage (VJ Instruments, India) consisted of a clear plastic made running wheel (36 cm in diameter, 11 cm width) and an adjoining plastic square cage (30.5 × 24 × 29 cm). The rats could enter the running wheel freely throughout the experiment. A feeding box was attached to the cage. The number of wheel revolutions was automatically recorded with the software attached computer system.

2.4. Experimental procedure

Female rats were individually housed in cages with running wheels for an adaptation period of 10 days (from day −10 to day 0). During this period, food and water were available ad libitum. Running wheel activity (RWA) was continuously registered by software developed by VJ Instruments, India. Food intake, wheel revolutions, body weight, and estrous cyclicity were monitored daily in all rats.

Since energy balance is known to fluctuate across estrous cycle, its phases were examined. Daily between 0900 and 1000 h, rats' vaginal cytology samples were collected. A cotton swab moistened with physiological saline was inserted into the vaginal canal and the mucosal samples were collected daily. Phases of estrous cycle (diestrus 1, diestrus 2, proestrus or estrus) were then determined microscopically by examining the appearance and abundance of leucocytes, nucleated/non-nucleated epithelial cells, cornified cells within each sample, as described previously (Atchley and Eckel, 2006; Becker et al., 2005; Eckel et al., 2000). Using this strategy, proestrus included the light phase peak in estradiol secretion, and estrus included the subsequent dark phase when female rats ovulate and display increased sexual receptivity, locomotor activity and food intake (Becker et al., 2005). At study onset, all rats displayed a regular 4-day estrous cycle and those that showed a minimum of 2 regular cycles were selected.

Following 10 days basal study, the animals that did not differ much in the food consumption and body weight were selected. Rats within a selected range were randomly divided into groups based on their body weight (n = 6).

Animal were injected daily with saline or agmatine (10–40 mg/kg, ip) just before the onset of dark phase and shifted to experimental cage with wheel for ABA test for 10 days. A pre-weighed quantity of food pellets (30 g) was placed daily for restricted period of only 2 h and thereafter food was removed from the cage hopper. Food intake was monitored (g) manually by weighing the leftover food immediately after 2 h. Animals were maintained on this restricted scheduled feeding between 1700–1900 h for 10 days. Body weight of all animals was measured daily just prior to drug administration. For ethical reasons, it was decided that rats were to be removed from the experiment when they lost more than 25% of their initial body weight.

2.5. Plasma corticosterone

As ABA demonstrated activation of HPA-axis, we determined the levels of hormone corticosterone. 24 h after last experimental day (day 11, 0900 to 1000 h) blood samples were collected by retro-orbital method in sodium citrate (4% w/v) rinsed tubes and centrifuged at 13000 × *g* for 15 min at 4 °C. Separated plasma was stored at −20 °C for corticosterone estimation. A quaternary gradient HPLC system equipped with Crestpak C18T-5 column and PDA detector (MD2010 plus) (Jasco, Japan) was used for quantification of plasma corticosterone. Briefly, 50 μl of plasma was extracted with 1 ml of DCM-Ether mixture (DCM:ether — 50:50) on mechanical shaker for 15 min. Supernatant (50 μl) was transferred to Eppendorf tube and evaporated under the slow stream of nitrogen. After complete evaporation, 1 ml of mobile phase (water:methanol — 80:20) was added and 20 μl of this was injected into the HPLC (Flow rate — 1 ml/min and estimated at 243 nm).

2.6. Data analysis

Body weight, food intake, wheel revolutions and corticosterone levels are expressed as mean ± SEM. For all measurements, baseline levels were not significantly different between all experimental groups. The significance of the changes as a consequence of the subchronic treatment with different agmatine doses was evaluated by two way ANOVA and Bonferroni test. Within group differences were analyzed using the Student's *t*-test. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Baseline phase

Prior to food restriction and drug treatment, no significant differences in mean daily food intake, wheel running, or body weight were detected between the groups. All rats displayed regular 4 day estrous cycles. The average body weight of animals was 254.3 ± 4.5 at the beginning of the protocol and did not change during baseline phase (days −10 to 0). Baseline RWA was determined as average RWA four days prior to the start of drug administration (day −4 to day 0) and found to be 2431 ± 134 revolutions. One way ANOVA comparing the average food intake of baseline phase revealed no significant difference

between the groups. The average food intake between the groups was 18.35 ± 2.4.

3.2. Induction of activity based anorexia in restriction feeding

3.2.1. Running wheel activity

As shown in Fig. 1, rats subjected to running wheels and food restriction drastically and progressively increased the running wheel activity (RWA) which became significant on day 3 of the protocol and thereafter sustained up to completion of the experiment. One way ANOVA revealed a significant increase in RWA from day 3 onwards [$F(10, 65) = 357.4$, $P < 0.0001$]. On day 10 of experiment the daily wheel running was found to be increased by 117% relative to that observed during baseline.

3.2.2. Body weights

Fig. 1 demonstrates restricted-feeding schedule decreased body weight of the rats. One way ANOVA revealed a significant reduction in body weight [$F(10, 65) = 147.09$, $P < 0.001$]. Application of post hoc Bonferroni test showed significant decrease in body weight of animals subjected to ABA on day 2 ($P < 0.01$) of the protocol and progressively decreased till the end of experiment. On the last day of experiment the body weights of the rats were decreased by 23% relative to that observed during baseline.

3.2.3. Food intake

As shown in Fig. 2, during the restricted feeding schedule, all animals decreased their food intake compared to baseline value of day 0. One way ANOVA revealed a significant reduction in food intake on all the experimental days [$F(10, 65) = 35.95$, $P < 0.001$]. Application of post-hoc Bonferroni test showed maximum decline in food intake on day 5 ($P < 0.001$) of the protocol. On the last day of experiment the daily food intake was found to be decreased by 84%, relative to that observed during baseline.

3.3. Estrous cycle

The estrous cycle disruptions as characterized by a failure to go into estrus and perpetual vaginal cytology indicative of diestrus were apparent in 22 out of 24 rats subjected to ABA.

3.4. Effect of agmatine on activity based anorexia

3.4.1. Running wheel activity

The daily injection of agmatine reversed the elevated RWA induced by food restriction in rats. Application of two way ANOVA revealed a significant interaction between agmatine treatment and duration in days [$F(27, 220) = 25.14$, $P < 0.001$], treatment [$F(3, 220) = 980.38$, $P < 0.001$] and days [$F(9, 220) = 632.11$, $P < 0.001$]. The post hoc Bonferroni test revealed significant reduction in RWA in agmatine treated (20 and 40 mg/kg, ip daily) rats subjected to ABA as compared to that in saline treated animals on day 2 onward. This resulted in reduce wheel running in agmatine treated rats relative to saline, on the final day of the experiment. This shows that repeated agmatine (20 and 40 mg/kg) treatment significantly attenuated the running wheel activity in rats maintained on food restriction phase. However, its lower dose (10 mg/kg) was ineffective (Fig. 3).

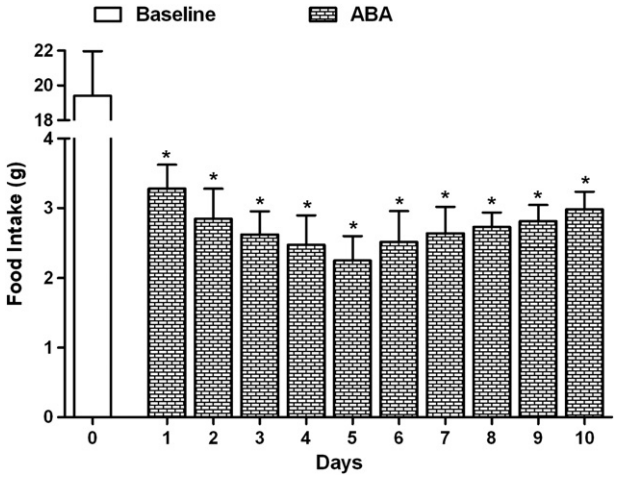

Fig. 2. Daily cumulative food intake in rats during food restriction. Results expressed as mean food intake (g) ± SEM (n = 6). *P < 0.001 vs baseline food consumption on day 0 (one way ANOVA post hoc Dunnett test).

3.4.2. Food intake

The agmatine (40 mg/kg, ip) treated food restricted rats showed significantly higher cumulative daily food intake than saline treated food restricted rats. Application of two-way ANOVA revealed an interaction between agmatine treatment and duration [$F(27, 220) = 0.14$, $P = 1.00$], treatment [$F(3, 220) = 39.87$, $P < 0.001$] and days [$F(9, 220) = 1.06$, $P = 0.39$]. The post hoc Bonferroni test revealed significant increased in food intake in agmatine treated (40 mg/kg, ip daily) rats subjected to ABA as compared to that in saline treated animals on day 2 onward ($P < 0.05$) and this effect was sustained up to the end of the

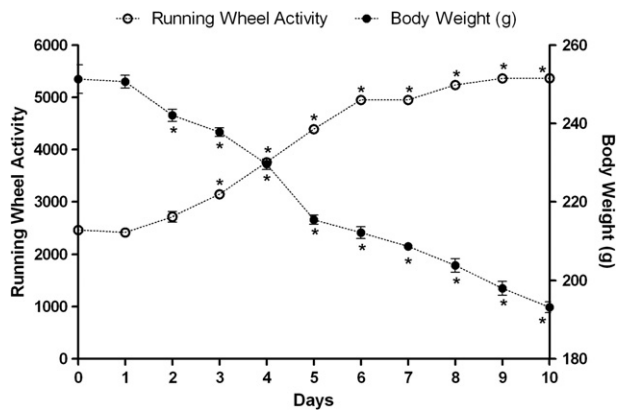

Fig. 1. Daily running wheel activity and body weights (g) in rats during food restriction. Results expressed as mean ± SEM (n = 6). *P < 0.01 vs baseline day 0 activity (one way ANOVA post hoc Dunnett test).

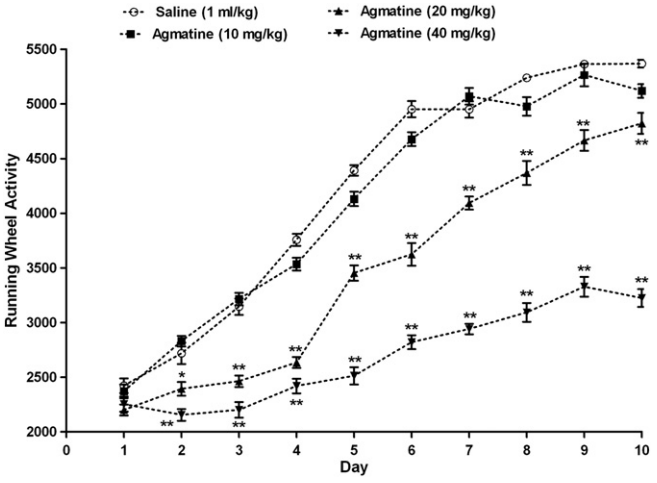

Fig. 3. Effect of agmatine (10, 20 and 40 mg/kg, ip) on running wheel activity in rats subjected to activity based anorexia. Results expressed as mean RWA ± SEM (n = 6). *P < 0.05, **P < 0.001 vs saline treated group (two way ANOVA post hoc Bonferroni test).

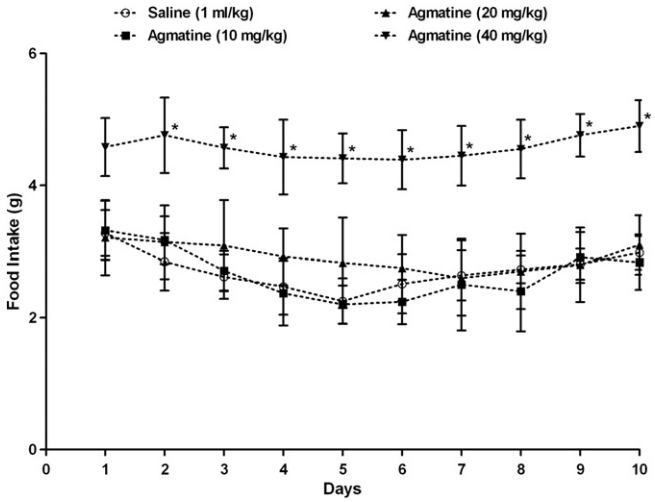

Fig. 4. Effect of agmatine (10–40 mg/kg, ip) on food intake in rats subjected to activity based anorexia. Results expressed as mean food intake (g) ± SEM (n = 6). *P < 0.001 vs saline treated group (two way ANOVA post hoc Bonferroni test).

protocol. However, its lower doses (10 and 20 mg/kg) were ineffective (Fig. 4).

3.4.3. Body weight changes

The pattern of changes obtained in the experiment of food intake, were entirely reflected on the body weight data (Fig. 5). While saline treated ABA rats showed persistent weight loss, subchronic agmatine treatment prevented it. Two-way ANOVA showed a significant interaction between variables agmatine treatment and duration in days [$F(27, 220) = 32.78$, $P < 0.001$], treatment [$F(3, 220) = 550.70$, $P < 0.001$] and days [$F(9, 220) = 429.14$, $P < 0.001$]. Subchronic agmatine (20 and 40 mg/kg, ip) treatment prevented the loss of body weights from day 4 onwards as compared to control rats. This shows that agmatine treated rats lost less weight than saline-treated rats during food restriction phase. However, its lower dose (10 mg/kg) was ineffective.

3.5. Estrous cycle

Repeated agmatine (40 mg/kg, ip) treatment normalized the disruptions of estrous cycle in all the rats subjected to ABA. These rats show the normal pattern of their estrous cycle during food restriction phase.

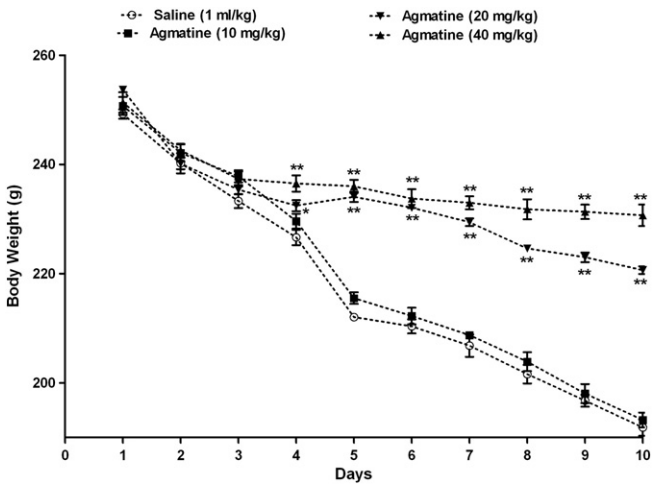

Fig. 5. Effect of agmatine (10–40 mg/kg, ip) on body weight in rats subjected to activity based anorexia. Results expressed as mean body weight (g) ± SEM (n = 6). *P < 0.01, **P < 0.001 vs saline treated group (two way ANOVA post hoc Bonferroni test).

However its lower doses (10 and 20 mg/kg) did not alter the estrous cycle disrupted by ABA.

3.6. Plasma corticosterone levels

As shown in Fig. 6, Subchronic agmatine [20 ($P < 0.05$) and 40 mg/kg ($P < 0.05$), ip)] treatment reversed the elevated levels of corticosterone in food restricted rats subjected to wheel running activity [$F(3, 21) = 4.89$, $P < 0.05$]. This demonstrated the normalization of HPA axis by agmatine. The corticosterone levels were not significantly different between saline and the lower dose of agmatine (10 mg/kg, ip).

4. Discussion

In agreement with previous reports (Atchley and Eckel, 2006; Liang et al., 2011), in the present study rats restricted to ABA paradigm, showed greater wheel running, weight loss, suppressed food consumption and disrupted estrous cycle. However, agmatine treatment significantly prevented the ABA as evident from pronounced increased food intake, restored body weights, decreased wheel running and resumption of estrous cycle as compared to saline treated animals. At the doses used in the study agmatine did not have any effect on locomotor activity (Taksande et al., 2010; Uzbay et al., 2010). Thus, the observed effect of agmatine could not be attributed to its locomotor component.

Activity based anorexia models the consequences of starvation and exercise behaviors. This experience with ABA could have long term physiological and neuronal effects that affect endocrine functions. For example, it has been demonstrated that rats with ABA experience during adolescence shows alterations in the HPA axis in adulthood (Burden et al., 1993). This is in agreement with the current study, as a result of restricted feeding and wheel running, the HPA axis is considerably activated in ABA rats. Sustained activation of HPA axis is associated with anorexia and abnormal body weight regulation. In our study, animals subjected to ABA show marked elevation in corticosterone levels exhibiting activation of HPA axis. Agmatine treatment decreased corticosterone levels in ABA rats, thereby stabilizing HPA axis. Further, agmatine has been reported to increase food intake; reduces depression-like behavior and also decreases obsessive compulsive behavior in rodents (Neis et al., 2014; Freitas et al, 2014; Dixit et al., 2014; Prasad and Prasad, 1996). We may note that endogenous agmatine get released during stress response as a self protective mechanism and its exogenous administration attenuated several effects of acute and chronic stress exposure in rodents. Thus, agmatine induced orexigenic, antistress and anticompulsive effect could have facilitated the inhibitory effect of agmatine in ABA.

In female rats, significant weight loss is often associated with disruptions in estrous cyclicity (Dixon et al., 2003). Thus, it is not surprising

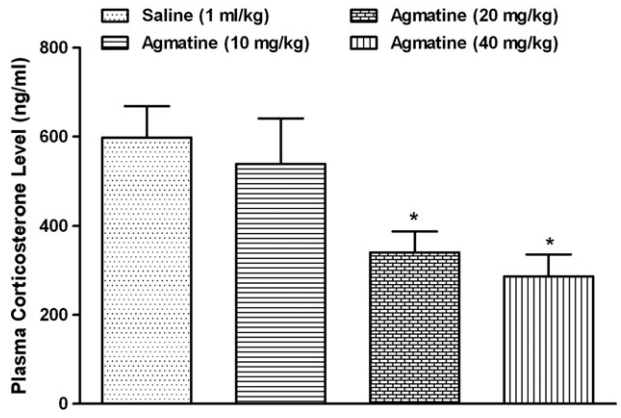

Fig. 6. Effect of agmatine (10–40 mg/kg, ip) on plasma corticosterone levels in rats subjected to activity based anorexia. Results expressed as mean plasma corticosterone level (ng/ml) ± SEM (n = 5–6). *P < 0.05 vs saline treated group (one way ANOVA post hoc Bonferroni test).

that many of the rats in the present study became acyclic during the restricted-feeding phase. This is supported by previous studies in which female rats displayed estrous cycle disruptions following weight loss between 13% and 17% of their baseline body weight (Knuth and Friesen, 1983; Tropp and Markus, 2001). Due to restricted food and high activity with wheels the availability of metabolic fuel is reported to be low to support disruption of estrous cyclicity. Subchronic agmatine treatment not only prevents the weight loss in ABA rats but also prevents the disruptions of estrous cycle. However, estrous cycling had ceased due to severe weight loss. Thus it is also possible that the effects on estrous cycling seen occur because of regaining of body weight, and agmatine may not have a direct effect on estrous cycling. This effect could be secondary to its effect on energy homeostasis and HPA axis regulation.

The pathophysiological mechanisms underlying AN and those for reduced food intake, increased exercise and the maintenance of low body weight in ABA are essentially different. Nevertheless, having experienced the consequences of such behaviors may serve to sustain the behaviors in both ABA and AN. The activity based anorexic rats have been shown to have significantly less adipose tissues and lower plasma levels of leptin and insulin (Pardo et al., 2010). Centrally, hypothalamic gene expressions of orexigenic neuropeptides (NPY and AGRP) are significantly elevated, while anorexigenic peptides (proopiomelanocortin and CART) are reduced in the ABA rats compared with ad lib or pair-fed controls (De Rijke et al., 2005). On the other hand, mesolimbic reward systems have been implicated in the hyperactivity and starvation paradox of AN (Kaye et al., 2009). In particular, studies have demonstrated that DA receptor antagonists can reduce food associated activity (Barbano and Cador, 2006; Scheurink et al., 2010) and body weight loss during ABA (Pardo et al., 2010; Verhagen et al., 2009). We have recently reported antipsychotic like effect of agmatine in rodent models of schizophrenia. Agmatine at the doses of 40 and 80 mg/kg blocked various dopamine mediated behavior like conditioned avoidance responding, apomorphine induced climbing and amphetamine hyperlocomotor activity and also augmented plasma prolactin levels (Kotagale et al., 2012). In fact, earlier studies from our laboratory and results of others clearly demonstrated an inhibitory effect of agmatine on hyperlocomotor activity induced by substances of abuse like alcohol, caffeine, morphine and nicotine without altering spontaneous locomotor activity (Kotagale et al., 2010; Ozden et al., 2011; Uzbay et al., 2010). Further, it is suggested that blockade of dopamine D2 receptors and activation 5-HT1A receptors by agmatine might be attributed to its favorable profile in rodent models of schizophrenia. Further studies are required to determine whether the specific neural systems involved in reward are influence by agmatine in animals exposed to ABA.

Patients with AN find little rewarding in life beyond their drive for thinness (Davis and Woodside, 2002) and abnormalities in dopaminergic signaling in AN may alter reward processing. Indeed, AN patients are more sensitive to reward and punishment (Harrison et al., 2010; Jappe et al., 2011). However, AN patients do not show the differential activation in the anterior ventral striatum distinguishing wins and losses following a reward task that control subjects' exhibit (Wagner et al., 2008). Thus, AN patients may not differentiate positive and negative feedback normally. They also do not find palatable foods as rewarding (Santel et al., 2006), have reduced novelty seeking (Harrison et al., 2007) and exhibit altered responses to taste stimuli in insular and striatal regions (Wagner et al., 2008). The suppression theory of ABA suggests that reward processes may also be altered in ABA because rodents press the lever more for access to a running wheel when food deprived, and find food less salient as activity levels increase (Pierce et al., 1986). The effects of agmatine on reward processes may contribute to its ability to reduce ABA. Agmatine has variable effects on reward anticipation and has been suggested to reduce substance abuse (Uzbay, 2012a, 2012b). Furthermore, agmatine has been shown to improve cognitive functioning in rodents. Several studies provide evidences for improvement in learning and memory following agmatine administration (Rushaidhi et al., 2013a, 2013b). Although it is unclear whether agmatine may enhance learning in ABA, agmatine treated rats housed with wheels exhibited increased adaptation to the food restriction paradigm, as illustrated by a trend for increased food intake in comparison with vehicle-treated rats. Thus, agmatine induced increase in food intake in ABA in comparison with vehicle-treated may be mediated through alterations in the reward saliency of the wheel and/or improved adaption to the food restriction paradigm via enhanced cognitive function.

In conclusion, the findings from this study not only support the assertion that experience with severe starvation and exercise enhances negative food associations, but also suggest that the consequences of these behaviors are not transitory. Subchronic agmatine treatment decreased RWA, improved food intake, prevented body weight reduction and normalized the dysregulation of estrous cycle in rats exposed to ABA. It also reduced corticosterone levels, thus stabilizing HPA-axis. In view of the therapeutic potential of agmatine, it would be interesting to know if it produced similar effects when administered by oral route and needs to be investigated. Considering the common resistance of AN to current therapy, the inhibition of ABA by agmatine treatment might have potential therapeutic importance in eating disorders like anorexia nervosa.

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