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Arcuate nucleus destruction does not block food deprivation-induced increases in food foraging and hoarding

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Abstract

The mechanisms underlying the control of food intake are considerably better understood than those underlying the appetitive ingestive behaviors of foraging and hoarding of food, despite the prevalence of the latter across species including humans. Neuropeptide Y (NPY) and agouti-related protein (AgRP), two orexigenic neuropeptides known to stimulate food intake in a variety of species, applied centrally to Siberian hamsters increases foraging and especially hoarding with lesser increases in food intake. Both are expressed in the arcuate nucleus (Arc) and their synthesis increases with food deprivation, a naturally-occurring stimulus that markedly increases foraging and hoarding in Siberian hamsters. Therefore, we tested whether destruction of Arc neurons blocks these ingestive behaviors. This was accomplished either by microinjecting NPY conjugated to saporin (NPY-SAP) bilaterally into the Arc to kill NPY receptor-bearing neurons or via neonatal monosodium glutamate (MSG) treatment. For both methods, Arc cresyl violet staining (cell density) and NPY and Y1 receptor immunoreactivity (ir) were significantly decreased. Although baseline foraging and food hoarding were not affected, food deprivation-induced increased food hoarding was surprisingly exaggerated ~100 % with both types of Arc destruction. We found a substantial amount of remaining NPY-ir fibers, likely emanating from the brainstem, and a significant upregulation of Y1 receptors in Arc NPY projections areas (hypothalamic paraventricular nucleus and perifornical area) after Arc denervation and their activation may have accounted for the exaggerated increases. The converging evidence from both Arc destruction methods suggests an intact Arc is not necessary for food deprivation-induced increases in food foraging and hoarding.

Keywords

Food hoarding; NPY; Siberian hamster

1. Introduction

We know little about the mechanisms underlying appetitive ingestive behavior (foraging and hoarding) even though all animals forage for food and most store (hoard) food for some period of time before eating it [for review see: (Bartness and Day, 2003)]. Because humans increase their foraging and food hoarding based on their energy status [*i.e.*, hungry people

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bring home and store more food than their sated counterparts (Beneke and Davis, 1985; Dodd, Stalling, and Bedell, 1977; Mela, Aaron, and Gatenby, 1996) and increases in these behaviors lead to increases in food intake (Ransley, Donnelly, Botham, Khara, Greenwood, and Cade, 2003) and potentially obesity (Lieberman, 2006), it is important to gain a deeper understanding of these appetitive ingestive behaviors to complement our knowledge of consummatory ingestive behavior (food intake).

We have been studying appetitive ingestive behaviors in an animal model using Siberian hamsters (*Phodopus sungorus*) that are housed in a foraging/hoarding apparatus. They earn food by wheel running and have a separate simulated burrow cage to hoard food (Day and Bartness, 2001). One of the most robust stimulators of foraging/hoarding in Siberian hamsters and other animals is food deprivation [for review see: (Bartness and Day, 2003; Vander Wall, 1990)]. Siberian hamsters are ideally suited for studies of appetitive ingestive behaviors triggered by food deprivation or other stimuli because when refed, they increase foraging and overhoard (Dailey and Bartness, 2008; Day and Bartness, 2003; Keen-Rhinehart and Bartness, 2007a; Keen-Rhinehart and Bartness, 2007b; Keen-Rhinehart and Bartness, 2008) instead of overeating as do all animal species tested except hamster species [for review see: (Bartness and Demas, 2004)]. Food deprivation in Siberian hamsters (Mercer, Moar, Ross, Hoggard, and Morgan, 2000) as well as laboratory rats (Brady, Smith, Gold, and Herkenham, 1990) and mice (Brady, Smith, Gold, and Herkenham, 1990; White and Kershaw, 1989) increases the synthesis of the orexigenic neuropeptides, neuropeptide Y (NPY) and agouti-related protein (AgRP), and decreases proopiomelanocortin (POMC), a precursor of the anorexigenic neuropeptide α -melanocyte stimulating hormone (α -MSH), in the arcuate nucleus of the hypothalamus (Arc). NPY and AgRP tend to be colocalized to a high degree in neurons of the Arc in laboratory rats [e.g., (Brady, Smith, Gold, and Herkenham, 1990)] and mice [e.g., (Bergen and Mobbs, 1996)], as well as Siberian hamsters (Mercer, Moar, Ross, Hoggard, and Morgan, 2000; Rousseau, Atcha, Cagampang, Le Rouzic, Stirland, Ivanov, Ebling, Klingenspor, and Loudon, 2002). Moreover, acute 3rd ventricular microinjections of NPY [e.g., (Clark, Kalra, Crowley, and Kalra, 1984; Stanley and Leibowitz, 1984)] or AgRP [e.g., (Rossi, Kim, Morgan, Small, Edwards, Sunter, Abusnana, Goldstone, Russell, Stanley, Smith, Yagaloff, Ghatei, and Bloom, 1998)] markedly stimulate food intake in laboratory rats. In Siberian hamsters, however, food foraging and especially hoarding are increased to a greater extent than food intake after intraventricular NPY (Day, Keen-Rhinehart, and Bartness, 2005) or AgRP (Day and Bartness, 2004). By contrast, α -MSH does not appear to play as significant a role as the orexigenic neuropeptides in controlling appetitive ingestive behaviors. Intraventricular injection of melanotan II (MTII), a synthetic analogue of α -MSH, does not decrease foraging and food hoarding in *ad libitum* fed Siberian hamsters (Keen-Rhinehart, 2007), but does decrease food intake just as α -MSH does in laboratory rats and mice [(Keen-Rhinehart, 2007; for review on rats and mice see: (Butler, 2006; Williams, 2005)]. In addition, MTII only partially inhibits post food deprivation induced increases in food hoarding normally seen in Siberian hamsters (Keen-Rhinehart, 2007). Thus, it is likely that the food deprivation-induced increases in expression of the orexigenic signals, NPY and AgRP, play a greater role than the decrease in expression of the anorexigenic signal, α -MSH, in stimulating appetitive ingestive behaviors after food deprivation in Siberian hamsters.

The Arc has been defined as a primary integrator of peripheral metabolic and hormonal signals (*i.e.*, leptin, ghrelin, insulin, and glucose) that may be responsible for relaying energy status information to other brain regions to drive changes in appetitive and consummatory ingestive behaviors (Sawchenko, 1998; Watts, 2000). Despite the evidence for the role of NPY and AgRP increasing food intake and α -MSH decreasing food intake (stated above), destruction of the Arc by neonatal treatment of MSG produces a negligible decrease in food intake in rats (Bunyan et al., 1976; Nikolettseas, 1977; Stricker-Krongrad et al., 1992). Thus,

it is possible that these Arc neuropeptides may play a greater role in appetitive ingestive behaviors than in consummatory ingestive behaviors. Therefore, we reasoned that elimination of Arc neurons might dampen or block food deprivation-induced increases in food foraging and hoarding. Because of the elongated shape of the Arc, complete and selective conventional lesions are difficult to achieve (Choi, Sparks, Clay, and Dallman, 1999); therefore, we chose two different strategies. For the first strategy (*experiment 1*), we injected NPY conjugated to saporin (NPY-SAP), a ribosomal inactivating toxin, to selectively destroy the NPY Y receptor (NPY-R) possessing Arc neurons, which include the NPY/AgRP-expressing neurons (Cowley, Pronchuk, Fan, Dinulescu, Colmers, and Cone, 1999; Larsen, Sheikh, Jakobsen, Schwartz, and Mikkelsen, 1993) and POMC neurons (Li, Dinh, and Ritter, 2008). For the second strategy (*experiment 2*), we treated neonatal hamsters with monosodium glutamate (MSG) to produce Arc lesions. MSG treatment is a well-established method to produce substantial destruction of the Arc in laboratory rats and mice [*e.g.*, (Alessi, Quinlan, and Khachaturian, 1988; Olney, 1969)], as well as in Siberian hamsters (Ebling, Arthurs, Turney, and Cronin, 1998; Leitner and Bartness, 2008; Leitner and Bartness, 2009; Pelz, Routman, Driscoll, Kriegsfeld, and Dark, 2008). We fully acknowledge that neither lesion method is specific for NPY/AgRP-expressing ARC neurons. We hypothesized, however, that these neurons play a larger role in the control of foraging/hoarding and food intake, for all the above reasons.

In both experiments, hamsters were housed in a running wheel-based, food delivery foraging system coupled with simulated burrow-housing (Day and Bartness, 2001). We collected baseline measures of foraging, hoarding and food intake from NPY-SAP-treated and neonatally MSG-treated adult hamsters and then food deprived half the hamsters and measured the same behaviors 1, 2, 4 and 24 h after refeeding.

2. Results

Experiment 1: Do Arc NPY-SAP lesions block food deprivation-induced increases in food hoarding by Siberian hamsters?

Histology—NPY-SAP-treatment significantly decreased Arc cellularity (cresyl violet staining) and Arc NPY- and Y1 receptor-immunoreactivity (ir) compared with B-SAP-treated animals ($P < 0.05$; Fig. 1A-C). This toxin treatment also significantly reduced PVH NPY-ir fibers -- an efferent projection destination of Arc-NPY cells (Broberger, Visser, Kuhar, and Hokfelt, 1999), compared with B-SAP controls ($P < 0.05$; Fig. 1E). By contrast, the NPY-SAP-treatment significantly increased PVH NPY Y1 receptor-ir compared with the B-SAP-treated animals in the PVH ($P < 0.05$; Fig. 1F).

Wheel revolutions—Baseline wheel revolutions were not affected by NPY-SAP treatment compared to the B-SAP animals for the FW group (Fig. 2D). The B-SAP group showed the typical food deprivation-induced increased wheel running with refeeding [*e.g.* (Dailey and Bartness, 2008; Day and Bartness, 2003; Keen-Rhinehart and Bartness, 2007a; Keen-Rhinehart and Bartness, 2007b; Keen-Rhinehart and Bartness, 2008)] at 2-4 h ($P < 0.05$; Fig 4A), whereas NPY-SAP treatment inhibited wheel running in refed hamsters at 2-4, 4-24 and cumulatively across the 24 h compared with B-SAP food deprived animals ($P < 0.05$; Fig. 4A). Food deprived and non-food deprived NPY-SAP-treated animals ran significantly less than did their respective B-SAP-treated controls at 1-2, 2-4 h and cumulatively ($P < 0.05$; Fig. 4A).

Foraging—Baseline foraging was not affected by NPY-SAP treatment compared with that of the B-SAP animals for the 10 Rev hamsters (Fig. 2A). Unexpectedly, the food deprivation-induced increase in foraging [*e.g.*, (Day and Bartness, 2003)] with refeeding did

not occur in food deprived control (B-SAP) or NPY-SAP treated hamsters (Fig. 3A). Moreover, food deprived NPY-SAP treated hamsters had significantly decreased foraging at 1-2 h compared with their non-food deprived counterparts ($P < 0.05$; Fig 3A).

Food hoarding—Baseline food hoarding was comparable among all NPY-SAP treated groups and their B-SAP counterparts (Figs. 2B, E and G). Rather than the predicted blockade or diminishment in food hoarding by NPY-SAP treatment, the normal refeeding-induced increase in hoarding after food deprivation was significantly exaggerated [*e.g.*, (Bartness, 1997; Bartness and Clein, 1994; Day and Bartness, 2003)] by the 10 Rev group compared with their B-SAP controls at 0-1, 2-4 h and cumulatively across the test ($P < 0.05$; Fig. 3B). Food hoarding was significantly increased at various times by the FW and BW groups regardless of toxin treatment after refeeding ($P < 0.05$; Figs. 4B and 5A). Unlike the NPY-SAP triggered exaggeration of post deprivation hoarding in the 10 Rev group, this did not occur for the NPY-SAP refed FW and BW groups ($P < 0.05$; Figs. 4B and 5A).

Food intake—Consistent with previous findings [*e.g.*, (Dailey and Bartness, 2008; Day and Bartness, 2003; Keen-Rhinehart and Bartness, 2007a; Keen-Rhinehart and Bartness, 2007b; Keen-Rhinehart and Bartness, 2008)], food intake was not increased after food deprivation in the B-SAP control animals of the 10 Rev and FW groups (Figs. 3C and 4C). Food intake by the BW NPY-SAP treated hamsters was significantly increased at 2-4 h after refeeding compared with their ad libitum-fed counterparts ($P < 0.05$; Fig. 5B). The FW NPY-SAP treated group had increased food intake 0-1 h post refeeding compared with their non-food deprived counterparts ($P < 0.05$; Fig. 4C). Subsequently (1-2 h post refeeding), food intake was decreased in NPY-SAP 10 Rev hamsters compared with their NPY-SAP non-food deprived controls ($P < 0.05$; Fig. 3C). Food intake was significantly decreased for NPY-SAP treated BW group hamsters 0-1 h and 2-4 h after refeeding which led to significantly decreased cumulative food intake compared with all other groups ($P < 0.05$; Fig. 5B).

Experiment 2: Does destruction of the Arc by neonatal MSG treatment block food deprivation-triggered increases in food hoarding by adult Siberian hamsters?

Histology—Neonatal MSG-treated animals had significantly decreased Arc cellularity (cresyl violet staining), Arc NPY-, Y1 receptor- and α -MSH-ir cells compared with their PBS-treated controls ($P < 0.05$; Figs. 6A-D). NPY-ir fibers in the PVH also were significantly decreased by MSG compared with PBS treatment ($P < 0.05$; Fig. 6E). Similar to the results after NPY-SAP Arc destruction in *experiment 1*, MSG-treated animals had significantly increased NPY Y1 receptor-ir compared with the PBS-treated animals in the PVH as well as in the PFA ($P < 0.05$; Figs. 6F-G).

Wheel Revolutions—During baseline, FW MSG-treated hamsters had similar wheel revolutions to that of the PBS-treated controls (Fig. 7A). After food deprivation and refeeding, there was no deprivation-induced increase in wheel revolutions in the PBS-treated controls typically seen in our animals [*e.g.*, (Day, Keen-Rhinehart, and Bartness, 2005)]. Wheel running was significantly decreased by MSG-treated hamsters compared with PBS-treated animals at 0-1 h and cumulatively ($P < 0.05$; Fig. 8A).

Food Hoarding—During baseline, there was no difference in food hoarding between the FW PBS-treated and MSG-treated hamsters (Fig. 7B). After food deprivation and refeeding, PBS and MSG-treated hamsters significantly increased food hoarding compared with their non-food deprived counterparts at 0-1 h, 1-2 h, 2-4 h and cumulatively, with MSG-treated hamsters also increasing food hoarding at 4-24 h ($P < 0.05$; Fig. 8B). In addition, MSG

significantly exaggerated the post-food deprivation-induced increased hoarding compared with their PBS controls at 0-1 h, 4-24 h and cumulatively ($P < 0.05$; Fig. 8B).

Food Intake—Under baseline conditions, MSG had no effect on food intake compared to PBS-treated hamsters (Fig. 7C). After food deprivation and refeeding, food intake was significantly increased in the PBS-treated hamsters at 1-2 h with subsequent decreased food intake by both the PBS and MSG-treated hamsters at 2-4 h when compared with their non-food deprived counterparts ($P < 0.05$; Fig. 8C). There were no significant differences between the food-deprived PBS and MSG-treated animals (Fig. 8C).

3. Discussion

The aim of these experiments was to test the role of the Arc in the control of appetitive, as well as, consummatory ingestive behaviors. Surprisingly, we found that an intact Arc was not necessary for post food deprivation-induced increased food hoarding. Arc destruction using either neonatal MSG treatment or Arc NPY-SAP injections significantly exaggerated the post deprivation-induced increase in food hoarding. An intact Arc also was not necessary for maintenance of the normal day-to-day control of the appetitive behaviors of food foraging and hoarding because neither behavior changed after Arc NPY-SAP injections or neonatal MSG treatment. There was, however, significantly decreased baseline food intake after Arc lesions produced by NPY-SAP, but not by MSG. Collectively, these findings suggest that the Arc may play a role in the normal, 'unchallenged' control of food intake, but is not necessary for the normal, 'unchallenged' control of food foraging and hoarding. Moreover, an intact Arc is not necessary for the increases in foraging and hoarding that accompany refeeding after food deprivation.

The robust exaggeration of food hoarding after food deprivation in NPY-SAP or MSG-treated animals (~100% greater than controls) demonstrated that the Arc, and likely the NPY/AgRP neurons located therein, may not be a primary site mediating changes in this behavior following refeeding. This exaggerated appetitive ingestive behavior is consistent with previous studies using the same or other means of testing NPY-mediated control of consummatory ingestive behavior (feeding). Specifically, there are exaggerated increases in food intake after lateral ventricular injections of NPY into adult laboratory rats previously treated with MSG as neonates (Stricker-Krongrad and Beck, 2004). Exaggerated increases in food intake after food deprivation also occur in mice after continuous icv infusions of NPY antibodies to neutralize the neuropeptide, a treatment that produces a similar loss of hypothalamic NPY signaling as in MSG-treated animals (Ishii, Muranaka, Tashiro, and Nishimura, 2007a). In the present study, we found exaggerated increases in food hoarding across all groups, but not food intake, when the food deprived hamsters were refed. This is likely because Siberian hamsters and other hamster species do not increase their food intake after food deprivation or if they do, the magnitude is small compared with laboratory rats and mice [for review see: (Bartness and Demas, 2004)] and may even be artifactual because refeeding after food deprivation synchronizes hamster food intake from the more variable pattern of eating approximately every 4 h (Bartness, Morley, and Levine, 1986). It also may be that if the aforementioned laboratory mouse and rat studies included the opportunity for these animals to explicitly express appetitive ingestive behaviors, then Arc destruction or blocking endogenous NPY after food deprivation might also affect these behaviors similarly to that seen here by Siberian hamsters. Indeed, NPY can increase appetitive ingestive behaviors in laboratory rats when given the opportunity to express these behaviors (Ammar, Sederholm, Saito, Scheurink, Johnson, and Sodersten, 2000; Sederholm, Ammar, and Sodersten, 2002). Whether it is appetitive or consummatory ingestive responses, however, it appears that chronic perturbation of the Arc and/or NPY synthesizing neurons exaggerates ingestive behaviors. Because our treatments were not specific to NPY producing Arc

neurons and because most Arc NPY producing neurons also synthesize AgRP (Mercer, Moar, Ross, Hoggard, and Morgan, 2000; Rousseau, Atcha, Cagampang, Le Rouzic, Stirland, Ivanov, Ebling, Klingenspor, and Loudon, 2002), another neuropeptide that stimulates food hoarding in this species (Day and Bartness, 2004), definitive conclusions concerning the importance, or lack thereof, of NPY in these ingestive behaviors cannot be made.

Compensatory mechanisms may develop when the Arc, or any other neural structure, is chronically perturbed such that energy balance can be maintained. Consistent with previous reports (Broberger, Johansen, Johansson, Schalling, and Hokfelt, 1998; Ishii, Muranaka, Tashiro, and Nishimura, 2007a; Kerkerian and Pelletier, 1986; Luquet, Perez, Hnasko, and Palmiter, 2005; Stricker-Krongrad and Beck, 2004), we found that for both chronic manipulations of the Arc there was substantial NPY innervation remaining in the PVH and PFA, two targets of Arc NPY that are known to contribute to the control of both appetitive and consummatory ingestive behaviors (Dailey and Bartness, 2009; Stanley, Daniel, Chin, and Leibowitz, 1985; Stanley, Magdalin, Seirafi, Thomas, and Leibowitz, 1993). Although the exact source of these remaining NPY fibers is unknown here, it is likely that they derive from brainstem NPY-producing neurons that project to hypothalamic sites (Bai, Yamano, Shiotani, Emson, Smith, Powell, and Tohyama, 1985; Sahu, Dube, Kalra, and Kalra, 1988; Sahu, Kalra, Crowley, and Kalra, 1988). When projections from the brainstem to the hypothalamus are severed by midbrain knife-cuts or neural transactions at the level of the dorsal tegmentum, NPY concentrations are decreased in the PVH (Sahu, Dube, Kalra, and Kalra, 1988; Sahu, Kalra, Crowley, and Kalra, 1988). In addition, lesions of the area postrema (AP)/medial NTS in laboratory rats result in increases in NPY mRNA in the Arc and NPY-ir in the PVH (Edwards, White, He, Dean, and Martin, 1997). Food intake also increases in these latter animals, suggesting that elevated hypothalamic NPY levels may compensate for the loss of brainstem NPY circuits (Edwards, White, He, Dean, and Martin, 1997) and perhaps in the case here, vice versa. Thus, NPY sources outside of the Arc may increase their activity or even degree of innervation when the Arc NPY contribution decreases and, consequently, NPY-induced ingestive behaviors may be triggered more easily.

A second compensatory mechanism that could contribute to the exaggerated appetitive ingestive behavioral response seen here after Arc NPY-SAP injections or neonatal MSG treatment is the up-regulation of NPY Y1 receptors in the PVH or PFA. Consistent with this notion is the up-regulation of these receptors in adult laboratory rats treated neonatally with MSG (Stricker-Krongrad and Beck, 2004) and mice given anti-NPY antibodies centrally (Ishii, Muranaka, Tashiro, and Nishimura, 2007b). Increases in receptor number at the target site after reduction of the source(s) of the naturally-occurring receptor agonist is a well-documented condition termed denervation supersensitivity [*e.g.*, (Bird and Aghajanian, 1975; Trulson and Jacobs, 1978; Zis and Fibiger, 1975)]. Such a condition may have occurred here with loss of Arc NPY inputs to the PVH/PFA after MSG- or NPY-SAP treatment. After food deprivation, an increase in the NPY release deriving from remaining Arc NPY fibers and/or from brainstem NPY sources could stimulate the increased number of Y1 receptors in the PVH or PFA and trigger the exaggerated increases in appetitive ingestive behaviors seen here. Indeed, after neonatal MSG treatment or NPY-SAP injections into the Arc, Y1 receptor-ir was significantly increased in the PVH and PFA compared with controls in the present study.

Although the literature cited above, as well as the present data, appear to support a key role of NPY in the exaggerated hoarding response after food deprivation and refeeding in hamsters suffering damage to the Arc by these two treatments, alteration in other Arc neuropeptides also could be involved. For example, AgRP is colocalized with NPY in a high

percentage of Arc neurons in many species including Siberian hamsters (Mercer, Moar, Ross, Hoggard, and Morgan, 2000; Rousseau, Atcha, Cagampang, Le Rouzic, Stirland, Ivanov, Ebling, Klingenspor, and Loudon, 2002). Therefore, AgRP-ir also was decreased after Arc destruction by both treatments (data not shown). The other predominant neurochemical phenotype of the Arc neurons are those co-expressing POMC and cocaine- and amphetamine-related transcript [CART; Vrang, 1999 10768 /id], and many of these neurons also appear to be ablated by NPY-SAP and MSG treatment, as suggested by the decreased α -MSH-ir. Although CART inhibits food intake when given centrally (Kristensen, Judge, Thim, Ribel, Christjansen, Wulff, Clausen, Jensen, Madsen, Vrang, Larsen, and Hastrup, 1998; Lambert, Couceyro, McGirr, Dall Vechia, Smith, and Kuhar, 1998), as does α -MSH (Shimizu, Shargill, Bray, Yen, and Gesellchen, 1989), CART has not been tested for its effects on appetitive ingestive behaviors in general, and in Siberian hamsters, specifically. In terms of α -MSH and AgRP, they show opposite effects on food intake -- α -MSH inhibits food intake and enhances energy expenditure by activating the melanocortin-4 receptor [MC4R; for review see: (Butler, 2006; Williams, 2005)], whereas, AgRP increases food intake and decreases energy expenditure by acting as an inverse agonist for the MC4R (Stutz, 2005). With MSG- or NPY-SAP- induced Arc destruction, α -MSH and AgRP-ir were decreased; however, how decreases in these two peptides that have opposing effects on ingestive behaviors could account for the present data is unclear. The exaggerated increases in food hoarding after food deprivation could be mediated by an increase in the expression of remaining Arc AgRP neurons because central AgRP injections markedly increase food hoarding for several days in Siberian hamsters (Day and Bartness, 2004). α -MSH does not appear to play as great of a role in decreasing food hoarding as does AgRP in increasing food hoarding in Siberian hamsters (Keen-Rhinehart and Bartness, 2007a); thus, a decrease in Arc α -MSH in the present study would appear unlikely to play a significant role in the exaggerated food hoarding. Finally, AgRP has prolonged effects on food intake and on food hoarding [up to a week or more; (Day and Bartness, 2004; Hagan, Rushing, Pritchard, Schwartz, Strack, Van Der Ploeg, Woods, and Seeley, 2000)], but the exaggerated increase in food hoarding after refeeding in animals treated with MSG or NPY-SAP was extremely short-lived (0-1 h). Collectively, it would seem most likely that the loss of NPY from Arc NPY/AgRP-producing neurons contributed to the exaggerated post-food deprivation increase in food hoarding more so than did the loss of AgRP from those neurons, or from destruction of POMC/CART neurons by these treatments.

By contrast to normal, day-to-day (baseline) foraging and hoarding, baseline food intake was decreased in the NPY-SAP-treated animals in the 10 Rev and BW groups with a trend towards a decrease in the FW group; however, there was no significant change in the food intake in the MSG-treated animals. This was the only major difference between these two treatments for all behaviors measured in the present study. The changes in day-to-day food intake after treatments designed to ablate Arc NPY-producing neurons are varied. For example, in laboratory rats, Arc injection of NPY conjugated to the toxin ricin (Burlet, Grouzmann, Musse, Fernet, Nicolas, and Burlet, 1995) or neonatal MSG treatment (Stricker-Krongrad and Beck, 2004) decreases food intake, whereas Arc NPY-SAP injections into laboratory rats, similar to the ones in the present study in Siberian hamsters that decreased food intake, increase daytime food intake (Bugarith, Dinh, Li, Speth, and Ritter, 2005). The differences in feeding responses across these studies could be due to one or more factors including the species used, the interval between treatment and the feeding measures, the degree of Arc ablation and/or the types of neurons destroyed the various toxins.

Collectively, the present results suggest that an intact Arc is not necessary for normal, day-to-day control of the appetitive ingestive behaviors of foraging and hoarding, but may be more involved in food intake under these conditions. Most importantly, instead neonatal

MSG treatment or Arc NPY-SAP injections blocking food deprivation-induced increases in food hoarding as was initially expected, a profound short duration (~1 hour) increase in this appetitive behavior occurred. This increased food hoarding was associated with significantly increased NPY Y1 receptor-ir in the PVH (after NPY-SAP and MSG treatments) and PFA (after MSG treatment) consistent with previous data showing up-regulation of these receptors after ablation of Arc NPY neurons in MSG-treated laboratory rats (Stricker-Krongrad and Beck, 2004) and mice given anti-NPY antibodies centrally (Ishii, Muranaka, Tashiro, and Nishimura, 2007b). Although the source of the natural ligand for these receptors is not precisely known for the present study, the two likely sources are remaining non-ablated Arc NPY/AgRP neurons and/or brainstem NPY [*e.g.*, catecholaminergic cell groups (Li and Ritter, 2004)]. Finally, it would be naïve to assume that only NPY is involved in the control of food foraging and hoarding as we have shown their modulation by AgRP (Day and Bartness, 2004), melanocortin receptor agonism (Keen-Rhinehart and Bartness, 2007a), as well as by the peripheral hormones ghrelin (Keen-Rhinehart and Bartness, 2005), cholecystokinin (Teubner and Bartness, 2009) and leptin (Keen-Rhinehart and Bartness, 2008). Moreover, it would be simplistic to focus on the Arc, hypothalamic or even solely forebrain control of appetitive ingestive behaviors, as the hindbrain continues to prove itself involved in ingestive behavior and related energy balance responses [for review see: (Grill and Kaplan, 2002)].

Finally, as we have previously noted (Dailey and Bartness, 2009), there has been little success in pharmacological and behavioral approaches to the obesity problem focused on the mechanisms underlying the control of consummatory ingestive behavior (food intake). Because most animals including humans forage for food (Burns, Williams, Secor, Owen-Smith, Bargmann, and Castellini, 2006; Illius, Tolkamp, and Yearsley, 2002; Kaun and Sokolowski, 2009) and many also hoard food for consumption later [for review see: (Bartness and Day, 2003; Vander Wall, 1990)], studying the underlying mechanisms of appetitive ingestive behaviors may provide another point of attack for pharmacological and/or behavioral intervention. The exaggeration of food deprivation-induced foraging/hoarding seen in the present study and previously in Siberian hamsters (Bartness, 1997; Bartness and Clein, 1994; Dailey and Bartness, 2008; Day and Bartness, 2003; Keen-Rhinehart and Bartness, 2005; Keen-Rhinehart and Bartness, 2007a; Keen-Rhinehart and Bartness, 2007b; Keen-Rhinehart and Bartness, 2008; Wood and Bartness, 1996) is seen in humans as well under less severe energy challenges. That is, if one goes to the grocery store hungry, more food is purchased and brought home than if you go to the grocery store when sated (Beneke and Davis, 1985; Dodd, Stalling, and Bedell, 1977; Mela, Aaron, and Gatenby, 1996). Given that obese people forage for more high fat and calorically dense foods at the grocery store than their normal weight counterparts (Dodd, Stalling, and Bedell, 1977), it is relatively easy to envision that pharmacological and/or behavioral treatments that would target this earlier stage of the ingestive behavioral sequence, perhaps affecting NPY, AgRP and/or their receptors, might be more efficacious in treating obesity than trying to control the consummatory aspects (eating) of the sequence.

4. Experimental Procedures

Experiment 1: Do Arc NPY-SAP lesions block food deprivation-induced increases in food hoarding by Siberian hamsters?

Animals and Housing—Adult male Siberian hamsters ~3 months old and weighing 35-46 g were obtained from our breeding colony. The colony was established in 1988 [see (Bowers, Festuccia, Song, Shi, Migliorini, and Bartness, 2004) for the genealogy of the colony]. Hamsters were group-housed and reared from birth in a 16:8 h light-dark cycle (lights-on at 2030). Room temperature was maintained at 21 ± 2 °C and relative humidity

was $50 \pm 10\%$. All procedures were approved by the Georgia State University Institutional Animals Care and Use Committee and were in accordance with the Public Health Service and United States Department of Agriculture guidelines.

Acclimation and Baseline Measures—Two replicates of 64 animals were transferred from group-housing and acclimated and trained for 1 wk in our hoarding/foraging apparatus [previously shown and described in (Day and Bartness, 2001)]. Briefly, two cages were connected with a convoluted polyvinyl-chloride tubing system (38.1 mm inner diameter and ~ 1.52 m long), with corner and straightways for both horizontal and vertical climbs. The top or “food cage” was $456 \times 234 \times 200$ mm (length $\times$ width $\times$ height) equipped with a water bottle and running wheel. The bottom or “burrow cage” was $290 \times 180 \times 130$ mm and was covered to simulate the darkness of a natural burrow. The burrow cage contained Alpha-Dri (Specialty Papers, Kalamazoo, MI) bedding and cotton nesting material. The animals were fed 75 mg pellets (Purified Rodent Diet; Research Diets, New Brunswick, NJ) throughout the experiment. During this period, animals were trained to forage for their food based on procedures previously established (Day and Bartness, 2001). In brief, hamsters were given free access to food for 2 d while they adapted to the running wheel. In addition to the free food, a 75 mg food pellet was dispensed upon completion of every 10 wheel revolutions. Wheel revolutions were counted using a magnetic detection system and monitored by a computer-based hardware/software system (Med Associated, Lancaster, NH). On the third day, the free food condition was replaced by a response-contingent condition in which only every 10 wheel revolutions triggered the delivery of a pellet. This condition was in effect for the remaining 5 d of the 1 wk-long training period. The hamsters were then separated into 12 groups: 2 neurotoxin groups (NPY-SAP or B-SAP), 2 feeding conditions (Food deprived or Non-food deprived), and 3 foraging groups that were matched for percent change in their body mass from their initial foraging/hoarding apparatus housing and average hoard size. The 3 foraging groups were 10 revolutions/pellet [10 Rev, Free Wheel/Free Food (FW); food was available non-contingently, that is not earned, but the running wheel was active serving as a locomotor activity control group or Blocked Wheel/Free Food (BW); food was available non-contingently, that is not earned, but the running wheel was blocked serving as a sedentary control group].

Measurement of Foraging, Food Hoarding, and Food Intake—Foraging (pellets earned) was defined as the number of pellets delivered upon completion of the requisite wheel revolutions. Food hoarding (pellets earned) was defined as the number of pellets found in the bottom “burrow” cage in addition to those removed from the cheek pouches. For the 10 Rev group, food intake (pellets eaten) was defined as pellets earned – surplus pellets – hoarded pellets = food intake. For the FW and BW groups, food intake (pellets eaten) was defined as pellets given – pellets left in the top cage – hoarded pellets = food intake. The electronic balance used to weigh the food pellets was set to “parts” measurement rather than obtaining fractions of a pellet in milligrams; thus, the smallest unit measured was one 75-mg food pellet and equal to 1; thus one 75 mg food pellet = 1. Fractions of a pellet were computed by the scale as well.

Intracranial injections—For Arc administration of NPY-SAP and the blank saporin control solution (B-SAP; Advanced Targeting Systems, San Diego, CA), hamsters were anesthetized with isoflurane and fur at the top of the head was removed to expose the area to be incised. A hole was trephined at the stereotaxic coordinates above the Arc and the injector was lowered into the Arc (AP -1.4 mm, ML -0.3 mm, DV -7.0 mm). These coordinates were chosen based on preliminary dye injections and NPY-SAP/colloidal gold injections in a subset of Siberian hamsters. Injections of 48 ng of NPY-SAP or B-SAP were delivered bilaterally (100 nl/side) to the Arc. The dose, injection parameters and time course

of NPY-SAP was chosen based on previously published data showing its effectiveness in producing significant destruction of NPY Y receptor-containing neurons within the Arc (Bugarith, Dinh, Li, Speth, and Ritter, 2005; Li, Dinh, and Ritter, 2008) and our own pilot studies. Injections were made through an internal cannula (26-gauge stainless steel; Plastics One, Roanoke, VA) connected to a 0.5 μ l microsyringe with polyethylene tubing. The solution was delivered slowly over a 5 min period. Fresh apple slices were given to facilitate fluid and caloric intake for the first 2-3 d post surgery. The animals also received a subcutaneous buprenorphine (0.05 mg/kg, s.c.) injection for 2 d after surgery. Animals then recovered for 7 d in single-shoebox cages with food and water available *ad libitum*. After recovery, hamsters were returned to their respective hoarding/foraging cages maintaining the same group membership.

Experimental protocol—The time course adopted to initiate and terminate behavioral testing for NPY-SAP destruction of NPY Y-R containing neurons was chosen based on previously published data for this methodology (Bugarith, Dinh, Li, Speth, and Ritter, 2005) and our pilot studies. In that study, a significant reduction of NPY terminals was present by the second wk after injection. Therefore, feeding studies were conducted beginning at approximately this time. After injection of NPY-SAP or B-SAP from Day 8 until Day 21, daily measurements of foraging, food hoarding and food intake were taken just before lights-out. On Day 22 after injection, a subset of animals in each group was food deprived for 56 h. In our previous studies of food hoarding, we have used food deprivation lengths ranging from 12 to 56 h (Institutional Animals Care and Use Committee approved), with the latter length appearing somewhat severe and/or non-physiological. In the laboratory, however, Siberian hamsters are almost 50 % body fat compared with ~25 % in nature (Weiner, 1987) and short food deprivation periods are without behavior effect. For example, food deprivation lengths of 12-24 h are minimally stimulate food hoarding (Clein MR and Bartness, TJ, unpublished results). Therefore, we selected 56 h food deprivation to trigger the behavior nearly maximally.

Two hours before the onset of the dark phase and refeeding, food was removed from the pouches of the non-food deprived hamsters and all animals were placed in clean burrow cages with access to the tubes blocked before refeeding. At the onset of the dark phase, access to the tubes and top cage was restored. Foraging, food hoarding, and food intake were then measured 1, 2, 4 and 24 h after refeeding.

Experiment 2: Does destruction of the Arc by neonatal MSG treatment block food deprivation-triggered increases in food hoarding by adult Siberian hamsters?

Animals and housing—Animals were obtained from our breeding colony as described in *experiment 1*. We adopted a previously published protocol to treat Siberian hamsters neonatally with MSG (Ebling, Arthurs, Turney, and Cronin, 1998) that we have used successfully recently in this species (Leitner and Bartness, 2008; Leitner and Bartness, 2009). Briefly, pups were injected subcutaneously in the dorsum using a 30 gauge needle with vehicle (0.1 M phosphate buffered saline, pH 7.4) or MSG (*L*-monosodium glutamate; 4 mg/g of body mass; Sigma, St. Louis, MO, USA). MSG was diluted to a concentration of 160 mg/ml such that a 1 g pup would receive an injection volume of 25 μ l to deliver 4 mg MSG. Injections occurred once daily for 5 consecutive days from post partum Day 4-8. All pups within a litter received a single treatment instead of mixing of treatments within a litter due to the possibility of cannibalism (unpublished observations). The pups were then reared as described in *experiment 1* in group housing with littermates of the same treatment group.

Acclimation and baseline measures—At ~3 m of age, two replicates of 28 male Siberian hamsters were transferred from group-housing and acclimated for 1 wk in our

hoarding/foraging apparatus. Because of reports that MSG treatment decreases spontaneous motor activity [e.g., (Menendez, McGregor, Healey, Atrens, and Leibowitz, 1990)], we reasoned that foraging for food might be too challenging for these animals and they may not earn enough food to survive. Therefore, unlike in *experiment 1*, where we had the three foraging groups, here all animals were kept in the Free Wheel condition (*i.e.*, food not earned, therefore foraging was not measured) and wheel revolutions were monitored. Data were collected as described in *experiment 1*. Half of the MSG or PBS-treated animals were separated into food deprived and non-food deprived groups that were matched for percent change in body mass from their initial introduction to the foraging/hoarding apparatus and average hoard size. The animals were food deprived for 56 h as described in *experiment 1* above. Food hoarding and intake were defined and measured as in *experiment 1*.

Methods shared between experiments 1 and 2

Tissue preparation—After the 24 h data were collected in both experiments, animals were deeply anesthetized with pentobarbital sodium (300 mg/kg ip) and perfused transcardially with 100 ml of 0.15 M saline followed by 150 ml of 4 % paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were extracted and post-fixed for 48 h at 4 °C, followed by a submersion in 30 % sucrose for at least 24 h at 4 °C. Brains were then frozen and sectioned on a microtome into 30 µm sections through the hypothalamus and distributed serially into five sets. The first set was mounted immediately onto gelatinized glass slides and counter-stained with cresyl violet. The remaining sections were stored in cryoprotectant at -20 °C until immunohistochemically processed.

Lesion verification using immunohistochemistry—Free-floating sections were removed from the cryoprotectant and washed with PBS. All washes and incubations occurred at room temperature. Sections were then incubated in 0.03 % H₂O₂ (Fisher Scientific, Houston, TX) and 0.25 % Triton X-100 (Sigma, St. Louis, MO) in PBS for 15 min. Sections were then transferred to wells containing primary antisera, either rabbit anti-Y1 receptor (1:1000; ImmunoStar Inc., Hudson, WI), sheep anti-NPY (1:10,000, Chemicon, Temecula, CA), rabbit anti-tyrosine hydroxylase (1:10,000; Chemicon, Temecula, CA), or rabbit anti- α melanocyte stimulating hormone (α -MSH; 1:10,000; ImmunoStar, Hudson, WI), diluted in 3 % normal donkey serum and 0.25 % Triton X-100 in PBS for 48 h. Sections were then washed with PBS and incubated in secondary antisera for 2 h using either donkey anti-goat or donkey anti-sheep (1:500; Jackson ImmunoResearch, West Grove, PA). Secondary antibodies were diluted in 3 % normal donkey serum corresponding to the host species of the secondary antibody and 0.25 % Triton X-100 in PBS. After washing with PBS, sections were incubated in a 1:222 solution of the avidin-biotin-peroxidase complex (Vector Elite kit; Vector laboratories, Burlingame, CA) in 0.3 % Triton X-100 in PBS for 1 h at RT. Sections were then washed with PBS, followed by a chromogen reaction using 3, 3'-diaminobenzidine (0.2 mg/ml; Sigma Chemicals, St. Louis, MO) as a peroxidase substrate in the presence of 0.025 % H₂O₂ in PBS for 5-10 min. The reaction was terminated with a PBS wash. Sections were mounted on gelatinized slides and dehydrated with increasing concentration of alcohol followed by xylenes (Fisher Scientific, Fair Lawn, NJ) and then coverslipped with Permount (Fisher Scientific, Fair Lawn, NJ). Note that all sections for each antibody were processed simultaneously to eliminate differences in staining that would compromise accurate quantification by the imaging system.

Image analysis—For quantification of cresyl violet staining or immunohistochemistry labeling, two bilateral anterior, two bilateral middle and two bilateral posterior sections from the Arc or PVH were matched and analyzed from each brain. A mouse brain atlas was used to guide in the identification of brain regions (Paxinos and Franklin, 2007) as there is no

Siberian hamster brain atlas commercially available and the size and shape of most mouse brain areas are similar to that of Siberian hamsters. Section levels were defined as follows: anterior Arc (bregma -1.34 mm), middle Arc (bregma -1.58 mm) and posterior Arc (bregma -1.82 mm); anterior PVH (bregma -0.58 mm), middle PVH (bregma -0.82 mm) and posterior PVH (bregma -1.06 mm). Two sections at the level of the PFA (bregma -1.70 mm and -1.82 mm) were analyzed from each brain. Relative optical density was obtained using a microscope (Nikon eclipse E800, 34357) connected to a camera (Retiga EXi Qimaging, 32-0062A-143) and imaging software (IPLab Scientific Image Processing, version 3.9.1, Mac OS 10.4.7; Scanalytic, Fairfax, VA).

Statistical analysis—Because the data were analyzed for each time interval in order to identify intervals within which any experimental effects occur, rather than cumulatively, no statistical comparisons were made across time (*i.e.*, there was no Time factor analysis). In addition, comparisons among time intervals would be inappropriate, as the intervals are of differing durations. Therefore, the data from one time interval only are compared with data within that time interval. Measures for both *experiments 1 and 2* were analyzed using a one-way ANOVA (Drug; B-SAP/NPY-SAP) using Number Crunching Statistical Software (NCSS v 2000; Kaysville, UT). Behavioral measures after food deprivation and refeeding in *experiment 1* were analyzed using a three-way ANOVA; $3 \times 2 \times 2$ [Foraging Group (10 Rev, FW or BW) $\times$ Drug (B-SAP/NPY-SAP) $\times$ Feeding (Non-food deprivation/food deprivation)] at each time point using NCSS. Behavioral measures after food deprivation and refeeding in *experiment 2* were analyzed using a two-way ANOVA: 2×2 [Drug (PBS/MSG) $\times$ Feeding (Non-food deprivation/food deprivation)] at each time point. For all behavioral analyses, Duncan's New Multiple Range tests were used for post hoc tests when appropriate. Relative optical density scores were evaluated using a one-way ANOVA (Drug) for both *experiments 1 and 2* and Bonferroni's post hoc tests when appropriate. Differences among groups were considered statistically significant if $P < 0.05$. Exact probabilities and test values were omitted for simplicity and clarity of the presentation.

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Abbreviations

Arc	arcuate nucleus
NPY	neuropeptide Y
AgRP	Agouti-related protein
NPY-SAP	neuropeptide Y conjugated to saporin
B-SAP	blank-saporin
MSG	monosodium glutamate

Figure 1.

Photomicrographs of representative arcuate nucleus (Arc; A-D), paraventricular nucleus of the hypothalamus (PVH; E-F) and perifornical area (PFA; G) brain sections from Siberian hamsters treated with blank saporin (B-SAP; *left*) or neuropeptide Y conjugated to saporin (NPY-SAP; *right*) and the relative optical density (*middle*) of Arc staining for cresyl violet (A), neuropeptide Y (B and E), neuropeptide Y1 receptor (C, F and G) and α -melanocyte stimulating hormone (D). Values are Mean $\pm$ SEM. *Ps<0.05 compared with B-SAP-treated animals.

Figure 2.

Mean±SEM baseline foraging for the 10 Revolutions/pellet group (10 Rev; A), wheel revolutions for the Free Wheel group (FW; D), food hoarding (B, E and G) and food intake (C, F and H) for the 10 Rev, FW and Blocked Wheel (BW) Siberian hamsters treated with blank saporin (B-SAP) or neuropeptide Y conjugated to saporin (NPY-SAP). * $P < 0.05$ compared with B-SAP-treated animals.

Figure 3.

Mean $\pm$ SEM foraging (A), food hoarding (B) and food intake (C) after food deprivation and refeeding for the 10 Revolutions/pellet group (10 Rev) of Siberian hamsters treated with blank saporin (B-SAP) or neuropeptide Y conjugated to saporin (NPY-SAP). ^aP_s<0.05 compared with B-SAP/Non-Food deprived animals. ^bP_s<0.05 compared with B-SAP/Food deprived animals. ^cP_s<0.05 compared with NPY-SAP/Non-Food deprived animals. ^dP_s<0.05 compared with NPY-SAP/Food deprived animals.

Figure 4. Mean $\pm$ SEM wheel revolutions (A), food hoarding (B) and food intake (C) after food deprivation and refeeding for the Free Wheel group (FW) of Siberian hamsters treated with blank saporin (B-SAP) or neuropeptide Y conjugated to saporin (NPY-SAP). * P s<0.05 compared with B-SAP-treated animals. ^a P s<0.05 compared with B-SAP/Non-Food deprived animals. ^b P s<0.05 compared with B-SAP/Food deprived animals. ^c P s<0.05 compared with NPY-SAP/Non-Food deprived animals. ^d P s<0.05 compared with NPY-SAP/Food deprived animals.

Figure 5.

Mean±SEM food hoarding (A) and food intake (B) after food deprivation and refeeding for the Blocked Wheel group (BW) of Siberian hamsters treated with blank saporin (B-SAP) or neuropeptide Y conjugated to saporin (NPY-SAP). ^aP_s<0.05 compared with B-SAP/Non-Food deprived animals. ^bP_s<0.05 compared with B-SAP/Food deprived animals. ^cP_s<0.05 compared with NPY-SAP/Non-Food deprived animals. ^dP_s<0.05 compared with NPY-SAP/Food deprived animals.

Figure 6.

Photomicrographs of representative arcuate nucleus (Arc; A-D), paraventricular nucleus of the hypothalamus (PVH; E-F) and perifornical area (PFA; G) brain sections from Siberian hamsters treated with phosphate buffered saline (PBS; *left*) or monosodium glutamate (MSG; *right*) and the relative optical density (*middle*) of Arc staining for cresyl violet (A), neuropeptide Y (B and E), neuropeptide Y1 receptor (C, F and G) and α -melanocyte stimulating hormone (D). Values are Mean $\pm$ SEM. * P s<0.05 compared with PBS-treated animals.

Figure 7. Mean $\pm$ SEM baseline wheel revolutions (A), food hoarding (B) and food intake (C) after for the Free Wheel group (FW) of Siberian hamsters treated with phosphate buffered saline (PBS) or monosodium glutamate (MSG).

Figure 8.

Mean $\pm$ SEM wheel revolutions (A), food hoarding (B) and food intake (C) after food deprivation and refeeding for the Free Wheel group (FW) of Siberian hamsters treated with phosphate buffered saline (PBS) or monosodium glutamate (MSG). ^aP_s<0.05 compared with PBS/Non-Food deprived animals. ^bP_s<0.05 compared with PBS/Food deprived animals. ^cP_s<0.05 compared with MSG/Non-Food deprived animals. ^dP_s<0.05 compared with MSG/Food deprived animals.