Amylin Acts in the Lateral Dorsal Tegmental Nucleus to Regulate Energy Balance Through Gamma-Aminobutyric Acid Signaling

Supplemental Information

Supplemental Methods

Drugs

The amylin receptor agonist salmon calcitonin (sCT; Bachem, Torrance, CA, USA) was dissolved in artificial cerebrospinal fluid (aCSF; Harvard Apparatus, Holliston, MA, USA) for central injections or 0.9% sterile saline for peripheral injections. Amylin (Bachem Torrance, CA, USA), the amylin receptor antagonist AC187 (R&D Systems, Minneapolis, MN, USA), and saclofen (Sigma Aldrich, St. Louis, MO, USA) were dissolved in aCSF. Bicuculline (Sigma Aldrich, St. Louis, MO, USA) was dissolved in 50% DMSO in aCSF-0.1M NaOH.

Stereotaxic Surgery

Animals were anesthetized with a cocktail (KAX) composed of ketamine (90 mg/kg), acepromazine (0.64 mg/kg), and xylazine (2.7 mg/kg; IM), and placed into a stereotaxic apparatus. Bilateral guide cannulae (Plastics One, Roanoke, VA, USA) targeting the LDTg (coordinates: ±0.5 mm lateral to midline, 8.7 mm posterior to bregma, 4.1 mm ventral to skull; injector aimed 6.6 mm ventral to skull) were implanted and affixed to the skull with bone screws and dental cement. Analgesia was provided for all surgeries (meloxicam, 2 mg/kg). Rats were allowed ~1 week recovery from surgery before the beginning of an experiment. LDTg injection placements were verified histologically

postmortem by either visualization of green fluorescent protein (GFP) in the calcitonin receptor (CTR) knockdown experiment or by intraparenchymal injections of pontamine sky blue (100 nl) in all other behavioral experiments. Animals with missed cannula placements were eliminated from analyses.

For lateral ventricle cannulae placements, rats were deeply anesthetized with a KAX cocktail composed of ketamine (90 mg/kg), acepromazine (0.72 mg/kg), and xylazine (2.8 mg/kg; IM), and placed in a stereotaxic apparatus. A stainless steel guide cannula (26-gauge Plastics One, Roanoke, VA) was surgically implanted toward the lateral ventricle using the following coordinates: 1.8 mm lateral to bregma, 0.9 mm posterior to bregma, and 2.6 mm ventral from the surface of the skull, injector aimed 4.6 mm ventral from the skull. Following one week of recovery, placement of the cannula was verified by the elevation of cytoglucopenia resulting from a single injection of 2 µl of 105µg/µl 5-thio-D-glucose (1). Animals that did not have at least 100% elevation of baseline blood glucose by 2 hours post injection were not used for colchicine injections.

Quantitative PCR

Chow-maintained rats (n=6) were euthanized 1-2h into the dark phase. Brains were rapidly removed, flash-frozen in −70°C isopentane, and stored at −80°C until processing. To examine the relative expression of the components of amylin receptor complex in the LDTg via quantitative real-time PCR (qPCR), bilateral 1 mm^3 micropunches/hemisphere of LDTg-enriched tissue (approximately 8.2-9.2 mm posterior to bregma) were collected from each brain. Tissue samples were processed for the

gene expression of components of the amylin receptor, the CTR and receptor activity modifying protein (RAMP) as previously described (2).

Total RNA was extracted from the tissue samples using TRIzol (Invitrogen, Grand Island, NY, USA) and the RNeasy kits (Qiagen, Valencia, CA, USA) and used to synthesize cDNA with the Advantage RT-for-PCR kit (Clontech, Mountain View, CA, USA). Relative mRNA expression of each CTR and RAMP subtype was quantified using Taqman gene expression kits (CTR-A: Rn01526770_m1, CTR-B: Rn01526768_m1, RAMP1: Rn01427056_m1, RAMP2: Rn00571815_m1, RAMP3: Rn00824652_g1, GapDH: Rn01775763_g1) and PCR reagents from Applied Biosystems (Grand Island, NY, USA). Samples were analyzed with the Eppendorf Mastercycler ep realplex2. Relative mRNA expression calculations were completed using the comparative threshold cycle method (2, 3). One sample was eliminated due to a technical error during RNA isolation.

Immunohistochemical Analyses

Rats were deeply anesthetized with an IM injection of the surgical dose of KAX used for the LDTg cannulation surgeries and transcardially perfused with 0.1 M PBS, followed by 4% paraformaldehyde in 0.1 M PBS. Brains were removed, postfixed in 4% paraformaldehyde for 6h and subsequently cryoprotected in 20% sucrose in 0.1 M PBS at 4°C for ~3 days. Brains were sectioned coronally (30 µm) with a cryostat. Sections were blocked in 0.1M PBS with 5% donkey serum and 0.2% Triton-X at room temperature and incubated in primary antibodies overnight in 5% normal donkey serum with 0.2% Triton-X. The following primary antibodies used were at a 1:1000 dilution:

rabbit anti-CTR (ab11042, Abcam, Cambridge, MA, USA), mouse anti-NeuN (MAB377, Millipore, Billerica, MA, USA), chicken anti-glial fibrillary acidic protein (GFAP, AB5541, Millipore, Billerica, MA, USA), and goat anti-choline acetyltransferase (ChAT, AB144P, Millipore, Billerica, MA, USA) (4-7). Following a PBS rinse, sections were incubated in appropriate donkey Alexa Fluor 488, 594, and AMCA secondary antibodies (Jackson Immunoresearch, West Grove, PA, USA) at room temperature (all secondary antibodies 1:500 in 5% normal donkey serum with 0.2% Triton-X). Sections were mounted on slides and visualized with a Leica SP5 X confocal microscope using objectives and the 405, 488, and 594 laser lines. All images were collected sequentially to avoid contamination of signals from other fluorophores.

Colchicine Treatment and Co-localization of CTR and GABAergic Markers

To enhance labeling for glutamate decarboxylase 67 (Gad67), a GABAergic marker, a rat (n=1) was injected with colchicine (400 µg, dissolved in 4 µl of DMSO), a neurotoxin that blocks neurotransmission, directly in the lateral ventricle (8, 9). Twenty-eight hours after colchicine treatment, the rat was anesthetized with the surgical dose of KAX used in the lateral ventricle cannulation, perfused transcardially with ice-cooled 0.9% saline, followed by 4% paraformaldehyde in 0.1 M borate buffer of pH 9.5. The brain was removed and immersed in 4% paraformaldehyde containing 12% sucrose for 20–24h at 4°C. The brain was then blocked transversely at the level of the caudal midbrain, and the block was flash-frozen in dry-ice cooled hexane before being sectioned frozen on a sliding microtome (transverse plane, 30 µm thickness, 5 series). Sections were stored in antifreeze solution (30% ethylene glycol, 20% glycerol in 0.02 M potassium phosphate-

buffered saline—KPBS) at -20°C until further processing. Sections were incubated in primary antibodies at 4°C overnight in KPBS containing 1% donkey serum and 0.1% Triton X-100. The following primary antibodies were used: rabbit anti-CTR (1:1000, ab11042, Abcam, Cambridge, MA, USA) and mouse anti-GAD67 (1:10000, MAB5406, Millipore, Billerica, MA, USA) (5, 10). Following washes in KPBS, sections were incubated in appropriate secondary antibodies at 4°C overnight diluted at 1:500 in KPBS containing 0.1% Triton X-100 (donkey AffiniPure Cy3 and Alexa Fluor 488, Jackson Immunoresearch, West Grove, PA, USA). Following washes in KPBS, sections were mounted onto glass slides, allowed to dry, and coverslipped, using 50% glycerol in KPBS mountant. Coverslip edges were sealed with clear nail polish. Photomicrographs were acquired as optical slices using a Zeiss LSM 700 UGRB Confocal System (controlled by Zeiss Zen software). All photomicrographs in the figures are oriented such that: up = dorsal, down = ventral, left = medial, right = lateral.

Behavioral Testing

Intra-LDTg amylin and salmon calcitonin dose-response feeding experiments

To assess the dose-response of amylin receptor activation in the LDTg on food intake and body weight gain, rats (n=10) received unilateral LDTg injections of amylin (0, 0.2, 0.4, and 0.8 µg; 100 nl aCSF) shortly before the onset of the dark cycle. The doses of amylin for this experiment were selected based on previous dose response experiments in the 3rd ventricle and VTA (11, 12). In order to compare the hypophagic effects of LDTg administration of amylin with the long acting amylin receptor agonist sCT, we injected sCT unilaterally into the LDTg just before the onset of the dark cycle in a

separate cohort of rats (n=6). The sCT doses for this series of experiments (0, 0.01, 0.04, and 0.1 µg; 100 nl aCSF) were selected based on previous dose response experiments in the 3rd ventricle and VTA (2). Given that previous studies have shown that lower doses of sCT are required to produce a feeding effect compared to amylin (2, 5, 11, 13), the doses of amylin were considerably lower than that of sCT.

For meal pattern experiments, rats (n=5) were housed in a custom-made automated feedometer system that consists of hanging wire mesh cages with a small hole allowing access to a food cup that rests on an electronic scale (2, 14, 15). Computer software (LabView) recorded the weights of the food cups every 10s for 24h. A meal was defined as at least 0.25g of food intake with at least 10 minutes in between feeding bouts (2, 14, 15). Meal patterns were assessed at 24h post-LDTg injection (0, 0.01, 0.04, or 0.1 µg sCT).

To test if nausea/malaise contributes to the intake suppression following LDTg amylin receptor activation by measuring pica, rats (n=6) were given access to both chow and kaolin clay (Research Diets, New Brunswick, NJ, USA) for ~1 week prior to the experiment. Chow intake, kaolin clay intake, and body weight were measured at 24h after injection of sCT (0, 0.01, 0.04, or 0.1 µg) into the LDTg.

Intra-LDTg amylin receptor activation sucrose self-administration experiment

Experiments were conducted in ventilated, sound-attenuating operant chambers (Med Associates, St Albans, VT, USA). Rats (n=8) were initially food restricted to ~80% of daily intake and trained to lever press for 45 mg sucrose pellets (Research Diets) on a fixed ratio 1 (FR1) schedule of reinforcement during 1 h operant sessions. Once animals achieved stable responding for sucrose (defined as <20% variation in responding over

three consecutive days) on the FR1 schedule of reinforcement, the response requirement was increased to FR3 for two days, followed by three days on FR5. Animals were then given access to *ad libitum* chow and maintained on FR5 for an additional seven days. Motivation to self-administer sucrose pellets was then assessed using a progressive ratio (PR) schedule of reinforcement. Under a PR schedule, the response requirement for the *i*th reinforcement of each subsequent delivery of a sucrose pellet increases exponentially, according to the formula $R(i) = [5e^{0.2i} - 5]$, until the rat fails to meet the requirement in a 30 minute time period (16). Using a within-subjects design, rats were injected with aCSF (100 nl), amylin (0.4 µg; 100 nl), or sCT (0.04 µg; 100 nl) into the LDTg 30 minutes prior to a PR self-administration test session. We chose these doses based on previous literature showing that lower doses of sCT are needed to produce a feeding effect compared to amylin (5, 11, 13, 17). Treatments were counterbalanced and test days were separated by at least 2 days of responding for sucrose on the FR5 schedule of reinforcement. The responding of one rat declined prior to completion of the experiment due to equipment malfunction, and he was subsequently removed from behavioral analyses.

Intra-LDTg acute amylin receptor blockade feeding experiment

We examined whether acute pharmacological blockade of LDTg amylin receptors was sufficient to attenuate the intake suppressive effects of a systemic sCT injection. The amylin receptor antagonist, AC187, was bilaterally injected in the LDTg (0.8 µg/100 nl/hemisphere; dose chosen from pilot experiments to be subthreshold for food intake effect on its own) 1h prior to the onset of the dark cycle (2). Fifteen minutes prior to the onset of the dark cycle, rats (n=11) were injected systemically with the amylin receptor

agonist, sCT (5 μg/kg, IP), and subsequent food intake and body weight were measured.

Intra-LDTg amylin receptor knockdown experiment

A validated AAV1-shRNA construct for CTRa/b knockdown was used as previously described (5). Rats were maintained on chow for 1 week prior to surgery to establish baseline intakes and body weight and were divided into two weight-matched groups. Animals were assigned to receive a bilateral LDTg injection (200 nl/hemisphere) of either an AAV control vector expressing GFP (AAV-Control, n=7) or the AAV-shRNA for the CTR-a/b expressing GFP (AAV-CTR KD, n=7). Beginning post-surgery day 1, food intake, food spillage and body weight were measured every 48h for a total of 30 days (days 1-31 post-viral injection). One rat was removed from the behavioral analyses due to technical errors in data collection. After conclusion of the behavioral study, rats were anesthetized with a surgical dose of KAX used in the LDTg cannulation (IM) and euthanized by decapitation. The brains were rapidly removed and flash frozen. Correct placement of AAV injections was verified by collecting a section containing the LDTg (30 μm) from each brain and visualizing GFP. Micropunches of the LDTg were collected and the LDTg CTRa knockdown was confirmed with qPCR, with GapDH as the internal control.

Intra LDTg GABA receptor blockade feeding experiment

To test the role of GABA receptors in mediating LDTg amylin-mediated intake suppression, a cocktail composed of bicuculline (GABA-A receptor antagonist, 100 ng; 100 nl) and saclofen (GABA-B receptor antagonist, 500 ng; 100 nl) was unilaterally injected in the LDTg (100 nl, 50% DMSO in aCSF) 30 minutes prior to the onset of the

dark cycle. Fifteen minutes prior to the onset of the dark cycle, rats (n=8) were unilaterally injected with sCT (0.04 µg; 100 nl, aCSF vehicle) in the ipsilateral LDTg, and food intake and body weight were measured.

Statistical Analyses

All data are represented as mean ± SEM. The α level was set to $p \leq 0.050$ for all studies. Statistical analyses were performed using Statistica (StatSoft). For all feeding and body weight studies, binned data were analyzed using separate repeated measures ANOVAs that accounted for the within-subjects experimental design, while assessing between-subjects effects (drug treatment, AAV condition) when applicable, except when noted. Body weight for the GABA-A/B receptor blockade experiment was analyzed as a repeated measures one-way ANOVA. Sucrose self-administration was analyzed using separate ANOVAs for each behavioral measure, accounting for the within-subject experimental design. CTR/RAMP expression was analyzed, relative to CTRb and RAMP3, using separate ANOVAs for each gene that accounted for the within-subject experimental design. For the CTR knockdown experiment, gene expression was analyzed using an ANOVA that accounted for the between-subject experimental design. Statistically significant effects were probed using Student-Neuman-Keuls *post hoc* analyses or planned comparisons when noted.

Supplemental References

1. Ritter RC, Slusser PG, Stone S (1981): Glucoreceptors controlling feeding and blood glucose: location in the hindbrain. *Science*. 213:451-452.
2. Mietlicki-Baase EG, Rupprecht LE, Olivos DR, Zimmer DJ, Alter MD, Pierce RC, et al. (2013): Amylin receptor signaling in the ventral tegmental area is physiologically relevant for the control of food intake. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology*. 38:1685-1697.
3. Bence KK, Delibegovic M, Xue B, Gorgun CZ, Hotamisligil GS, Neel BG, et al. (2006): Neuronal PTP1B regulates body weight, adiposity and leptin action. *Nature medicine*. 12:917-924.
4. Lopez NE, Krzyzaniak MJ, Blow C, Putnam J, Ortiz-Pomales Y, Hageny AM, et al. (2012): Ghrelin prevents disruption of the blood-brain barrier after traumatic brain injury. *Journal of neurotrauma*. 29:385-393.
5. Mietlicki-Baase EG, Reiner DJ, Cone JJ, Olivos DR, McGrath LE, Zimmer DJ, et al. (2015): Amylin modulates the mesolimbic dopamine system to control energy balance. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology*. 40:372-385.
6. Saunders A, Granger AJ, Sabatini BL (2015): Corelease of acetylcholine and GABA from cholinergic forebrain neurons. *eLife*. 4.
7. Wang HL, Morales M (2009): Pedunculopontine and laterodorsal tegmental nuclei contain distinct populations of cholinergic, glutamatergic and GABAergic neurons in the rat. *The European journal of neuroscience*. 29:340-358.
8. Wang X, Gao F, Zhu J, Guo E, Song X, Wang S, et al. (2014): Immunofluorescently labeling glutamic acid decarboxylase 65 coupled with confocal imaging for identifying GABAergic somata in the rat dentate gyrus-A comparison with labeling glutamic acid decarboxylase 67. *Journal of chemical neuroanatomy*. 61-62:51-63.
9. Halasy K, Hajszan T, Kovacs EG, Lam TT, Leranth C (2004): Distribution and origin of vesicular glutamate transporter 2-immunoreactive fibers in the rat hippocampus. *Hippocampus*. 14:908-918.
10. Deidda G, Allegra M, Cerri C, Naskar S, Bony G, Zunino G, et al. (2015): Early depolarizing GABA controls critical-period plasticity in the rat visual cortex. *Nature neuroscience*. 18:87-96.
11. Mietlicki-Baase EG, Olivos DR, Jeffrey BA, Hayes MR (2015): Cooperative interaction between leptin and amylin signaling in the ventral tegmental area for the control of food intake. *American journal of physiology Endocrinology and metabolism*. 308:E1116-1122.
12. Rushing PA, Hagan MM, Seeley RJ, Lutz TA, Woods SC (2000): Amylin: a novel action in the brain to reduce body weight. *Endocrinology*. 141:850-853.

13. Lutz TA, Tschudy S, Rushing PA, Scharrer E (2000): Amylin receptors mediate the anorectic action of salmon calcitonin (sCT). *Peptides*. 21:233-238.

14. Hayes MR, Leichner TM, Zhao S, Lee GS, Chowansky A, Zimmer D, et al. (2011): Intracellular signals mediating the food intake-suppressive effects of hindbrain glucagon-like peptide-1 receptor activation. *Cell metabolism*. 13:320-330.

15. Kanoski SE, Zhao S, Guarnieri DJ, DiLeone RJ, Yan J, De Jonghe BC, et al. (2012): Endogenous leptin receptor signaling in the medial nucleus tractus solitarius affects meal size and potentiates intestinal satiation signals. *American journal of physiology Endocrinology and metabolism*. 303:E496-503.

16. Hopkins TJ, Rupprecht LE, Hayes MR, Blendy JA, Schmidt HD (2012): Galantamine, an acetylcholinesterase inhibitor and positive allosteric modulator of nicotinic acetylcholine receptors, attenuates nicotine taking and seeking in rats. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology*. 37:2310-2321.

17. Mietlicki-Baase EG, Ortinski PI, Rupprecht LE, Olivos DR, Alhadeff AL, Pierce RC, et al. (2013): The food intake-suppressive effects of glucagon-like peptide-1 receptor signaling in the ventral tegmental area are mediated by AMPA/kainate receptors. *American journal of physiology Endocrinology and metabolism*. 305:E1367-1374.