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Amylin-Induced Central IL-6 Production Enhances Ventromedial Hypothalamic Leptin Signaling

Diabetes 2015;64:1621–1631 | DOI: 10.2337/db14-0645

Amylin acts acutely via the area postrema to reduce food intake and body weight, but it also interacts with leptin over longer periods of time, possibly via the ventromedial hypothalamus (VMH), to increase leptin signaling and phosphorylation of STAT3. We postulated that amylin enhances VMH leptin signaling by inducing interleukin (IL)-6, which then interacts with its gp130 receptor to activate STAT3 signaling and gene transcription downstream of the leptin receptor. We found that components of the amylin receptor (RAMPs1–3, CTR1a,b) are expressed in cultured VMH astrocytes, neurons, and microglia, as well as in micropunches of arcuate and ventromedial hypothalamic nuclei (VMN). Amylin exposure for 5 days increased IL-6 mRNA expression in VMH explants and microglia by two- to threefold, respectively, as well as protein abundance in culture supernatants by five- and twofold, respectively. Amylin had no similar effects on cultured astrocytes or neurons. In rats, 5 days of amylin treatment decreased body weight gain and/or food intake and increased IL-6 mRNA expression in the VMN. Similar 5-day amylin treatment increased VMN leptin-induced phosphorylation of STAT3 expression in wild-type mice and rats infused with lateral ventricular IgG but not in IL-6 knockout mice or rats infused with ventricular IL-6 antibody. Lateral ventricular infusion of IL-6 antibody also prevented the amylin-induced decrease of body weight gain. These results show that amylin-induced VMH microglial IL-6 production is the likely mechanism by which amylin treatment interacts with VMH leptin signaling to increase its effect on weight loss.

Amylin is synthesized by pancreatic β-cells and is co-released with insulin in response to food intake and increasing glucose concentrations (1). While insulin stimulates nutrient disposal and storage, amylin limits nutrient availability by inhibiting food intake, gastric emptying, and digestive secretions (2,3). The amylin receptor is composed of a calcitonin receptor (CTR) heterodimerized with a receptor activity–modifying protein (RAMP) (4,5). There are two splice variants of CTR, 1a and 1b (4,6), and three known RAMP subtypes (RAMP1, 2, 3), providing six possible combinations for expressing the amylin receptor (7). The CTR can be activated by peptides such as calcitonin and amylin (8); however, CTR has an enhanced affinity for amylin when combined with RAMPs (9). Amylin binds to its receptors, which are distributed throughout the brain. These include the area postrema (AP), nucleus of the solitary tract, the lateral hypothalamic area, ventromedial (VMN) and arcuate (ARC) hypothalamic nuclei, and the ventral tegmental area (VTA) (10–14).

Several studies have documented the satiating effect of amylin via its action on the AP (3,15–17). The VTA has also been demonstrated as a site of action by amylin (18). However, suggestions that amylin acts in the VMN and ARC to enhance leptin signaling and synergistically decrease food intake and body weight when coadministered with leptin in obese rats and humans, as well as lean rats, have also been made (19–22). Systemic amylin administration increases expression of the intracellular signaling form of the leptin receptor, Lepr-b, as well as binding of leptin to its receptors in the ARC and

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Received 22 April 2014 and accepted 14 November 2014.
This article contains Supplementary Data online at http://diabetes.diabetesjournals.org/lookup/suppl/doi:10.2337/db14-0645/-/DC1.
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See accompanying article, p. 1498.

VMN. This is associated with an increase in VMN leptin-induced pSTAT3 (19,20); STAT3 is one of the major signaling pathways downstream of the leptin receptor (23,24).

Since there is currently no evidence that amylin acting at the AP increases VMN leptin signaling, we postulated that amylin might act independently in the ventromedial hypothalamus (VMH; the ARC plus the VMN) to stimulate the production of interleukin (IL)-6, which then acts on its receptor signaling complex, the IL-6 receptor (IL-6R) coupled to gp130, to activate STAT3 as a means of increasing downstream leptin signaling. This hypothesis is based on the finding that endogenous IL-6 increases leptin sensitivity (25) and that increased IL-6 production in the VMH increases leptin signaling and anorectic sensitivity in swim-stressed rats, an effect that is blocked by intraventricular administration of IL-6 antibodies (26). Using in vivo and in vitro methods, we found that amylin causes VMH microglia to produce IL-6 and increases IL-6 mRNA expression in VMN micropunches from rats treated with amylin. Amylin treatment increased VMN leptin-induced pSTAT3 expression in wild-type (WT) mice and rats, but it failed to do so in IL-6 knockout (KO) mice or rats infused in their lateral ventricles (LVs) with IL-6 antibody. These results strongly suggest that amylin enhances VMH leptin signaling by directly stimulating microglia IL-6 production, which then acts on VMH neurons to increase leptin-induced pSTAT3.

RESEARCH DESIGN AND METHODS

Animals

Outbred male Sprague-Dawley rats were purchased from Charles River Laboratories (Wilmington, MA). IL-6 KO (B6;129S6-*Il6*^{tm1Kopf}/J) and WT (C57BL/6J) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Rats were housed at 23–24°C on a reverse 12-h light/12-h dark cycle (lights off at 0800) with ad libitum access to chow (3.36 kcal/g, 13.5% fat; Purina #5001) and water. Mice were fed mouse chow (3.81 kcal/g, 25% fat; Purina #5015) and housed on a conventional 12-h light/12-h dark schedule with lights off at 0900. All work was in compliance with the Institutional Animal Care and Use Committee of the East Orange Veterans Affairs Medical Center.

In Vitro Amylin Effects

VMH Explants

Sprague-Dawley male rats were killed on postnatal days (P) 21–28, and 350-μm sections of the VMH (from bregma −2.30 to −3.60 mm [27]) were cut with a vibratome in oxygenated slushed artificial cerebrospinal fluid (containing 118 mmol/L NaCl, 3 mmol/L KCl, 1 mmol/L $MgCl_2$, 2.5 mmol/L $NaHCO_3$, 1.5 mmol/L $CaCl^2$, 1.2 mmol/L NaH_2PO_4, 5 mmol/L HEPES, 2.5 mmol/L glucose, 15 mmol/L sucrose [pH 7.4]). Explant slices were transferred to individual wells and maintained in Neurobasal (Invitrogen, Grand Island, NY) containing 10% FBS, 5 mmol/L glucose, 10 μg/mL gentamicin, and 10,000 U/mL penicillin/streptomycin at 37°C for 5 days. They were exposed twice daily to 10 μmol/L amylin (Bachem, Torrance, CA) or PBS control (n = 9 rats/group). On day 5, media were collected and stored at −80°C for cytokine assays. Slices were placed in RNA Later (Ambion, Grand Island, NY), the VMH was punched under microscopic guidance, and mRNA expression was assayed by quantitative reverse transcriptase PCR (QPCR; Applied Biosystems, Grand Island, NY) (28,29).

Primary VMN Neuronal Cultures

On P21–28, rats were perfused with a 4% sucrose solution, and neurons were dissociated from VMN punches, as previously described (28,29). Neurons were cultured in growth media (Neurobasal plus 2.5 mmol/L glucose) for 5 days and exposed twice daily to 10 μmol/L amylin (Bachem) or PBS (n = 9 rats/group). On day 5, media were collected and kept at −80°C for cytokine assays. Neurons were exposed to 120 μL of lysis buffer (Ambion) from which mRNA was extracted and gene expression assayed by QPCR (Applied Biosystems) (28).

Primary VMH Astrocyte Cultures

The VMH was dissected from rats at P21–28 and triturated in Neurobasal-A (Invitrogen) containing 2.5 mmol/L glucose, 0.23 mmol/L sodium pyruvate, 10,000 U/mL penicillin/streptomycin, 10 μg/mL gentamicin, and 10% FBS at pH 7.4. Astrocytes were dissociated, as previously described (30). The day before amylin treatment, astrocytes were washed with PBS, and serum-free Neurobasal-A was added overnight. Astrocytes then were exposed to vehicle alone (PBS) or 10 μmol/L amylin twice daily for 5 days (n = 9 rats/group). Terminally, media were collected and stored at −80°C for cytokine assays. Astrocytes were exposed to 120 μL of lysis buffer (Ambion), followed by mRNA extraction, reverse transcription, and quantification by QPCR (Applied Biosystems) (28).

Primary Cortical and Hypothalamic Microglia Cultures

Primary mixed glial cortical and hypothalamic cultures were generated from cortical or hypothalamic tissue from rats at P2. Intact brains were removed and dissected free of meninges. Tissue samples were placed in 2% glucose/PBS and digested in 0.25% trypsin for 20 min. Complete Minimum Essential Media (Invitrogen) containing 10% FBS, 1% glutamine, 10,000 U/mL penicillin/streptomycin, and 6% glucose then were added. The tissue was gently triturated with a 10-mL pipet and passed through a 130-μm screen. Cells were pelleted at 1,200 rpm for 5 min, and the pellet was suspended in 10 mL Complete Minimum Essential Media and passed through a 35-μm screen. Cells were counted and plated at a density of 1.5×10^6 cells/mL. Cells were cultured in 75-cm^2 tissue culture flasks and maintained at 37°C in 5% carbon dioxide. When cultures reached confluence, microglia cells were harvested by shaking at 250 rpm for 90 min, then were

pelleted at 1,200 rpm for 5 min, suspended in DMEM and Ham's F-12 Nutrient Mixture (Invitrogen) containing 10% FBS, and plated at a density of 4×10^5 cells/mL. At 90% confluence, microglia were treated with vehicle (PBS) or 1 μmol/L amylin twice daily for 5 days (n = 6/group). Terminally, media were collected and stored at −80°C for cytokine assays. Microglia were treated with 120 μL of lysis buffer (Ambion); mRNA was extracted and assayed by QPCR.

Tissue Culture Cytokine Measurement

IL-1β, IL-6, IL-10, and tumor necrosis factor (TNF)-α were measured in culture supernatants using a customized V-Plex proinflammatory assay for rats (K15044D; Meso Scale Discovery [MSD], Gaithersburg, MD). For all assays, culture supernatants were diluted in the diluent provided (1:2). Samples collected from VMH explants and neuronal cultures were incubated in the coated MSD plate for 2 h at room temperature, whereas samples from astrocyte and microglia cultures were incubated overnight at 4°C to increase the sensitivity of the assay. Assays were otherwise completed per the manufacturer's instructions. Detection limits of the assay were 6.92 pg/mL for IL-1β, 13.8 pg/mL for IL-6, 16.4 pg/mL for IL-10, and 0.72 pg/mL for TNF-α. Plates were read using the MESO QuickPlex SQ 120 (MSD) and analyzed using MSD's Discovery Workbench analyzer and software package. Cytokine concentrations were corrected against cyclophilin mRNA to normalize sample size.

In Vivo Amylin Effects

After 1 week of acclimation, 9- to 10-week-old (300–350 g) male rats were randomized by body weight into experimental groups, anesthetized, and implanted with subcutaneous, intrascapular minipumps (Alzet Model 2001; Durect Corp., Cupertino, CA) (20).

Experiment 1 (Fig. 2 and Tables 3 and 4): Rats were divided into three groups: amylin (dissolved in 0.9% saline infused at 100 μg/kg/day; Bachem), treated and fed ad libitum; pair-fed (0.9% saline infusion with intake matched to intake by amylin rats the previous day); and those fed ad libitum (0.9% saline infusion). Body weight and food intake were monitored every 2 days. Terminally, food was removed at lights on (2000) the evening before, and rats were rapidly decapitated at lights off the next morning. Brains were removed, snap frozen on dry ice, and stored at −80°C for mRNA analysis by QPCR (31). Resulting target gene expression was expressed as a ratio of the constitutively expressed gene, cyclophilin.

Experiment 2 (Fig. 3): A cannulae linked to a subcutaneous osmotic minipump (Azlet Model 2002 and Brain Infusion Kit 2) were implanted in the LV (X = 1.6 mm, Y = 0.9 mm, Z = 4 mm, relative to the dura) of 9- to 10-week-old male rats to infuse either IL-6 antibody (400 ng/kg/day; Santa Cruz sc-7920) or IgG control (400 ng/kg/day; Santa Cruz sc-2027). After 5 days of recovery, rats were implanted with a second subcutaneous osmotic minipump (Alzet 2001) for saline (0.9%) or amylin (100 μg/kg/day) for an additional 5 days. Body weight and food intake were monitored daily. Terminally, food was removed 2 h before lights off (0700). Rats were injected intraperitoneally with murine leptin (5 mg/kg in PBS; NHPP, Torrance, CA) at lights off, anesthetized (ketamine/xylazine) 45 min later, and rapidly perfused with 0.9% saline followed by 4% paraformaldehyde (PFA) in PBS. Brains were removed, postfixed overnight in 4% PFA, and transferred the next day to potassium phosphate-buffered saline containing 20% sucrose for 24 h. The brains were then frozen in 2-methylbutane.

Amylin Effects on IL-6 KO Mice

Male IL-6 KO and WT mice (9 weeks old) were fed mouse chow ad libitum throughout the experiment. After 1 week of acclimation, mice were randomized by body weight into four groups then anesthetized and implanted with subcutaneous intrascapular minipumps (Alzet Model 1002; Durect Corp.). Pumps contained amylin (Bachem) in 0.9% saline (WT-A and IL-6 KO-A infused at 1 mg/kg/day) or 0.9% saline vehicle (WT-S and IL-6 KO-S). Body weight and food intake were monitored biweekly for 2 weeks. Terminally, food was removed 2 h before lights off. Mice were injected intraperitoneally with murine leptin (5 mg/kg in PBS; NHPP) at lights off, anesthetized (ketamine/xylazine) 45 min later, and rapidly perfused with 0.9% saline followed by 2% PFA in PBS (32). Brains were removed, postfixed overnight in potassium phosphate-buffered saline containing 20% sucrose, and frozen with 2-methylbutane.

^{125}I Amylin Receptor-Binding Autoradiography

Brains from nonfasted rats were removed and frozen on powdered dry ice; 12-μm sections were cut through the midpoint of the ARC, VMN, and dorsomedial nucleus (DMN) pars compacta (27), mounted on gel-coated slides, desiccated, and stored at −80°C. Amylin receptor binding was carried out by methods adapted from Sexton et al. (33). Briefly, sections were thawed and rinsed in incubation buffer (20 mmol/L HEPES containing 100 mmol/L NaCl, 1 mg/mL BSA, and 0.5 mg/mL bacitracin). Sections then were incubated at room temperature for 1 h in incubation buffer containing 70–75 pmol/L ^{125}I amylin (NEX44; Perkin Elmer, Boston, MA) plus 1 μmol/L unlabeled rat amylin (nonspecific "binding"; Bachem). Slides were rinsed in incubation buffer at 4°C and rinsed two more times in modified incubation buffer (20 mmol/L HEPES containing 100 mmol/L NaCl) at 4°C. After a brief dip in dH_2O, sections were dried under forced cold air and desiccated for 24 h. Sections were then exposed to BioMax MR Film (Kodak, Rochester, NY) at −80°C for 7–14 days.

pSTAT3 Immunohistochemistry

Brain sections (30 μm) were cut through the mid-VMH (27) from saline- and amylin-treated WT and IL-6 KO

mice and mounted on Superfrost Plus slides. Free-floating (30-μm) sections were cut from brains of control and amylin-treated rats that received LV infusion of IgG or IL-6 antibody. Slides and free-floating sections were stored in antifreeze at −20°C until processed for leptin-induced pSTAT3 immunohistochemistry using rabbit anti-pSTAT3 antibody (1:1,000; Cell Signaling Technologies, Danvers, MA) using previously described methods (31). Three consecutive sections per brain were counted using an image analysis system (Bioquant, Nashville, TN) by an experimentally naive observer.

Statistics

Statistical comparisons among variables for in vivo studies were made by one-way ANOVA with Bonferroni post hoc analysis. Body weight gain and food intake were analyzed by one-way ANOVA with repeated measures. Food efficiency was calculated by dividing the body weight gain in grams by the total food intake in kilocalories and multiplying the total by 1,000. All data are expressed as mean ± SEM. Statistical analysis was performed using SYSTAT software (SYSTAT, Chicago, IL). Comparisons between control and amylin-treated groups in in vitro studies were assessed using the *t* test for nonparametric statistics (GraphPad Prism, La Jolla, CA).

RESULTS

Distribution of ^{125}I Amylin Binding

We confirmed previous findings (33,34) that ^{125}I amylin binds to the ventromedial portion of the VMN, as well as the ARC, DMN, perifornical and medial tuberal hypothalamus, and the medial amygdalar nucleus (Fig. 1). There was little binding in cerebral cortex or hippocampal structures at the rostrocaudal level through the midpoint of the VMH. Hindbrain structures were not examined because the emphasis here was on the effects of amylin on forebrain structures. No amylin binding occurred in sections co-incubated with unlabeled amylin (Supplementary Fig. 1).

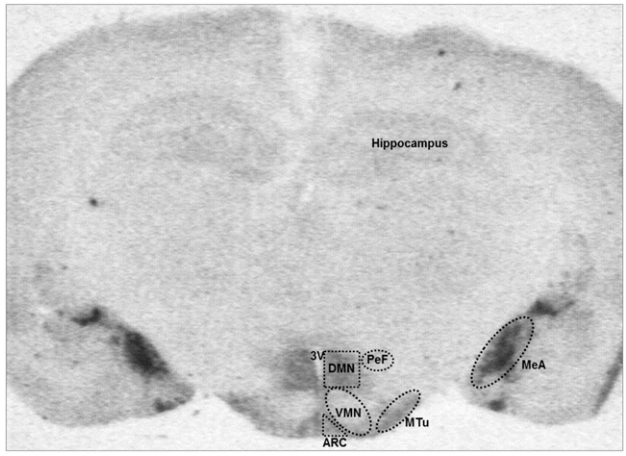

Figure 1— ^{125}I amylin binding in chow-fed rats. *Dotted areas* represent the hypothalamic ARC, VMN, DMN, perifornical (PeF) and medial tuberal nucleus (MTu), and medial amygdala (MeA), as identified on the cresyl violet–stained slides used to generate the autoradiogram.

In Vitro Effects of Amylin on Hypothalamic Explants, Neurons, Astrocytes, and Microglia

Exposing VMH explants to 10 μmol/L amylin for 5 days increased IL-6 mRNA expression by 320% (Table 1) and secretion of IL-6 protein 5.5-fold (Table 2). Amylin also increased mRNA expression of RAMP1 and two subunits of the amylin receptor by 122% and 103%, respectively, whereas it decreased expression of the CTR1b subunit of the amylin receptor by 72% (Table 1). In addition, amylin increased IL-10 secretion sevenfold (Table 2).

To assess the specific cellular source of IL-6 production within the VMH, primary cultures of VMH neurons, microglia, and astrocytes, as well as cerebral cortical microglia, were incubated with amylin (1–10 μmol/L) for 5 days. Exposure of primary hypothalamic microglial cultures from rats (P2) to 1 μmol/L amylin increased IL-6 mRNA expression by 211% (Table 1) and IL-6 protein production by 204% (Table 2). Amylin also increased microglial CTR1b mRNA expression by 56% and decreased both leukemia inhibitory factor (LIF), a member of the IL-6 cytokine family that acts though gp130, and gp130 mRNA expression by 29% (Table 1). The amylin-induced increase in IL-6 mRNA expression was not specific to hypothalamic microglia; amylin also increased cerebral cortex microglial IL-6 mRNA expression by 140% (Table 1) and IL-6 media secretion by 310% (Table 2). Amylin increased the secretion of TNF-α by cortical microglia by 158% (Table 2). Amylin exposure had no effect on neuronal cytokine mRNA or protein production (Tables 1 and 2), although it did increase neuronal SOCS3 (an inhibitor of Janus kinase [JAK]/STAT3 signaling) mRNA expression by 33% (Table 1). Similarly, while amylin had no effect on IL-6 mRNA expression in cultured astrocytes, it did increase TNF-α mRNA by 113%, IL-1β by 211%, and ciliary neurotrophic factor by 74%, while decreasing LIF expression by 61% (Table 1).

In Vivo Effects of Amylin on VMH Cytokine Production (Experiment 1)

Male, 9- to 10-week-old rats were infused subcutaneously with either amylin or vehicle for 5 days. Vehicle-treated rats pair-fed to amylin-treated rats served as additional controls. Amylin-treated rats consumed 24% fewer kilocalories overall (P = 0.001; Fig. 2*B* and Table 3) and gained 86% less body weight compared with ad libitum-fed controls over 5 d of treatment (Fig. 2*A* and Table 3). This resulted in an 82% lower overall feed efficiency in amylin-treated rats, suggesting an amylin-induced increase in energy expenditure (Table 3). In VMN micropunches from these rats, expression of IL-6 mRNA was increased by 46% in amylin-treated rats versus ad libitum controls, whereas pair-feeding had no effect on IL-6 expression (Table 4). Associated with the increase in VMN IL-6 expression, VMN Lepr-b mRNA expression was increased by 60% (Table 4) compared with pair-fed controls. Also, expression of VMN CTR1a and b were increased by

Table 1—Amylin-induced changes in VMH explant, neuron, astrocyte, hypothalamic, and cerebral cortex microglia gene expression

	Explant		Neurons		Astrocytes		Hypothalamic microglia		Cortical microglia	
Genes	Control	Amylin	Control	Amylin	Control	Amylin	Control	Amylin	Control	Amylin
IL-6	0.77 ± 0.35	3.24 ± 0.87*	1.39 ± 0.21	1.89 ± 0.17	1.01 ± 0.11	0.78 ± 0.07	0.53 ± 0.07	1.65 ± 0.39*	0.68 ± 0.10	1.63 ± 0.25*
IL1-β	1.30 ± 0.29	1.71 ± 0.20	1.17 ± 0.20	1.23 ± 0.19	0.47 ± 0.05	1.46 ± 0.16*	0.76 ± 0.09	1.32 ± 0.22	0.94 ± 0.14	1.21 ± 0.12
IL-10	Und	Und	Und	Und	Und	Und	0.89 ± 0.13	0.97 ± 0.19	0.57 ± 0.11	1.11 ± 0.14*
TNF-α	1.20 ± 0.30	1.86 ± 0.40	1.24 ± 0.20	1.40 ± 0.21	0.67 ± 0.12	1.43 ± 0.17*	2.16 ± 0.28	1.66 ± 0.31	1.07 ± 0.10	0.95 ± 0.12
LIF	0.78 ± 0.28	0.41 ± 0.15	1.01 ± 0.11	1.08 ± 0.11	1.40 ± 0.10	0.55 ± 0.06*	1.07 ± 0.07	0.76 ± 0.07*	0.90 ± 0.06	1.08 ± 0.08
CNTF	0.88 ± 0.19	0.69 ± 0.16	1.34 ± 0.26	1.50 ± 0.34	0.74 ± 0.08	1.29 ± 0.16*	1.16 ± 0.08	0.93 ± 0.05	0.95 ± 0.07	0.95 ± 0.12
gp130	0.17 ± 0.04	0.21 ± 0.10	1.44 ± 0.27	1.20 ± 0.18	1.02 ± 0.05	0.95 ± 0.02	1.21 ± 0.13	0.86 ± 0.07*	1.00 ± 0.07	1.16 ± 0.20
CTR1a	2.41 ± 0.54	3.80 ± 1.35	1.26 ± 0.05	1.30 ± 0.03	0.71 ± 0.17	0.65 ± 0.1	0.87 ± 0.12	1.13 ± 0.18	0.78 ± 0.12	1.35 ± 0.21
CTR1b	5.92 ± 0.53	1.65 ± 0.63*	1.03 ± 0.04	1.01 ± 0.06	Und	Und	0.75 ± 0.11	1.17 ± 0.10*	Und	Und
RAMP1	1.23 ± 0.32	2.74 ± 0.49*	0.94 ± 0.05	0.96 ± 0.09	0.78 ± 0.06	1.03 ± 0.12	1.14 ± 0.10	0.87 ± 0.12	1.07 ± 0.09	0.92 ± 0.08
RAMP2	0.29 ± 0.04	0.59 ± 0.10*	1.03 ± 0.06	1.01 ± 0.07	0.93 ± 0.08	0.97 ± 0.1	1.07 ± 0.09	1.02 ± 0.05	0.95 ± 0.04	1.10 ± 0.04*
RAMP3	0.89 ± 0.27	1.15 ± 0.15	1.00 ± 0.04	0.96 ± 0.04	0.99 ± 0.07	1.13 ± 0.06	0.74 ± 0.15	1.07 ± 0.13	0.80 ± 0.05	1.24 ± 0.05*
Lepr-b	1.89 ± 0.60	1.73 ± 0.70	0.75 ± 0.06	0.55 ± 0.06						
SOCS3	0.59 ± 0.20	0.47 ± 0.15	0.98 ± 0.07	1.30 ± 0.12*						

VMH explants, neurons, astrocytes, and microglia were incubated with amylin (1–10 μmol/L) vs. vehicle (PBS) for 5 days. Data are mean ± SEM of duplicate determinations expressed relative to the amount of the mRNA expression of cyclophilin (n = 9/group). *$P \leq 0.05$ control vs. amylin in each type of cell culture. Und, undetectable.

Table 2—Cytokine production after 5 days of treatment with amylin (1–10 μmol/L) in VMH explant, neurons, and astrocytes and cortex and hypothalamic microglia from male SD rats

	Explant		Neurons		Astrocytes		Hypothalamic microglia		Cortical microglia	
Cytokines	Control	Amylin	Control	Amylin	Control	Amylin	Control	Amylin	Control	Amylin
IL-6	368 ± 83	2,058 ± 241*	64.4 ± 6.6	82.1 ± 7.2	15.1 ± 1.82	11.3 ± 1.50	28.6 ± 6.59	86.9 ± 21.6*	18.6 ± 3.92	76.5 ± 10.21*
IL-10	2.46 ± 0.95	18.1 ± 4.62*	2.21 ± 0.41	3.02 ± 0.61	3.75 ± 0.72	3.95 ± 0.57				
IL-1β			7.22 ± 1.17	8.27 ± 2.40	11.9 ± 0.75	10.8 ± 1.52	9.19 ± 2.23	12.54 ± 2.81	11.24 ± 3.54	15.3 ± 1.80
TNF-α	5.27 ± 1.51	12.4 ± 3.32	8.83 ± 0.56	9.88 ± 0.96	0.47 ± 0.05	0.56 ± 0.05	2.44 ± 0.56	3.15 ± 0.72	1.48 ± 0.44	3.82 ± 0.55*

Cytokine concentrations in the supernatant of cultures (picograms per milliliter) were normalized to the amount of cyclophilin mRNA expression in each respective tissue; n = 6–9 rats/group. *$P \leq 0.05$, t test, vehicle vs. amylin.

120% and 176%, respectively, compared with pair-fed rats (Table 4). The amylin-induced changes appeared to be specific to IL-6 as amylin had no effects on the mRNA expression of any other VMN or ARC cytokine. Despite the lack of significant amylin-induced changes in IL-6 or Lepr-b expression in the ARC, amylin-treated rats had significant increases in both NPY and AgRP mRNA expression compared with ad libitum or pair-fed controls (Table 4).

Amylin Effects on Rat VMH Leptin Signaling of LV IL-6 Antibody Infusions (Experiment 2)

To confirm the hypothesis that the amylin-sensitizing effect on leptin signaling is caused by an amylin-induced increase in IL-6 activation of JAK/STAT3 signaling, IgG or IL-6 antibodies were infused into the LV of rats for 5 days. Rats then were additionally infused subcutaneously with either amylin or vehicle for 5 days more. Neither IgG nor IL-6 antibodies altered food intake or body weight gain over the first 5 days of LV infusion (Fig. 3*A* and *B*). After an additional 5 days of amylin treatment, LV IgG-infused rats decreased their body weight gain and food intake by 96% and 27%, respectively, compared with IgG-saline rats (Fig. 3*C* and *D*). On the other hand, LV IL-6 antibody infusion attenuated the amylin-induced decrease in body weight gain by 37% (Fig. 3*C*) but had no effect on amylin-induced reduction in food intake (Fig. 3*D*). Most important, 10 days of IL-6 antibody treatment and 5 days of amylin infusion prevented the amylin-induced enhancement of leptin-induced VMN pSTAT3 expression that occurred in IgG amylin rats by 25% (Fig. 3*E*). However, IL-6 antibody infusion had no effect on the enhancement of leptin-induced pSTAT3 expression by amylin in the ARC (Fig. 3*E*). These data strongly suggest that IL-6 is required for the amylin-sensitizing effects on VMH leptin signaling, through which it contributes to amylin-induced reductions in body weight gain, but not food intake.

Effects of Amylin on Leptin Signaling in IL-6 KO Mice

To further confirm the hypothesis that the amylin-sensitizing effect on leptin signaling is caused by an amylin-induced increase in IL-6 activation of JAK/STAT3 signaling, WT and IL-6 KO mice were infused with either amylin or vehicle by minipumps for 2 weeks. Although there were no significant differences in body weight gain or food intake among the groups, there was a trend toward decreased body weight gain in amylin-treated WT controls (Supplementary Fig. 2). Most important, 2 weeks of amylin treatment was selectively associated with a 67% increase in leptin-induced pSTAT3 expression in the VMN of WT but not IL-6 KO mice (Fig. 4*A* and *B*). These data strongly suggest that IL-6 is required for the amylin-sensitizing effects of VMH leptin signaling.

DISCUSSION

The goal of this study was to identify the mechanism by which systemic amylin administration increases VMH leptin signaling to produce a synergistic effect on weight

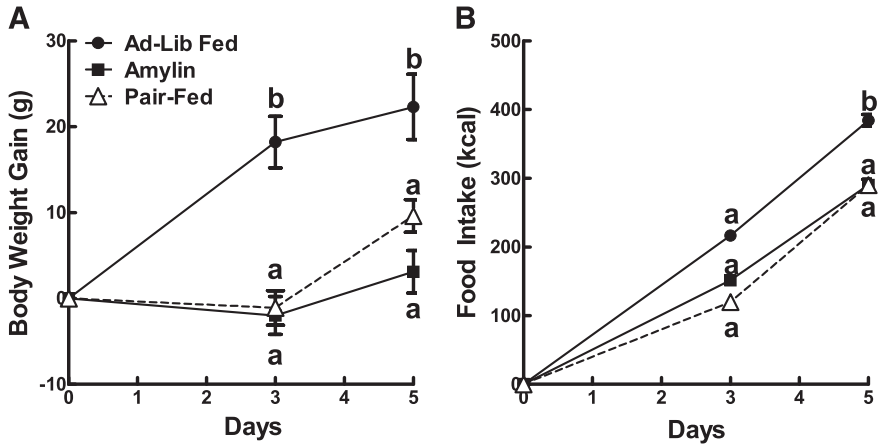

Figure 2—Body weight gain (*A*) and food intake (*B*) in 9- to 10-week-old male rats fed ad libitum (Ad-Lib), amylin-treated, and pair-fed after 5 days of systemic amylin (100 μg/kg/day) vs. vehicle (0.9% saline) infusion with an osmotic minipump (experiment 1). Vehicle was infused in Ad-Lib-fed and pair-fed groups. Values are mean ± SEM; n = 9–10 rats/group. Parameters with differing letters (a,b) differ from each other by $P \leq 0.05$.

loss in obese individuals (19,20). We first confirmed that ^{125}I amylin binds in the VMH (as well as other forebrain areas) and demonstrated, for the first time, that CTR1a and b, along with RAMP1–3 components of the amylin receptor complex, are variably expressed in VMH microglia, astrocytes, and neurons. We postulated that amylin interacts with leptin signaling by causing cells within the VMH to produce IL-6, which is known to phosphorylate STAT3, a common downstream mediator of leptin signaling (35,36), via its IL-6R complex. Amylin increased IL-6 production in both VMH explants and VMN micropunches from rats treated in vivo with amylin; this occurred selectively in microglia but not astrocytes or neurons. As previously demonstrated (19,20), amylin reduced body weight gain and food intake in adult rats; this reduced body weight gain was partially reversed by LV infusions of IL-6 antibody. While high doses of amylin failed to reduce body weight gain or food intake in WT or IL-6 KO mice, the enhancement of VMN leptin-induced pSTAT3 expression by amylin was completely inhibited in IL-6 KO mice and rats with LV IL-6 antibody infusions. The failure of LV IL-6 antibody infusions to completely prevent amylin-induced reductions in body weight gain or food intake in rats was not unexpected because amylin is known to produce weight loss and anorexia via its actions in the AP (37,38), as well as in the VTA (18). The failure of LV IL-6 antibody to reverse the amylin-induced reduction in food intake suggests that the primary role of amylin-induced enhancement of VMH leptin signaling via microglial IL-6 production is in affecting energy expenditure. This also suggests that the effects of amylin on reducing food intake are not mediated via its actions on VMH leptin signaling, but rather via its actions on other brain sites; however, it is also possible that the dose of IL-6 antibody used was not sufficient to prevent the effect of amylin on food intake. Therefore, because the partial blockade of amylin-induced body weight reduction by LV IL-6 antibody infusion was not paired with a decrease in food intake, this suggests that the enhancement of leptin signaling in the VMH by amylin likely resulted in increased energy expenditure.

Table 3—Effects of 5 days of systemic amylin (100 μg/kg/day) vs. vehicle (0.9% saline) infusions in rats

	Fed ad libitum	Amylin	Pair-fed
Body weight, g			
Initial	346 ± 2.9	346 ± 2.6	346 ± 3.8
Final	369 ± 3.6^{a}	349 ± 3.2^{b}	356 ± 3.2^{b}
5-Day body-weight gain	22 ± 3.8^{a}	3.1 ± 2.5^{b}	9.6 ± 1.9^{ab}
5-Day food intake, kcal	384 ± 8.6^{a}	290 ± 8.3^{b}	290 ± 7.5^{b}
5-Day feed efficiency*	51 ± 10^{a}	9 ± 9^{b}	32 ± 6^{ab}

Values are mean ± SEM; n = 9–10 rats/group. *Feed efficiency was calculated using the following formula: (body weight gain [g]/food intake [kcal]) × 1,000. ^{a,b}Parameters with differing letters differ from each other by $P \leq 0.05$.

Our data strongly support the hypothesis that the enhancement of VMH leptin signaling by amylin (20) is attributable to its direct action on VMH microglia to produce IL-6, which then acts on its IL-6R/gp130 receptor complex (39) to activate pSTAT3, which is also downstream of Lepr-b signaling (40). Activation of STAT3 results in its dimerization and translocation into the nucleus, where it then alters gene transcription (24). Given the fact that systemic amylin treatment increases VMH *Lepr-b* gene expression, binding of leptin to its cell surface receptor in both the ARC and VMN and leptin-induced pSTAT3 expression in the VMN (19,20), our data support the hypothesis that convergence of amylin-induced microglial production of IL-6 on STAT3 activation is an important route by which amylin enhances leptin signaling

Table 4—ARC and VMN gene expression after 5 days of systemic amylin (100 μg/kg/day) vs. vehicle (0.9% saline) infusion in rats

	ARC			VMN		
Genes	Fed ad libitum	Amylin	Pair-fed	Fed ad libitum	Amylin	Pair-fed
IL-6	1.29 ± 0.20	1.44 ± 0.08	1.25 ± 0.17	1.66 ± 0.21[a]	2.43 ± 0.15[b]	1.81 ± 0.21[ab]
IL-1β	0.81 ± 0.18	0.74 ± 0.12	0.80 ± 0.15	1.54 ± 0.27	1.06 ± 0.17	1.27 ± 0.24
TNF-α	1.96 ± 0.18	1.60 ± 0.21	1.64 ± 0.16	1.28 ± 0.18	1.10 ± 0.14	1.15 ± 0.11
LIF	1.24 ± 0.13	1.05 ± 0.11	1.21 ± 0.08	0.91 ± 0.05	0.89 ± 0.03	1.04 ± 0.06
CNTF	1.56 ± 0.13	1.52 ± 0.10	1.82 ± 0.13	1.16 ± 0.07	1.25 ± 0.04	1.15 ± 0.05
gp130	2.53 ± 0.21	2.35 ± 0.15	2.36 ± 0.15	1.40 ± 0.07	1.49 ± 0.06	1.27 ± 0.06
Lepr-b	0.81 ± 0.06	0.83 ± 0.07	0.82 ± 0.08	0.93 ± 0.11[ab]	1.20 ± 0.10[a]	0.75 ± 0.06[b]
SOCS3	1.33 ± 0.14	1.17 ± 0.06	1.42 ± 0.10	1.15 ± 0.16	1.05 ± 0.13	0.87 ± 0.11
RAMP1	0.71 ± 0.07	0.74 ± 0.05	0.99 ± 0.10	1.14 ± 0.08	1.05 ± 0.07	1.20 ± 0.08
RAMP2	1.22 ± 0.08	1.23 ± 0.06	1.23 ± 0.08	1.09 ± 0.04[a]	1.04 ± 0.04[ab]	0.95 ± 0.03[b]
RAMP3	0.87 ± 0.04	0.90 ± 0.07	1.04 ± 0.11	0.89 ± 0.10	1.08 ± 0.11	0.75 ± 0.09
CTR1a	1.17 ± 0.10	1.23 ± 0.07	1.14 ± 0.07	0.72 ± 0.14[ab]	1.01 ± 0.15[a]	0.46 ± 0.10[b]
CTR1b	1.06 ± 0.09	1.17 ± 0.07	0.97 ± 0.08	0.89 ± 0.15[ab]	1.27 ± 0.18[a]	0.46 ± 0.08[b]
InsR	1.10 ± 0.07	1.03 ± 0.04	1.07 ± 0.05	0.94 ± 0.06	1.06 ± 0.05	0.91 ± 0.05
NPY	0.84 ± 0.12[a]	1.33 ± 0.10[b]	0.96 ± 0.07[a]			
AgRP	0.84 ± 0.10[a]	1.20 ± 0.07[b]	0.80 ± 0.04[a]			
POMC	1.12 ± 0.11	1.10 ± 0.12	1.06 ± 0.10			

Values are mean ± SEM of duplicate determined mRNA concentrations compared with relative mRNA concentrations of cyclophilin; n = 9–10 rats/group. [a,b]Parameters with differing letters differ from each other by $P \leq 0.05$.

in the VMH. Here we confirmed the previous finding that systemic amylin treatment increases leptin-induced pSTAT3 expression in the VMN of WT mice (20) and show, for the first time, that amylin had no effect on VMN pSTAT3 expression in IL-6 KO mice. The enhancement of VMN leptin-induced pSTAT3 expression by amylin was also inhibited by LV IL-6 antibody infusion in rats. Such a direct action in the VMH requires that amylin cross the blood–brain barrier, which has been shown to occur (41,42). Since amylin exposure did not increase Lepr-b expression in isolated VMH explants as it does when given in vivo (20), there may be an additional

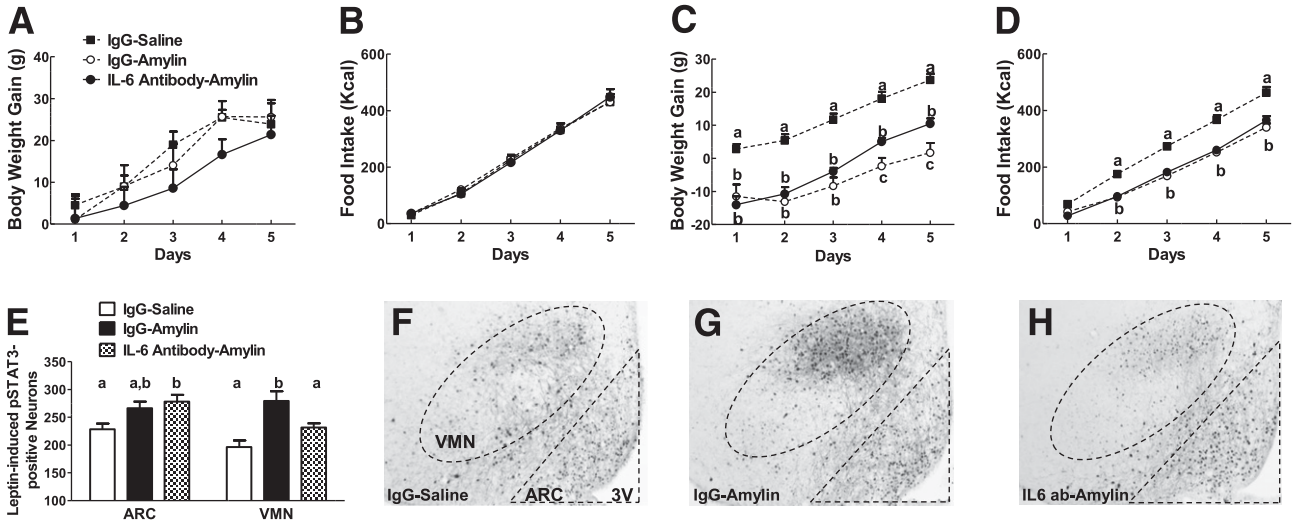

Figure 3—Body weight gain (*A* and *C*) and food intake (*B* and *D*) of LV infusions of IgG vs. IL-6 antibody for 5 days (*A* and *B*), followed by 5 days of systemic amylin (100 μg/kg/day) vs. vehicle (0.9% saline) with an osmotic minipump (*C* and *D*) in 9- to 10-week-old rats (experiment 2). *E*: The effect of LV infusions of IgG vs. IL-6 antibody and systemic amylin vs. saline on leptin-induced (5 mg/kg, intraperitoneal) pSTAT3 immunohistochemistry in the ARC and VMN. Values are mean ± SEM; n = 8 rats/group. Parameters with differing letters (a, b, and c) differ from each other by $P \leq 0.05$. pSTAT3 immocytochemistry of the VMN and ARC in IgG saline–treated (*F*), IgG-amylin–treated (*G*), and IL-6 ab-amylin–treated (*H*) rats. Images taken at 10× magnification.

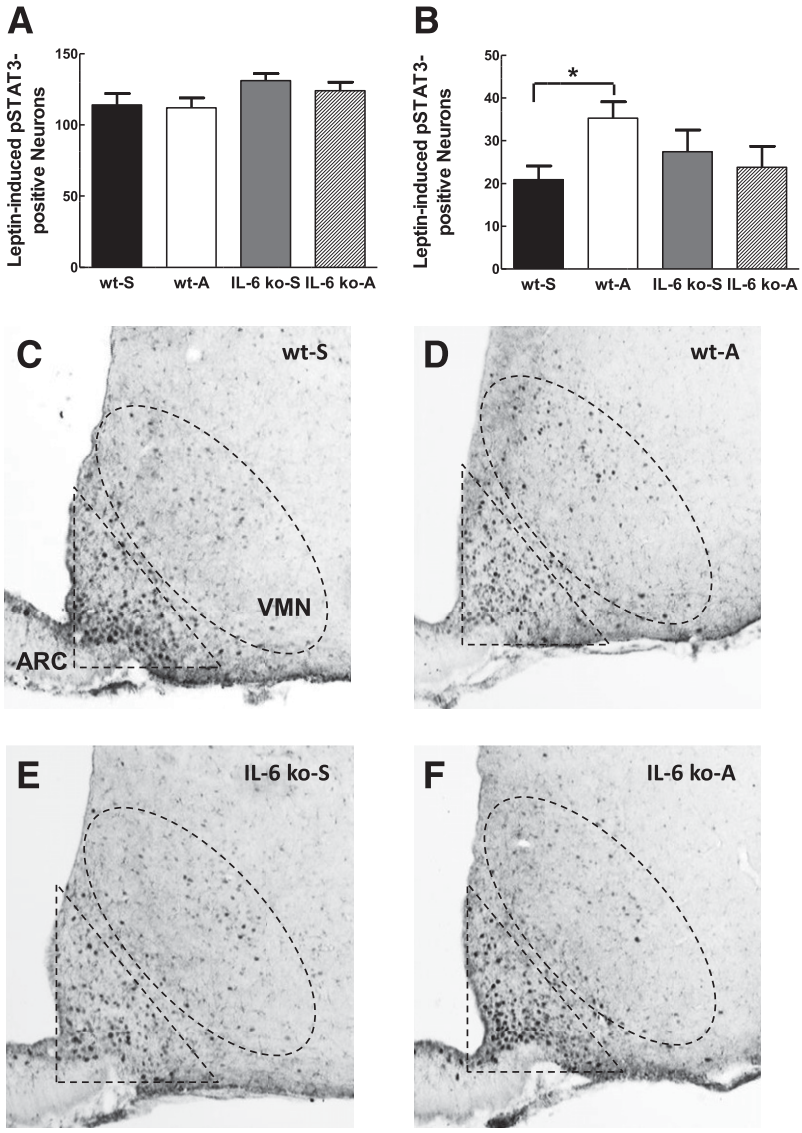

Figure 4— Effect of systemic amylin or saline on leptin-induced (5 mg/kg, intraperitoneal) pSTAT3 immunohistochemistry in the ARC (*A*) and VMN (*B*) of WT mice treated with saline (WT-S) vs. amylin (1 mg/kg/day; WT-A) and saline-treated KO (IL-6 KO-S) vs. amylin-treated IL-6 KO mice (IL-6 KO-A) after 2 weeks of systemic amylin (1mg/kg/day) vs. vehicle (0.9% saline) infusion with an osmotic minipump. Images of WT-S (*C*) vs. IL-6 KO-S (*E*) and WT-A (*D*) vs. IL-6 KO-A (*F*) (original magnification ×20). Values are mean ± SEM; $n = 8$ mice/group. *$P \leq 0.05$ WT-S vs. WT-A mice.

component of the effect of amylin on leptin signaling that is required to alter Lepr-b expression in the intact animal.

Interestingly, amylin exposure had differential effects on CTR1 and RAMP expression. For example, amylin exposure increased RAMP1 and 2 in VMH explants and RAMP2 and 3 in cortical, but not VMH microglial, cultures. However, systemic amylin administration in vivo had no consistent effect on ARC or VMN RAMP expression. Similarly, CTR1b expression was differentially altered depending on the tissue examined and the type of exposure. These results suggest that there are clear differences between the responses of cortical and VMH microglia to amylin, just as cortical and hypothalamic astrocytes differ in their characteristics (43). Given the fact that amylin affected CTR and RAMP expression only in cultured microglia—not astrocytes or neurons—these results demonstrate selective feedback by amylin on its own receptor in microglia. Similarly, while amylin stimulated VMH microglial IL-6 expression, it also had a negative feedback effect (44) on the expression of the gp130 component of the IL-6 receptor complex (45). In fact, the gp130 family of receptors can be activated by other cytokines such as LIF (39,46), although in our case amylin altered LIF expression only in cultured hypothalamic astrocytes, and this was an inhibitory rather than a stimulatory effect.

Although numerous studies (2,19,20,47–49), including this one in rats, clearly show that amylin acts alone to decrease food intake and body weight in obese and lean rats and obese humans, it had no such effects in WT

mice treated for 2 weeks with amylin doses that were 10 times higher than those used in rats, despite showing a clear enhancement of WT leptin signaling in the VMN. There is no ready explanation for this lack of effect on body weight or food intake in amylin-treated WT mice. It is possible that amylin treatment reduced their adiposity, but this could not be assessed because of methodological requirements for later immunohistochemistry. Also, although 5 days of pair feeding to the level of amylin-treated rats had no effect on ARC NPY or AgRP expression, amylin treatment actually increased the expression of these orexigenic peptides. Since all of the changes in amylin-induced IL-6 production and leptin signaling occurred selectively in the VMN, to the exclusion of ARC Lepr-b-expressing neurons, it is possible that the upregulation of these peptides was indirectly mediated by alterations in VMN leptin signaling.

In conclusion, we demonstrated that, in addition to the well-known direct effect of amylin on AP and VTA neurons, which mediates much of its anorectic effects (3,15–18), amylin also acts directly to stimulate VMH microglia production of IL-6. This IL-6 is released into the interstitial space, where it acts on its IL-6/gp130 receptor on Lepr-b-expressing neurons in the VMN to enhance the activation of pSTAT3 by leptin. While amylin acts directly in the AP to decrease food intake and body weight, especially acutely (15,47,50), its interaction with leptin on weight loss in obese rats and humans seems to depend on its ability to stimulate VMN microglial IL-6 production to increase leptin signaling (2,19,47,48). This novel discovery provides a potential avenue for the discovery of new leptin sensitizers in the treatment of obesity.

Acknowledgments. The authors thank Antoinette Moralishvili, Charlie Salter, and Sunny Lee (all from the VA Medical Center) for their technical assistance.

Funding. This work was supported by the American Heart Association Founders Affiliate Predoctoral Fellowship (M.D.J.), the Swiss National Science Foundation (C.N.B. and T.A.L.), the Research Service of the Department of Veterans Affairs (B.E.L.), and the National Institute of Diabetes and Digestive and Kidney Diseases (grant no. DK-030066 to B.E.L.).

Duality of Interest. No potential conflicts of interest relevant to this article were reported.

Author Contributions. C.L.F., M.D.J., A.A.D.-M., and C.N.B. performed the research, designed the experiments, and wrote the manuscript. T.A.L. and B.E.L. designed the experiments and wrote the manuscript. C.L.F., M.D.J., and B.E.L. are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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