ASSESSING THE INVOLVEMENT OF ALTERED NEUROTENSIN SIGNALING IN ANOREXIA NERVOSA

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ABSTRACT

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Anorexia Nervosa (AN), characterized by a persistent and detrimental drive to lose weight via restriction of food intake and excessive exercise, is the psychiatric disorder with the highest mortality rate. Very few options exist when considering pharmacotherapies used to treat AN patients, and no drugs have been demonstrated to significantly improve weight gain. This highlights the need to not only find better drug-based therapies for AN but to also find druggable targets for this disorder. While AN is thought to be highly heritable, with heritability estimates ranging between 50-80%, it has been challenging to identify significant genetic contributors. Thus, determining the genetic risk factors of AN will first be required for development of better therapeutics.

In an effort to better understand the genetic basis of AN, recent work has been performed to uncover rare genetic variants that confer high risk of disease development. Loss-of-function variants in Neurotensin (Nts) and Nts Receptor 1 (NtsR1) were identified in individuals with eating disorders. Nts is a neuropeptide known to regulate ingestive and locomotor behavior. Nts modulates these behaviors centrally, and a subset of dopamine (DA) neurons with the ventral tegmental area (VTA) that coexpress NtsR1 are known to contribute to DA-mediated weight loss behaviors. Ablation of all NtsR1 VTA neurons was shown to promote excessive locomotor activity without a sufficient increase in feeding, leading to low body weight. Finally, increased fiber densities have been found within the lateral hypothalamic area (LHA) of individuals with

AN, and the LHA is a region with a significant population of Nts neurons known to modulate both feeding and activity. We therefore hypothesized that Nts populations in feeding centers, such as the LHA, receive altered input from structures associated with AN and that alterations and/or disruption of Nts signaling promotes AN-like behaviors.

This hypothesis was explored via three different approaches. First, the location and density of Nts populations within the brains of *Nts^{Cre}; Floxed GFP* mice were mapped, and this revealed the presence of Nts in regions implicated in regulation of feeding and AN. The next approach involved determining if disrupted Nts signaling increases risk for development of AN-like behaviors. This was accomplished by characterizing NtsR1-deficient mice both at baseline and after exposure to an adolescent-stress model of AN. This study revealed that deficiency of NtsR1 is a genetic risk factor that, when interacting with risks of being female and exposure to adolescent stress, promotes aberrant feeding, excessive locomotor behaviors, and compulsive anxiety behaviors analogous to those observed in AN. Finally, a rabies virus-based method was used to identify direct inputs to LHA Nts neurons, and this highlighted the existence of afferents, and thus top-down control, from structures implicated in AN. In addition, densities of these inputs were determined in mouse models of AN, and this demonstrated that afferent inputs to LHA Nts neurons are increased from sites associated with AN.

Altogether, the data presented in this thesis highlight the possible genetic and neurocircuitry alterations to the Nts-NtsR1 system that may promote and/or be the result of development of AN. These data also indicate the need for future studies to better understand the mechanism by which such alterations in Nts signaling promote this disease.

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KEY TO ABBREVIATIONS

3N: oculomotor nucleus

3PC: oculomotor nucleus, parvicellular part

4N: trochlear nucleus

4V: floor of the 4th ventricle

5ADi: motor trigeminal nucleus, anterior digastric part

5-HIAA: 5-hydroxyindoleacetic acid

5N: spinal/ motor trigeminal nucleus

5SoI: trigeminal-solitary transition zone

5Tr: trigeminal transition zone

5TT: motor trigeminal nucleus, tympani part

6N: abducens nucleus

6RB: abducens nucleus, retractor bulbi part

7N: facial nucleus

7VM/ 7DM/ 7DI/ 7DL/ 7L/ 7VI: facial nucleus subnuclei

8N: vestibulocochlear nerve

10N: dorsal motor nucleus of the vagus

12N: hypoglossal nucleus

α-MSH: α-melanin-stimulating-hormone

A5: A5 noradrenaline cells

A14: A14 dopamine cells

AA: anterior amygdalar area

AAV: adeno-associated virus

ABA: activity-based anorexia model

ACA: anterior cingulate area

Acb: nucleus accubmens

AcbC: nucleus accumbens, core

AcbSh: nucleus accumbens, shell

AD: anterodorsal thalamic nucleus

AgRP: agouti-related peptide

AHA: anterior hypothalamic area, anterior part

AHC: anterior hypothalamic area, central part

AHiAL: amygdalohippocampal area, anterolateral part

AHP: anterior hypothalamic area, posterior part

AI: agranular insular area

Amb: nucleus ambiguus

AM: anteromedial thalamic nucleus

AOM: anterior olfactory area, medial part

AON: anterior olfactory nucleus

AP: area postrema

APir: amygdalopiriform transition area

APT: anterior pretectal nucleus

APTD: anterior pretectal nucleus, dorsal part

AN: anorexia nervosa

ANS: accessory neurosecretory nucleus

Arc: arcuate nucleus

ArcD: arcuate nucleus, dorsal part

ArcL: arcuate nucleus, lateral part

ArcLP/ ArcMP: caudal arcuate hypothalamic nucleus

Ast: amygdalostriatal transition

ATg: anterior tegmental nucleus

AV: anteroventral thalamic nucleus

AVDM: anteroventral thalamic nucleus, dorsomedial part

AVPe/ AVPV: anteroventral periventricular nucleus

BA/ BAOT: bed nucleus of the accessory olfactory tract

BAC: bed nucleus of the anterior commissure

BDNF: brain-derived neurotrophic factor

bic: brachium of the inferior colliculus

BIC: nucleus of the brachium of the inferior colliculus

BL: basolateral amygdalar nucleus

BLA: basolateral amygdalar nucleus, anterior part

BLP: basolateral amygdalar nucleus, posterior part

BLV: basolateral amygdalar nucleus, ventral part

BM: basomedial amygdalar nucleus

BMA: basomedial amygdalar nucleus, anterior part

BMP: basomedial amygdalar nucleus, posterior part

BNST: bed nucleus of the stria terminalis

Bo: Botzinger complex

BSTLD: bed nucleus of the stria terminalis, lateral division, dorsal part

BSTLI: bed nucleus of the stria terminalis, lateral division, intermediate part

BSTLJ: bed nucleus of the stria terminalis, lateral division, juxtacapsular part

BSTLP: bed nucleus of the stria terminalis, lateral division, posterior part

BSTLV: bed nucleus of the stria terminalis, lateral division, ventral part

BSTMA: bed nucleus of the stria terminalis, medial division, anterior part

BSTMAL: bed nucleus of the stria terminalis, medial division, anterolateral part

BSTMPI: bed nucleus of the stria terminalis, medial division, posterointermediate part

BSTMPL: bed nucleus of the stria terminalis, medial division, posterolateral part

BSTMPM: bed nucleus of the stria terminalis, medial division, posteromedial part

BSTMV: bed nucleus of the stria terminalis, medial division, ventral part

CAT: nucleus of the central acoustic tract

CC: corpus callosum

CCK: cholecystokinin

CEA: central amygdala

CeC: central amygdalar nucleus, central part

CeCv: central cervical nucleus of the spinal cord

CeL: central amygdalar nucleus, lateral part

CeM: central amygdalar nucleus, medial part

Cent: caudomedial entorhinal cortex

Cg: cingulate cortex

CGA: central gray, alpha part

CGB: central gray, beta part

CGRP: calcitonin gene-related peptide

CIC: central nucleus of the inferior colliculus

cic: commissure of the inferior colliculus

CL: centrolateral thalamic nucleus

CLi: caudal linear nucleus of the raphe

CM: central medial nucleus of the thalamus

CN: cochlear nuclei

CnF: cuneiform nucleus

CNO: clozapine-n-oxide

CNS: central nervous system

COA/ PMCo/ PLCo/ Aco: cortical amygdalar nucleus

CPu: caudate putamen

CRH/ CRF: corticotropin releasing hormone/ factor

csc: commissure of the superior colliculus

CSF: cerebrospinal fluid

Cu/ cu: cuneate nucleus/ cuneate fasciculus

CuR: cuneate nucleus, rotundus part

CVL: caudoventrolateral reticular nucleus

Cx: cerebral cortex

CxA: cortex-amygdala transition

D1R: dopamine receptor 1

D2R: dopamine receptor 2

DA: dopamine

DB: nucleus of the diagonal band of broca

DC: dorsal cochlear nucleus

DCDp: dorsal cochlear nucleus, deep layer

DCFu: dorsal cochlear nucleus, fusiform layer

DCIC: dorsal cortex of the inferior colliculus

DEN: dorsal endopiriform nucleus

df: dorsal fornix

DG: hippocampus, dentate gyrus

DIEnt: Dorsointermedial entorhinal cortex

DK: nucleus of Darkschewitsch

DLEnt: dorsolateral entorhinal cortex

DLL: dorsal nucleus of the lateral lemniscus

DLPAG: dorsolateral periaqueductal gray

DM: dorsomedial hypothalamic nucleus

DMPAG: dorsomedial periaqueductal gray

DMSp5: dorsomedial spinal trigeminal nucleus

DMTg: dorsomedial tegmental area

DMV: dorsomedial hypothalamic nucleus, ventral part

DP: dorsal peduncular cortex

DpG: deep gray layer of the superior colliculus

DPGi: dorsal paragigantocellular nucleus

DpWh: deep white layer of the superior colliculus

DR: dorsal raphe nucleus

DRC: dorsal raphe nucleus, caudal part

DRD: dorsal raphe nucleus, dorsal part

DRI: dorsal raphe nucleus, interfascicular part

DRL: dorsal raphe nucleus, lateral part

DRV: dorsal raphe nucleus, ventral part

DS: dorsal subiculum

DTg: dorsal tegmental nucleus

DTgC: dorsal tegmental nucleus, central part

DTgP: dorsal tegmental nucleus, pericentral part

DTT: dorsal tenia tecta

EAC/ EAM/ EA: sublenticular extended amygdala

ECIC: external cortex of the inferior colliculus

ECT: ectorhinal area

ENT: entorhinal area

EP: endopiriform nucleus

Ep/ MGP: entopeduncular nucleus

ESO: episupraoptic nucleus

Eve: nucleus of the origin of the efferent fibers of the vestibular nerve

EW: edinger-westphal nucleus

f: fornix

FF: fields of forel

Fr: fasciculus retroflexus

Fve: F cell group of the vestibular complex

Gal: Galanin

GFP: green fluorescent protein

GHRH: growth hormone releasing hormone

Gi: gigantocellular reticular nucleus

GiA: gigantocellular reticular nucleus, alpha part

GiV: gigantocellular reticular nucleus, ventral part

GP: globus pallidus

Gr: gracile nucleus

GrC: granule cell layer of the cochlear nucleus

GU: gustatory area

HDB: nucleus of the horizontal limb of the diagonal band

HVA: homovanillic acid

IA: Intercalated amygdalar nucleus

IAD: interanterodorsal thalamic nucleus

IAM: interanteromedial thalamic nucleus

IC: inferior colliculus

ICj: islands of cajella

ICjM: island of cajella, major island

ICV: intracerebroventricular

IF: interfascicular nucleus

IL: infralimbic cortex

ILA: infralimbic area

ILL: intermediate nucleus of the lateral lemniscus

IM: intercalated amygdalar nucleus, main part

IMD: intermediodorsal nucleus of the thalamus

InC/ InCSh: Interstitial nucleus of cajal with shell region

InG: intermediate gray layer of the superior colliculus

Ins: insular cortex

Int: internal capsule

InWh: intermediate white layer of the superior colliculus

IO: inferior olivary complex

IOA/ IOB: inferior olive, subnucleus A and B of the medial nucleus

IOC: inferior olive, subnucleus C of the medial nucleus

IOD: inferior olive, dorsal nucleus

IODM: inferior olive, dorsomedial cell group

IOM: inferior olive, medial nucleus

IOPr: inferior olive, principal nucleus

IP: interpeduncular nucleus

IPAC: interstitial nucleus of the posterior limb of the anterior commissure

IPC: interpeduncular nucleus, caudal subnucleus

IPDL: interpeduncular nucleus, dorsolateral subnucleus

IPDM: interpeduncular nucleus, dorsomedial subnucleus

IPF: interpeduncular fossa

IPI: interpeduncular nucleus, intermediate subnucleus

IPL: interpeduncular nucleus, lateral subnucleus

IPR: interpeduncular nucleus, rostral subnucleus

IR: immunoreactivity

Irt: intermediate reticular nucleus

IS: inferior salivatory nucleus

ISH: *In situ* hybridization

KF: koelliker-fuse nucleus

LA: lateral amygdalar nucleus

LAcbSh: nucleus accumbens, lateral shell

LAH: lateroanterior hypothalamic nucleus

LC: locus coeruleus

LDTg: laterodorsal tegmental nucleus

LDTgV: laterodorsal tegmental nucleus, ventral part

LEnt: lateral entorhinal cortex

LepRb: long form of the leptin receptor

LG: lateral geniculate nucleus

LHA: lateral hypothalamic area

LHb: lateral habenula

LL: lateral lemniscus

LM: lateral mammillary nucleus

LOT: nucleus of the lateral olfactory tract

LPAG: lateral periaqueductal gray

LPBC: lateral parabrachial nucleus, central part

LPBD: lateral parabrachial nucleus, dorsal part

LPBE: lateral parabrachial nucleus, external part

LPBI: lateral parabrachial nucleus, internal part

LPBS: lateral parabrachial nucleus, superior part

LPBV: lateral parabrachial nucleus, ventral part

LPGi: lateral paragigantocellular nucleus

LPLR: LP thalamic nucleus, laterorostral part

LPMC: LP thalamic nucleus, mediocaudal part

LPMR: LP thalamic nucleus, mediorostral part

LPO: lateral preoptic nucleus

LPS: lipopolysaccharide

LRt: lateral reticular nucleus

LS: lateral septal nucleus

LSD: lateral septal nucleus, dorsal part

LSI: lateral septal nucleus, intermediate part

LSV: lateral septal nucleus, ventral part

LT: lateral terminal nucleus (pretectum)

LVe: lateral vestibular nucleus

MA3: medial accessory oculomotor nucleus

MBO: mammillary body

MCH: melanin-concentrating hormone

MCPC: magnocellular nucleus of the posterior commissure

MCPO: magnocellular preoptic nucleus

MD/ MDL/ MDC/ MDM: mediodorsal thalamic nucleus

MdD: medullary reticular nucleus, dorsal part

MdV: medullary reticular nucleus, ventral part

Me: medial nucleus of the amygdala

me5: mesencephalic trigeminal tract

MeAD: medial amygdalar nucleus, anterodorsal part

MeAV: medial amygdalar nucleus, anteroventral part

MEBS: Minnesota eating behavior survey

MEE: median eminence, zona externa

MEI: median eminence, zona interna

MEnt: medial entorhinal cortex

MePD: medial amygdalar nucleus, posterodorsal part

MePV: medial amygdalar nucleus, posteroventral part

mfb: medial forebrain bundle

MG: medial geniculate nucleus

MGM: medial geniculate nucleus, medial part

MGV: medial geniculate nucleus, ventral part

MHb: medial habenula

MiTg: microcellular tegmental nucleus

ml: medial lemniscus

ML: medial mammillary nucleus, lateral part

mlf: medial longitudinal fasciculus

MM: medial mammillary nucleus, medial part

MnM: medial mammillary nucleus, median part

MnPO: median preoptic nucleus

MnR: median raphe nucleus

MO: medial orbital cortex

MOB: main olfactory bulb

MOp: primary motor area

MOs: secondary motor area

MPA: medial preoptic area

MPB: medial parabrachial nucleus

MPBE: medial parabrachial nucleus, external part

MPL: medial paralemniscal nucleus

MPO: medial preoptic nucleus

MPT: medial pretectal area

MRN: mesencephalic reticular nucleus

mRt: mesencephalic reticular nucleus/ formation

MS: medial septal nucleus

MSN: medium spiny neurons

mtg: mammillotegmental tract

MVe: medial vestibular nucleus

MVeMC: medial vestibular nucleus, magnocellular part

MVePC: medial vestibular nucleus, parvicellular part

Mx: matrix region of the medulla

NAc: nucleus accumbens

NPC: nucleus of the posterior commissure

NPY: neuropeptide Y

NRM: nucleus raphe magnus

ns: nigrostriatal bundle

Nts: neurotensin

NTS: nucleus of the solitary tract

NtsR1: neurotensin receptor 1

NtsR1^{++}: neurotensin receptor 1 wildtype

NtsR1KO/ *NtsR1*^{KOKO}: neurotensin receptor 1 knockout

NtsR2: neurotensin receptor 2

NtsR3: neurotensin receptor 3/ sortilin

NR: not reported

Nv: navicular postolfactory nucleus

OCD: obsessive-compulsive disorder

Op: optic nerve layer of the superior colliculus

OPT: olivary pretectal nucleus

opt: optic tract

OX: orexin

p1PAG: prosomere 1 periaqueductal gray

p1Rt: p1 reticular formation

P5: peritrigeminal zone

P7: perifacial zone

PA: posterior amygdalar nucleus

Pa4: paratrochlear nucleus

Pa6: paraabducens nucleus

PAG: periaqueductal gray

PaR: pararubral nucleus

PaXi: paraxiphoid nucleus of the thalamus

PB: parabrachial area/nuclei

PBG: parabigeminal nucleus

PBP: parabrachial pigmented nucleus of the ventral tegmental area

PBS: phosphate-buffered saline

PBQ: phenyl-p-benzoquinone

PC: paracentral thalamic nucleus

pc: posterior commissure

PCG: pontine central gray

PCR: polymerase chain reaction

PCRt: parvicellular reticular nucleus

PCRtA: parvicellular reticular nucleus, alpha part

PD: parkinson’s disease

PDR: posterodorsal raphe nucleus

Pe: periventricular hypothalamic nucleus

PeF: perifornical nucleus

PERI: perirhinal area

PF: parafascicular thalamic nucleus

PG: pregeniculate nucleus

PGi: paragigantocellular reticular nucleus

PH: posterior hypothalamic nucleus

PHD: posterior hypothalamic area, dorsal part

PIF: parainterfascicular nucleus of the ventral tegmental area

PIL: posterior intralaminar thalamic nucleus

PIR: piriform area/cortex

PKC-δ: protein kinase C-δ

PL: prelimbic area

PLi: posterior limitans thalamic nucleus

PLV: perilemniscal nucleus, ventral part

PMCo: posteromedial cortical amygdalar nucleus

PMnR: paramedian raphe nucleus

PMV: premammillary nucleus, ventral part

PN: paranigral nucleus of the ventral tegmental area

PnC: pontine reticular nucleus, caudal part

PnO: pontine reticular nucleus, oral part

PnR: pontine reticular/raphe nucleus

Po: posterior thalamic nuclear group

POA: preoptic area

PoMn: posteromedian thalamic nucleus

PoT: posterior thalamic nucleus, triangular part

PP: peripeduncular nucleus

PPTg: pedunculopontine nucleus

Pr: prepositus nucleus

PR: prerubral field

PR: progressive ratio

PR5: principal sensory trigeminal nucleus

PR5DM: principal sensory trigeminal nucleus, dorsomedial part

PR5VL: principal sensory trigeminal nucleus, ventrolateral part

PRC: precommissural nucleus

PrCnF: precuneiform area

PrEW: pre-edinger-westphal nucleus

PRh: perirhinal cortex

PrL: prelimbic cortex

PS: parastrial nucleus

PSTh: parasubthalamic nucleus

PT: paratenial thalamic nucleus

PTg: pedunculotegmental nucleus

PTLp: posterior parietal association areas

Pv: paraventricular fiber system

PV: paraventricular thalamic nucleus

PVA: paraventricular thalamic nucleus, anterior part

PVH/ PVN: paraventricular hypothalamic nucleus

PVP: paraventricular thalamic nucleus, posterior part

Py: pyramidal cell hippocampus

Py3 CA3: pyramidal field CA3 hippocampus

qRT-PCR: quantitative reverse transcription-polymerase chain reaction

R: red nucleus

Ramb: retroambiguus nucleus

RCh: retrochiasmatic area

RChL: retrochiasmatic area, lateral part

Re: reuniens thalamic nucleus

REth: retroethmoid nucleus

Rh: rhomboid thalamic nucleus

RLi: rostral linear nucleus

RM: retromammillary nucleus

RMg: nucleus raphe magnus

RML: retromammillary nucleus, lateral part

RMM: retromammillary nucleus, medial part

rmx: retromammillary decussation

Ro: nucleus of roller

Rob: Raphe obscurus nucleus

RPA: nucleus raphe pallidus

RPC/ RMC: red nucleus

RR: retrorubral nucleus

RRF: retrorubral field

RS: retrosplenial area

Rs: rubrospinal tract

RSG/ RSD: retrosplenial granular/ dysgranular cortex

RSPv: granular retrosplenial area

RT: reticular thalamic nucleus

RVL: rostroventrolateral reticular nucleus

RVRG: rostral ventral respiratory group

S: somatosensory cortex

S/ Sub: subiculum

Sag: sagulum nucleus

SC: superior colliculus

SCh: suprachiasmatic nucleus

Scp: superior cerebellar peduncle

SFi: septofimbrial nucleus

SFO: subfornical organ

SG: suprageniculate thalamic nucleus

Sge: supragenual nucleus

SGN: suprageniculate nucleus

SH/ SHi: septohippocampal nucleus

SHy: septohypothalamic nucleus

SI: substantia innominata

SM: nucleus stria medullaris

SNC: substantia nigra pars compacta

SNR: substantia nigra pars reticulata

SO: supraoptic nucleus

SOc: superior olivary complex

SolC: solitary nucleus, commissural part

SolCe: solitary nucleus, central part

SolDL: solitary nucleus, dorsolateral part

SolDM: solitary nucleus, dorsomedial part

SolG: solitary nucleus, gelatinous part

SolI: solitary nucleus, interstitial part

SolL: solitary nucleus, lateral part

SolIM: solitary nucleus, intermediate part

SolM: solitary nucleus, medial part

SolV: solitary nucleus, ventral part

SolVL: solitary nucleus, ventrolateral part

Sp5C: spinal trigeminal nucleus, caudal part

Sp5I: spinal trigeminal nucleus, interpolar part

Sp5O: spinal trigeminal nucleus, oral part

SPF: subparafascicular nucleus

SPO: superior paraolivary nucleus

SPTg: subpeduncular tegmental nucleus

SpVe: spinal vestibular nucleus

Sst: somatostatin

st: stria terminalis

StA: strial part of the preoptic area

STh: subthalamic nucleus

StHy: striohypothalamic nucleus

STIA: ST, intraamygdalar division

STr: subiculum transition area

Su3: supraoculomotor periaqueductal gray

Su3C: supraoculomotor cap

Su5: supratrigeminal nucleus

SubB: subbrachial nucleus

SubCD: subcoeruleus nucleus, dorsal part

SubCV: subcoeruleus nucleus, ventral part

SubP: subpostrema area

SuG: superficial gray layer of the superior colliculus

SuM: supramammillary nucleus

SuVe: superior vestibular nucleus

Te: terete hypothalamic nucleus

Th: tyrosine hydroxylase

TrLL: triangular nucleus, lateral lemniscus

TRN: tegmental reticular nucleus

TS: triangular septal nucleus

TT: taenia tecta

Tu: olfactory tubercle

Tz: trapezoid nucleus

VA: ventral anterior thalamic nucleus

VCA: ventral cochlear nucleus, anterior part

VCl: ventral part of the claustrum

VDB: nucleus of the vertical limb of the diagonal band

Ve: vestibular nuclei

VeCb: vestibulocerebellar nucleus

VEN: ventral endopiriform nucleus

vhc: ventral hippocampal commissure

VIEnt: ventral intermediate entorhinal cortex

VISC: visceral area

VLH: ventrolateral hypothalamic nucleus

VLL: ventral nucleus of the lateral lemniscus

VLPAG: ventrolateral periaqueductal gray

VLPO: ventrolateral preoptic nucleus

VMH: ventromedial hypothalamic nucleus

VMPO: ventromedial preoptic nucleus

VO: ventral orbital cortex

VOLT: vascular organ of the lamina terminalis

VP: ventral pallidum

VS: ventral subiculum

VTA: ventral tegmental area

VTg: ventral tegmental nucleus

VTT: ventral tenia tecta

X: nucleus X

Xi: xiphoid thalamic nucleus

Y: nucleus Y

ZI: zona incerta

ZIC: zona incerta, caudal part

ZID: zona incerta, dorsal part

ZIR: zona incerta, rostral part

ZIV: zona incerta, ventral part

CHAPTER 1. Role of Central Neurotensin in Regulating Feeding: Implications for the Development and Treatment of Body Weight Disorders

Part of this chapter represents a manuscript published in *Biochemica et Acta* (*BBA)-Molecular Basis of Disease* (2018) **1864** (3): 900-916.

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Abstract

The peptide neurotensin (Nts) was discovered within the brain over 40 years ago and is implicated in regulating analgesia, body temperature, blood pressure, locomotor activity and feeding. Recent evidence suggests, however, that these disparate processes may be controlled via specific populations of Nts neurons and receptors. The neuronal mediators of Nts anorectic action are now beginning to be understood, and, as such, modulating specific Nts pathways might be useful in treating feeding and body weight disorders. This review considers mechanisms through which Nts normally regulates feeding and how disruptions in Nts signaling might contribute to the disordered feeding and body weight of schizophrenia, Parkinson's disease, anorexia nervosa, and obesity. Defining how Nts specifically mediates feeding vs. other aspects of physiology will inform the design of therapeutics that modify body weight without disrupting other important Nts-mediated physiology.

Keywords: Neurotensin receptor, dopamine, energy balance, obesity, anorexia

Ingestive Behavior Impacts Health

The physiological processes that sustain life constantly tap bodily energy reserves, which must be replaced via ingestion; hence, feeding is a compulsory behavior for survival. Decades of research have proven that the brain is the master-organizer of feeding behavior, vigilantly monitoring energy status and coordinating appropriate ingestive behavior. For example, fasting-induced hunger increases the motivation to find and ingest food, while stomach fullness or increased body fat cue the cessation of feeding. However, despite recognition that these processes take place, and the fact that eating and drinking are perhaps the most commonly performed behaviors in animals and humans, the precise mechanisms by which the brain orchestrates these processes remain incompletely understood.

Defining the biology of ingestion is necessary not only to understand immediate survival but also to treat, and ultimately prevent, feeding dysregulation that endangers health and well-being. For example, intake of excess calories, along with insufficient physical activity and metabolic rate to consume them, results in increased adiposity. The rise in highly palatable, energy-dense foods, their ease of acquisition and the widespread increase in sedentary lifestyles have contributed to the worldwide rise in the overweight and obese [1]. Increased body weight, as assessed via body mass index, elevates risk of developing severe chronic conditions, including cardiovascular disease, type-2 diabetes, kidney disease, cancer and disability, and has been accountable for 4 million annual deaths [2]. Though lifestyle intervention is safe and somewhat effective in promoting weight loss, it is difficult to maintain and, as a result, has not been

sufficient to counteract the overweight and obesity epidemic. Bariatric surgery is currently the most effective option to treat obesity; however, not all patients are able to undergo such procedures because of cost, complications, or restrictive guidelines [3]. In addition, the search for both safe and efficacious pharmacological therapies to treat obesity has proven difficult. For example, serotonin reuptake inhibitors were found to have serious cardiopulmonary side effects that limited their usage [3]. Cannabinoid type 1 receptor antagonists appeared to hold promise as effective weight loss medications without adverse cardiovascular-related events, but these drugs caused severe psychiatric side effects that precluded their usage [3]. This stresses the need to find efficacious pharmacological interventions with suitable safety profiles that both support weight loss and prevent debilitating chronic conditions that diminish life span.

Insufficient feeding can be equally deleterious. This is evidenced by the wide array of medical complications that arise with the self-imposed feeding restriction that defines the eating disorder anorexia nervosa (AN) [4]. This “relentless pursuit of thinness” has the highest mortality rate of any psychiatric illness [4], and there is an urgent need to find therapies that improve outcomes. AN is often accompanied by other psychiatric illness, including mood, anxiety and substance use disorders, and comorbidity is present in about 50% of all adolescents with AN [4]. Such comorbidity dictates the types of medications these patients receive. While use of antidepressants and antipsychotics can improve psychiatric symptoms, they fail to restore body weight [4]. As with obesity, finding efficacious pharmacotherapies for these patients has proven particularly difficult and has been limited by an incomplete understanding of how

the brain regulates feeding. Thus, there is a crucial need to elucidate the neural signals that regulate feeding to direct discovery of interventions to treat eating disorders.

Neuropeptides have emerged as important regulators of body weight, with some promoting feeding (orexigenic) and others suppressing it (anorexigenic). The field has learned much about orexigenic neuropeptides. Yet, many anorexigens are comparatively less well characterized, though they may hold particular therapeutic promise for treating body weight disorders. Recently there has been increasing attention directed at how the neuropeptide neurotensin (Nts) modifies body weight. Nts signaling appears to play a pivotal, yet still poorly understood, role in intestinal fat absorption [5], but pharmacological data suggest that Nts may act centrally to suppress feeding. This review will focus on the growing understanding of how Nts signals within the brain, its contribution to regulation of energy balance and how disruption of central Nts signaling may underlie disordered feeding and body weight in disease.

Neurotensin (Nts) Structure and Expression Pattern

Nts was first isolated from purified bovine hypothalamus by Carraway and Leeman. Injecting the isolated peptide intravenously into rats led to the initial characterization of Nts as a powerful hypotensive agent, an inducer of vascular permeability and a regulator of intestinal and uterine contraction [6]. These data suggested that Nts may not strictly exist as a central neuropeptide. Indeed, Nts was subsequently found within epithelial cells of the stomach and intestine [7]. Cloning of the Nts gene revealed that it contains coding sequences for both Nts and the Nts-related peptide Neuromedin N and led to the discovery that it produces a 169 amino acid precursor protein (pro-neurotensin, [pro-Nts]), which has an N-terminal signal sequence and is processed into both peptides [8]. Furthermore, two different-sized mRNA products, a 1.0 kb or 1.5 kb mRNA species, may be produced, and these mRNAs differ in their 3' untranslated regions. Both transcripts are present in approximately equal ratios throughout the brain; however, the 1.0 kb mRNA species is 10 times more prevalent than the 1.5 kb mRNA within the intestine [8], suggesting tissue-specific regulation and perhaps differential peptide functions in the brain and periphery. Pro-Nts is subsequently cleaved by prohormone convertases to produce Neuromedin N and the Nts tridecapeptide (Glu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-OH) [9]. Intriguingly, a truncated form of Nts (Nts 8-13) has superior Nts Receptor binding affinity compared to the full-length peptide, and this fragment is often used for *in vitro* studies of Nts action [9].

Nts is produced centrally and peripherally, but these pools of Nts are thought to mediate distinct actions. The abundant amount of Nts peptide found within the plasma may originate from the adrenal gland [10] and from the subset of intestinal enteroendocrine cells termed N-Cells [11]. Central vs. systemic administration of Nts produces different physiological responses. For instance, with intracisternal administration (a method of direct infusion into the subarachnoid space that allows drugs to bypass the blood-brain barrier), Nts induces antinociceptive and hypothermic effects. By contrast, intravenous (systemic) administration fails to produce either of these responses and, in fact, has been shown to result in elevated body temperature [12]. Intestinal Nts has recently been shown to be necessary for fat absorption via yet to be established mechanisms [5]. Circulating Nts levels are increased after bariatric surgery and weight loss, raising the possibility that Nts exerts some peripheral regulation of body weight [13]. Though the differential effects of central vs. peripheral Nts administration have led to the consensus that the blood-brain barrier is impermeable to Nts, new data suggests that there is some Nts transport to and from the brain [14]. This is further supported by the fact that peripheral Nts can activate brainstem structures in vagotomized mice and can induce gene expression of the anorectic peptide POMC within the arcuate nucleus of the hypothalamus [15]. However, given that the doses of Nts used in this study resulted in supraphysiological concentrations of circulating Nts [15], and given that Nts has a relatively short half-life in the blood [14], it is likely that central actions of Nts are primarily mediated via Nts produced within the brain.

Immunohistochemical labeling of the specific brain cells that express Nts requires pre-treatment with the axonal transport-inhibitor colchicine, and this necessity has complicated detection and subsequent study of these populations. Without colchicine treatment, Nts immunolabeling identifies Nts fibers while failing to detect the Nts-containing cell bodies of origin. **Table 1.1** summarizes the reports of colchicine-mediated Nts-immunoreactive cell bodies and fibers in the rat brain, which are present within specific nuclei ranging from the hindbrain to the forebrain [16–21]. Many brain regions contain Nts cell bodies as well as fibers, which may signify Nts neurons that project locally to regulate adjacent cells. In some cases, however, Nts fibers are observed in regions that lack cell bodies. These may be axons of passage, or may identify terminal projection sites where Nts is released. Most of the sites with immunolabeled Nts cell bodies are consistent with the distribution of Nts neurons identified via *in situ* hybridization (ISH), as reported in the Allen Brain Atlas [22]. Both of these methods indicate substantial populations within the septal nuclei, preoptic area (POA), bed nucleus of the stria terminalis (BNST), central amygdala (CeA), lateral hypothalamic area (LHA), parabrachial area (PB) and the nucleus of the solitary tract (NTS). An important species difference, however, is noted within the ventral tegmental area (VTA). Rats have a large population of VTA Nts neurons, most of which also contain dopamine (DA), but mice have very few VTA Nts cells [22]. In the rat, VTA Nts neurons also co-express the satiety-inducing neuropeptide cholecystokinin (CCK), which suppresses feeding and elicits similar effects to intracerebroventricular (ICV) Nts treatment, including hypothermia, antinociception and neuroleptic-like activity [23]. Thus, while there is ample evidence to suggest that VTA Nts neurons directly overlap

Table 1.1. Distribution of Nts Cells in the Central Nervous System.
Summary of structures reported to contain Nts immunoreactivity in brains from colchicine-treated rats [16-20] and guinea pigs [21]. Results convey the relative density of Nts-labeled cell bodies or fibers. NR not reported; + few or sparse; ++ some; +++ many; ++++ very dense.

KEY

NR	Not Reported
+	Few
++	Some
+++	Many
++++	Very Dense

Structures Reported to Contain Nts	Supporting Literature	Density of Cell Bodies	Density of Fibers
SPINAL CORD			
Spinal cord: laminae I and II	18	NR	+++
Spinal cord: lamina III and IV	18	NR	++
Spinal cord: lamina X	18	+	++
HINDBRAIN			
Spinal trigeminal nucleus (Sp5C)	16, 18, 21	+++ / ++++	+++
Cuneate nucleus (Cu)	18	NR	++
Nucleus ambiguus (Amb)	18	++	+++
Pontine reticular nucleus (PnR)	18	NR	++++
Pontine central gray (PCG)	18	NR	++++
Mesencephalic Trigeminal Tract (me5)	18	NR	++++
Trapezoid Nucleus (Tz)	18, 21	+++	+++
Gigantocellular reticular nucleus (Gi)	18	NR	++++
Paragigantocellular reticular nucleus (PGi)	21	+++	NR
Parvocellular reticular nucleus	18	NR	++
Lateral reticular nucleus (LRt)	21	++	NR
Ventrolateral reticular formation	18	+	+
Ventral reticular formation	18	NR	++++

Table 1.1 (cont'd)

Nucleus linearis	18	NR	++
Nucleus of the solitary tract (NTS)	16, 18, 21	++ / +++	+++ / ++++
Nucleus raphe magnus (RMg)	18, 21	++	++++
Nucleus raphe pallidus (RPA)	21	++	NR
Nucleus raphe obscurus (RO)	21	++	NR
Dorsal Cochlear nucleus (DC)	16	+	+
Area Postrema (AP)	18	+	++
Floor of the 4th Ventricle (4V)	16, 18	++	++
Parabrachial nuclei (PB)	16, 18, 21	++ / +++	++ / ++++
Locus coeruleus (LC)	16, 18, 21	++	++
MIDBRAIN			
Dorsal raphe nucleus (DR)	16, 18, 21	++ / +++	++
Pontine raphe nucleus (PnR)	18	++	++++
Median raphe nucleus (MnR); also known as "nucleus centralis superior"	18	++	++
Periaqueductal gray (PAG); also known as "central gray"	16, 18, 21	++	++ / +++
Pretectal nucleus (APT)	18	NR	+ / ++
Medial pretectal area (MPT)	18	NR	++
Lateral lemniscus (LL)	18	NR	+++
Ventral tegmental area (VTA); also known as "Paranigral nucleus"	16, 18, 21	+++ / ++++	++
Interpeduncular fossa (IPF)	18	NR	++
Substantia nigra pars compacta (SNC)	18	NR	+++
THALAMUS & NEARBY REGIONS			
Periventricular nuclei of Thalamus	18	++	++
Medial Thalamic nuclei	18	NR	++
Rhomboid thalamic nucleus (Rh)	18	NR	+++
Reuniens thalamic nucleus (Re)	18	NR	+

Table 1.1 (cont'd)

Posteromedian Thalamic nucleus	18	NR	+
Parafascicular Thalamic nucleus (PF)	21	+++	NR
Medial geniculate (MG)	18	NR	+ / ++
Lateral Habenula (LHb)	18	+	NR
HYPOTHALAMUS & NEARBY REGIONS			
Posterior hypothalamic nucleus	17, 19	+	+
Dorsal hypothalamus	18	NR	++++
Dorsomedial hypothalamic nucleus (DM)	17 - 19, 21	+ / +++	++
Ventromedial hypothalamic nucleus (VMH)	17	+ / ++	NR / ++
Arcuate nucleus (Arc); also known as "Infundibular nucleus"	17, 19 - 21	++/+++	NR
Posterior hypothalamus	19	+	NR
Median eminence, zona externa (MEE)	17 - 20	NR	+++ / ++++
Median eminence, zona interna (MEI)	17	NR	++
Posterior mammillary nucleus	17, 19	NR	+++ / ++++
Stria terminalis (st)	17, 19, 21	NR	+++ / ++++
Premammillary nucleus, ventral part (PMV)	17, 18, 21	+	+
Lateral hypothalamic area (LHA)	17-21	+++	++/++++
Paraventricular hypothalamic nucleus (PVN)	17-21	+++ / ++++	+++ / ++++
Anterior hypothalamus	17, 19	+	NR
Zona Incerta (ZI)	17, 18, 21	+ / ++	++
Medial forebrain bundle (mfb)	17, 18	+	++
Medial preoptic area (MPA)	16-19, 21	+ / +++	++++
Ventromedial preoptic nucleus (VMPO)	18	++	NR
Lateral preoptic area (LPO)	17, 18, 21	++	+++
Substantia innominata (SI)	21	++	NR

Table 1.1 (cont'd)

Anteroventral periventricular nucleus (AVPe)	18	++++	NR
Periventricular hypothalamic nucleus (Pe)	17-21	++	++++
Suprachiasmatic nucleus (SCh)	18, 21	++	+++
Posterior pituitary gland	17, 19	NR	+
Pituitary Stalk	19	NR	+++ / ++++
Hippocampus	18	NR	+
AMYGDALA			
Central nucleus of the amygdala (CeA)	17-19, 21	+++	"+++ / ++++
Medial nucleus of the amygdala (Me)	17, 18, 21	+/++	++
Basomedial amygdalar nucleus (BMA)	18, 21	+	+++
Cortical amygdalar nucleus (COA)	18	+	+++
CORTEX			
Prepiriform cortex	18	NR	+ / +++
Cerebral cortex (Cx)	18	NR	+++
STRIATUM AND FOREBRAIN			
Caudate putamen (CPu)	18	++	++++
Globus pallidus (GP)	18	NR	+
Bed nucleus of the stria terminalis (BNST)	17 - 19, 21	+++ / ++++	++ / ++++
Triangular septal nucleus (TS)	21	++	NR
Nucleus of the diagonal band of Broca (DB)	18, 21	++	+
Lateral septal nucleus (LS)	18, 21	++	NR
Medial septal nucleus (MS)	21	++	NR
Septum pellucidum	18	NR	++

with anorectic and DAergic mechanisms to modify feeding in the rat, this differs in mice. Given the important role of DA neurons in feeding and body weight [24], the species differences in VTA Nts expression suggest very different mechanisms for Nts to modify DA signaling and behavior in rats and mice. Thus, investigators should use caution when studying the Nts system and interpreting results from different rodent models.

Central Neurotensin Receptors (NtsRs)

Nts binds brain and gut tissue [9], which is primarily mediated via Neurotensin Receptor 1 (NtsR1) and Neurotensin Receptor 2 (NtsR2) [25]. Although the NtsR1 and NtsR2 isoforms share 64% sequence homology, they differ in binding properties, expression and function. NtsR1 has a much higher affinity for Nts (K_d = 0.3 nM) compared to NtsR2 (K_d 2-4 nM), but only the NtsR2 isoform binds levocabastine (a Histamine H1 receptor antagonist with no known Nts-analogous effects) [9,25,26] . NtsR1 and NtsR2 are both G protein-coupled receptors and engage second messenger systems, though the exact system seems to depend on cell type [25]. Indeed, NtsR1 and NtsR2 are differentially expressed within the brain: NtsR2 expression was identified in both neurons and glia, but NtsR1 is found only in neurons [27,28]. Intriguingly, experimental brain injury increases the number of NtsR2-expressing astrocytes and NtsR2 mRNA, suggesting a role for NtsR2 in the inflammatory response [27], though, this has yet to be fully understood. Recently, a third Nts receptor has been reported, Neurotensin Receptor 3 (NtsR3), which is identical to the previously identified sortilin protein [29]. NtsR3/sortilin is a single transmembrane receptor involved in membrane trafficking of ligands [29]. The N-glycosylated form of NtsR3/sortilin that resides on the plasma membrane internalizes upon Nts binding. Conversely, intracellular NtsR3/sortilin is recruited to the plasma membrane as a result of Nts binding. Together, these data suggest that NtsR3/sortilin may be involved in turnover of the Nts peptide [29]. However, since no data exists linking NtsR3 to the central regulation of energy balance, the remainder of this review will primarily focus on the NtsR1 and NtsR2 isoforms that have been explored in regulation of body weight.

Nts binding can be detected throughout the entire rostral-caudal axis of the brain, with enrichment in some specific regions. Cloning of the three Nts receptor isoforms and development of ISH probes enabled more precise assessment of receptor localization, which has primarily been investigated within the rat brain [30–35]. Interestingly, NtsR1 is broadly expressed throughout the rat brain during gestation, perhaps suggesting that it contributes to the formation of neural circuits. However, since NtsR1 expression is more restricted within adults, it likely exerts more specified signaling roles in the mature brain [36]. This differential expression of NtsR1 over life span may account for the fact that transgenic NtsR1^{Cre}-reporter mice bred to Cre-inducible reporter strains identify numerous "NtsR1" cortical pyramidal cells within layer 6, even though ISH suggests that these cells do not actively express NtsR1 in the adult brain [22]. Presumably, the burst of early NtsR1 and Cre expression during development causes recombination and permanent cell labeling, even in cells that no longer express NtsR1. By contrast, NtsR2 expression is modest during development but increases over life span [9]. The distribution of NtsR isoforms reported in the adult rat brain via ISH or immunolabeling are summarized in **Table 1.2**, but functional studies (e.g. site-specific injections) suggest additional sites of NtsR expression. A number of brain regions contain Nts fibers **(Table 1.1)** and NtsRs (**Table 1.2),** indicating places where Nts is endogenously released and can engage NtsR isoforms. Such sites include the suprachiasmatic nucleus, SN, VTA, BNST, and CeA. Both NtsR1 and NtsR2 isoforms are robustly expressed within the SN and VTA [32], and are also detected within the globus pallidus, BNST, substantia innominata, suprachiasmatic nucleus, habenula, CeA, arcuate nucleus, subiculum and the zona incerta [32].

Table 1.2. Distribution of NtsR1, NtsR2, and NtsR3 Cells in the Central Nervous System.

Summary of structures reported to contain NtsR1, NtsR2 or NtsR3 cell bodies, using Nts-immunoreactivity or *in situ* hybridization [30-35] in the rat central nervous system. Results convey the relative density of NtsR-labeled cell bodies. NR: not reported. + few or sparse; ++ some; +++ many; ++++ very dense.

KEY

NR	Not Reported
+	Few
++	Some
+++	Many
++++	Very Dense

Structures reported to contain NtsR	Supporting Literature	NtsR1	NtsR2	NtsR3/ Sortilin
SPINAL CORD				
Spinal cord: lamina I and II	35	++	NR	NR
HINDBRAIN				
Oculomotor nucleus (3N)	33, 34	NR	++	++ / +++
Trochlear nucleus (4N)	34	NR	+ / ++	NR
Spinal trigeminal nucleus (5N)	33, 34	NR	+	+ / ++
Abducens nucleus (6N)	33, 34	NR	+	++
Facial nucleus (7N)	33, 34	NR	+	++
Vestibulocochlear nerve (8N)	32	NR	++	NR
Dorsal motor nucleus of vagus (10 N or DMX)	33, 34	NR	- / +	+ / ++
Hypoglossal nucleus (12N)	33, 34	NR	+	+ / ++
Vestibular nuclei (Ve)	32 - 35	+	+	++
Cochlear nuclei (CN)	32 - 34	NR	++	+ / ++
Superior colliculus (SC)	31 - 34	+++	+	+ / ++
Inferior colliculus (IC)	33, 34	NR	+	++ / +++
Trapezoid Nucleus (Tz)	33, 34	NR	++	++
Mesencephalic reticular nucleus (MRN)	31, 35	++	NR	NR
Ventrolateral reticular formation	30	+++	NR	NR
Giganotcellular reticular nucleus (Gi)	34	NR	+	NR
Paragigantocellular reticular nucleus (PGi)	34	NR	+	NR
Parvocellular reticular nucleus	34	NR	-/+	NR

Table 1.2 (cont'd)

Medial reticular formation	33	NR	NR	++
Lateral reticular nucleus	33 - 35	++	+	+
Nucleus of the solitary tract (NTS)	33, 34	NR	- / +	+ / ++
Nucleus raphe magnus (RMg)	34	NR	- / +	NR
Nucleus raphe pallidus (RPA)	34	NR	- / +	NR
Medial lemniscus (ml)	33	NR	NR	+ / ++
Cuneate nucleus (Cu)	33, 34	NR	+	+ / ++
Gracile nucleus (Gr)	33	NR	NR	+ / ++
Inferior olivary complex (IO)	33, 34	NR	+ / ++	++ / +++
Superior olivary complex (SOc)	33, 34	NR	+ / ++	++
MIDBRAIN				
Subiculum (S)	31 - 34	++	++ / +++	+ / ++
Pontine nuclei	33, 34	NR	++	+ / ++
Periaqueductal gray (PAG)	31 - 35	+ / ++	+	+ / ++
Dorsal raphe nucleus (DR)	31, 33, 34	++	+	++
Median raphe nucleus (MnR)	33	NR	NR	+ / ++
Rostral linear nucleus raphe (RLi)	31, 34	+ / ++	- / +	NR
Tegmental reticular nucleus (TRN)	31	+++	NR	NR
Precommissural nucleus (PRC)	33	NR	NR	+ / ++
Ventral tegmental area (VTA)	30 - 35	+++	+	+ / ++
Interfascicular nucleus (IF)	34	NR	- / +	NR
Interpeduncular nucleus (IPF)	31, 33, 34	++	- / +	+ / +++
Nucleus of the optic tract	31	+++	NR	NR
Nucleus of the posterior commissure (NPC)	31	+++	NR	NR
Substantia nigra pars compacta (SNC)	30 - 35	+++	++	++ / +++
Red nucleus (R)	33, 34	NR	++	++ / +++
Pedunculopontine nucleus (PPTg)	31	+ / ++	NR	NR
Peripeduncular nucleus (PP)	31	+	NR	NR
THALAMUS & NEARBY REGIONS				
Paraventricular thalamic nucleus	31, 33, 34	+	+	+ / +++
Rhomboid thalamic nucleus (Rh)	31	+	NR	NR
Reuniens thalamic nucleus (Re)	31, 33	++	NR	+ / ++

Table 1.2 (cont'd)

Reticular thalamic nucleus (RT)	33, 35	+	NR	++ / +++
Mediodorsal thalamic nucleus	33, 34	NR	+ / ++	+ / ++
Ventral medial nucleus of the thalamus	34	NR	+	NR
Posterior nuclear group of thalamus	33	NR	NR	++
Intermediodorsal nucleus of the thalamus (IMD)	31	+	NR	NR
Central medial nucleus of the thalamus (CM)	31	+	NR	NR
Anterodorsal thalamic nucleus (AD)	31, 33, 34	+++	- / +	++ / +++
Anteroventral thalamic nucleus (AV)	33, 34	NR	+	+ / ++
Supragenicular nucleus (SGN)	31	+	NR	NR
Lateral geniculate nucleus (LG)	31, 33, 34	++	+	+ / ++
Medial geniculate nucleus (MG)	31, 33, 34	++	+	++
Intergeniculate leaflet	31	++	NR	NR
HYPOTHALAMUS & NEARBY REGIONS				
fimbria of the fornix	33	NR	NR	+ / ++
Subparafascicular nucleus (SPF)	31	++	NR	NR
Supramammillary Area Nucleus (SuM)	30, 31	++ / ++++	NR	NR
Mammillary body (MBO)	33	NR	NR	++
Medial mammillary nucleus (MM)	32	NR	++	NR
Lateral mammillary nucleus (LM)	34	NR	+	NR
Posterior hypothalamus	31	+++	NR	NR
Dorsomedial hypothalamic nucleus (DM)	31	+++	NR	NR
Ventromedial hypothalamic nucleus (VMH)	31, 33	++	- / +	NR
Premammillary nucleus, ventral part (PMV)	31	++	NR	NR
Arcuate nucleus (Arc)	31 - 34	+++	+ / ++	++ / +++

Table 1.2 (cont'd)

Lateral Hypothalamic Area (LHA)	31, 33 - 35	+ / +++	- / +	+ / ++
Paraventricular hypothalamic nucleus (PVN)	31, 33, 34	+/++	- / +	++
Anterior hypothalamus	31, 33	++ / +++	NR	+
Zona Incerta (ZI)	31 - 35	+++	- / +	++
Subthalamic nucleus (STh)	33	NR	NR	++ / +++
Nucleus of the lateral olfactory tract (LOT)	31	+	NR	NR
optic tract (opt)	33	NR	NR	+ / ++
Suprachiasmatic preoptic nucleus	31	++	NR	NR
Preoptic periventricular nucleus	31	+	NR	NR
Supraoptic nucleus (SO)	31, 33 - 35	+	+	++ / +++
Subparaventricular zone	31	+ / ++	NR	NR
Posterior Periventricular nucleus	31	+	NR	NR
Intermediate Periventricular nucleus	31	+	NR	NR
Periventricular hypothalamic nucleus (Pe)	31 - 33	++	++	+ / ++
Anteroventral Periventricular nucleus	31	+	NR	NR
Anterior Periventricular nucleus	31	++	NR	NR
Nucleus circularis	31	++	NR	NR
Suprachiasmatic nucleus (SCh)	30 - 32	++ / +++	++	+ / +++
Retrochiasmatic area (RCh)	31, 34	++	++	NR
Hippocampus: Dentate gyrus (DG)	32 - 34	NR	-/+	+/++
Hippocampus: CA1 and CA2	32 - 34	NR	++ / +++	++ / +++
Hippocampus: CA3	31, 33	++	NR	++ / +++
Lateral Habenula (LHb)	31 - 34	+++	- / +	+ / ++
Medial Habenula (MHb)	31, 34, 35	++	- / +	NR
AMYGDALA				
Posterior amygdalar nucleus (PA)	31	+	NR	NR
Central nucleus of the amygdala (CeA)	31, 33 - 35	+++	++	++

Table 1.2 (cont'd)

Medial nucleus of the amygdala (Me)	31, 33 - 35	+ / ++	- / +	+ / ++
Lateral amygdalar nucleus (LA)	31	+	NR	NR
Basomedial amygdalar nucleus (BM)	33	NR	NR	+/++
Basolateral amygdalar nucleus (BL)	31, 33	+	NR	+/++
Cortical amygdalar nucleus (COA)	31, 33, 34	++	++	++
Intercalated amygdalar nucleus (IA)	31	++	NR	NR
Anterior amygdalar area (AA)	31	+	NR	NR
CORTEX				
Retrosplenial area (RS)	31, 33, 34	+	++	+ / +++
Granular retrosplenial area (RSPv)	31	+	NR	NR
Occipital cortex / visual cortex	31, 33, 34	+	+ / ++	+ / ++
Parietal cortex	33, 34	NR	++	++
Posterior parietal association areas (PTLp)	31	+	NR	NR
Somatosensory areas (SS)	31	+	NR	NR
Visceral area (VISC)	31	+	NR	NR
Primary motor area (MOp)	31	+	NR	NR
Secondary motor area (MOs)	31	++	NR	NR
Temporal cortex	33, 34	NR	+/++	++
Ventral temporal association area	31	+	NR	NR
Piriform area (PIR)	33-35	- / +	+	++ / +++
Entorhinal area (ENT)	31, 33 - 35	+++	++ / +++	++ / +++
Perirhinal area (PERI)	31, 34	+ / ++	+	NR
Ectorhinal area (ECT)	31	+ / ++	NR	NR
Prelimbic Area (PL)	31	++	NR	NR
Infralimbic Area (ILA)	31	++	NR	NR
Ventral orbital area (VO)	31	++	NR	NR
Frontal cortex	33, 34	NR	+ / ++	+ / ++
Anterior cingulate area (ACA)	31 - 34	++	+ / ++	+ / +++
Auditory areas	31, 33	+	NR	++
Gustatory areas (GU)	31	+	NR	NR
Agranular insular area (AI)	31	++	NR	NR
Insular Cortex (Ins)	33, 34	NR	+ / ++	++ / +++

Table 1.2 (cont'd)

Endopiriform nucleus (EP)	31, 33, 35	++	NR	++
STRIATUM AND FOREBRAIN				
Corpus callosum (CC)	32, 33	NR	++	+ / ++
Claustrum	31, 33	++	NR	+ / ++
Bed nucleus of the accessory olfactory tract (BA)	31	+	NR	NR
Nucleus accumbens (NAc)	31 - 34	+	- / ++	+ / ++
Internal capsule (int)	31	+	NR	NR
Caudate-putamen	33 - 35	+	+ / ++	+ / ++
Globus pallidus	31	+	++	+ / +++
Substantia innominata (SI)	31, 32, 35	+ / ++++	++	NR
Parastrial nucleus (PS)	31	+	NR	NR
Subfornical organ (SFO)	31, 32	+	++	NR
Bed nucleus of the stria terminalis (BNST)	31 - 35	+ / ++	++ / +++	+ / ++
Ventral pallidum	35	++	NR	NR
Islands of Cajella (ICj)	31, 33 - 35	+	+	++
Diagonal Band of Broca (DB)	30, 32 - 35	+++ / ++++	- / +	+ / +++
Taenia tecta (TT)	31, 33, 34	++	+	++ / +++
Septofimbrial nucleus (SFi)	31	+++	NR	NR
Triangular nucleus of septum (TS)	31	+	NR	NR
Septohippocampal nucleus (SH)	32, 33	NR	++	+ / ++
Lateral Septal nucleus (LS)	31, 33, 34	++ / +++	+ / ++	++ / ++++
Medial Septal nucleus (MS)	30, 31, 33 - 35	+++ / ++++	- / +	++ / +++
Olfactory tubercle (Tu)	34, 35	++	++	NR
Anterior olfactory nucleus (AON)	33	NR	NR	++ / +++
Main olfactory bulb (MOB)	31, 32, 34	+	+ / ++	NR

However, **Table 1.2** also identifies regional differences in the distribution of NtsR1 and NtsR2, hinting that they do not exert completely overlapping functions. Additionally, while NtsR2 ISH expression was identified in cells with architecture resembling either neurons or glia [27], this was not the case for NtsR1, which was found only on cells with neuronal morphology. Interestingly, stab-wound lesions in rat brain significantly increase NtsR2-expressing astrocytes and NtsR2 mRNA, suggesting that NtsR2 may be predominantly astrocytic and may play a role in the inflammatory response after brain injury [27]. The similar overlap of NtsR3/sortilin expression with that of NtsR2 suggests that NtsR3/sortilin is also predominantly expressed on glia [33]. Subsequent development of $NtsR1^{Cre}$ and $NtsR2^{Cre}$ mice, which permitted labeling of these cells via Cre-inducible reporter protein expression, confirmed that most NtsR2-expressing cells co-express the astrocyte marker S100 and are broadly distributed throughout the brain [28]. Additionally, consistent with NtsR1 ISH in the rat brain, these models revealed that mouse NtsR1 is confined to neurons, including a particularly dense population of DA-containing NtsR1 neurons found within the VTA [22,28,37]. Going forward, use of these mouse models will be helpful to visualize and selectively test the function of specific populations of NtsR1- and NtsR2-expressing cells.

Physiology Regulated By Central Nts Signaling

To date, pharmacological tools and genetic reagents have primarily been used to study how Nts functions via NtsR activation. Such pharmacological tools include the inhibitor SR142948A, which antagonizes both NtsR1 and NtsR2, and SR48692, which selectively antagonizes NtsR1. These antagonists have been particularly useful in distinguishing physiology regulated via NtsR1 vs. NtsR2. In addition to the biological agonist levocabastine (which acts as an agonist of NtsR2 but not NtsR1), many NtsR1- or NtsR2-biased agonists are in development. Such agonists are being used to dissect the physiological effects engendered by each receptor isoform, with the long-term goal of modifying isoform-specific physiology for health benefits [38,39]. Additionally, different lines of NtsR1 and NtsR2 knockout mice have been generated [40–42]; however, differences in the genetic design or background of these strains may account for some of the conflicting physiology attributed to NtsR1 or NtsR2. For example, **Table 1.3** compares the phenotypes between different lines of NtsR1 knockout mice [37,40–45]. Use of these mouse and pharmacological reagents has, however, provided some consensus on the role of central Nts via NtsR1 vs. NtsR2 in regulating analgesia, blood pressure, body temperature, locomotor activity, drug addiction, drinking and feeding/body weight. The physiologic effects of central Nts vs. site-specific Nts are depicted in **Figure 1.1 and Table 1.4** [6,41,46–63]**,** and are briefly described below.

Table 1.3. Characterization of NtsR1KO mice.
Summary of the current characterization of NtsR1KO mice, classified according to study [37,40–45] and origin. Observable differences in baseline physiology as well as in Nts-, leptin-, and drug-induced responses may be the result of differences in strain as well as in study design. Included are comparisons to wildtype controls. *Indicates response when raised on a high-fat, high-sucrose diet. #Though diminished, not significant. ⚹Opposite (intra-LHA Leptin induces hyperphagia). PBQ = phenyl-p-benzoquinone. NR: not reported.

Study	Remaury et al.	Maeno et al.	Mechanic et al.	Liang et al.	Pettibone et al.	Kim et al.	Opland et al.
Reference	**41**	**42**	**45**	**44**	**40**	**43**	**37**
Site Generated	**Remaury et al, France**	**Maeno et al, Japan**	**Roche, USA**	**Roche, USA**	**Deltagen, USA**	**Deltagen, USA-Jackson Labs**	**Deltagen, USA-Jackson Labs**
Genetic Background	**C57BL/6J**	**C57BL/6J**	**C57BL/6J**	**C57BL/6J**	**C57BL6/Sv 129J**	**C57BL/6J**	**C57BL/6J**
Body Weight	Increased	NR	NR	NR	NR	Increased	Same/ Increased*
Food Intake	Increased	NR	NR	NR	NR	Increased	Decreased/ Increased*
Body Temperature	Increased	NR	Same	NR	NR	NR	NR
Heat Production	NR	NR	NR	NR	NR	Increased	NR
Locomotor Activity	Same or Decreased	NR	Same	Increased	NR	Increased	Increased/ Increased*
Analgesia	NR	Same with hot plate, tail flick	Same	NR	NR	NR	NR
Nts-induced analgesia	Present with PBQ	NR	Absent with tail immersion	NR	Absent with hot plate	NR	NR
Nts-induced hypothermia	Absent	NR	Absent	NR	Absent	NR	NR
Nts-induced hypolocomotion	Diminished or Absent	NR	NR	NR	NR	NR	NR
Nts inhibition of fasting-induced refeeding	Absent	NR	NR	NR	NR	Absent	NR
Leptin-induced hypophagia	NR	NR	NR	NR	NR	#Diminished	★Opposite
Leptin-induced weight loss	NR	NR	NR	NR	NR	#Diminished	★Opposite
D-amphetamine-induced activity	NR	NR	NR	Increased	NR	NR	NR
Extracellular striatal DA	NR	NR	NR	Increased	NR	NR	NR
Striatal D2/D1 ratio	NR	NR	NR	Increased	NR	NR	NR
Prefrontal Glutamate	NR	NR	NR	Decreased	NR	NR	NR
Nts reductions in apomorphine-induced climbing	NR	NR	Absent	NR	NR	NR	NR

Figure 1.1. Differential physiological effects of ICV vs. site-specific Nts administration.
Central administration of Nts produces a wide range of physiological responses, including increases in analgesia, drinking, and hypothermia and decreases in blood pressure, locomotor activity, and feeding. Some of these effects are elicited when Nts is injected directly into specific brain regions. For example, reductions in feeding are observed when Nts is directly injected into the NTS, VMH, PVN, and VTA of rats. While decreases in locomotor activity are apparent upon administration of Nts into the NAc, centrally increased locomotor activity is observed with administration of Nts into the VTA, and this is likely due to the activation of the mesolimbic VTA DA neurons and the subsequent release of DA into the NAc that modulates motivated behaviors. In contrast, infusion of Nts directly into the NAc decreases DA release, and this is thought to be due to Nts-induced GABA release and D2R antagonism.

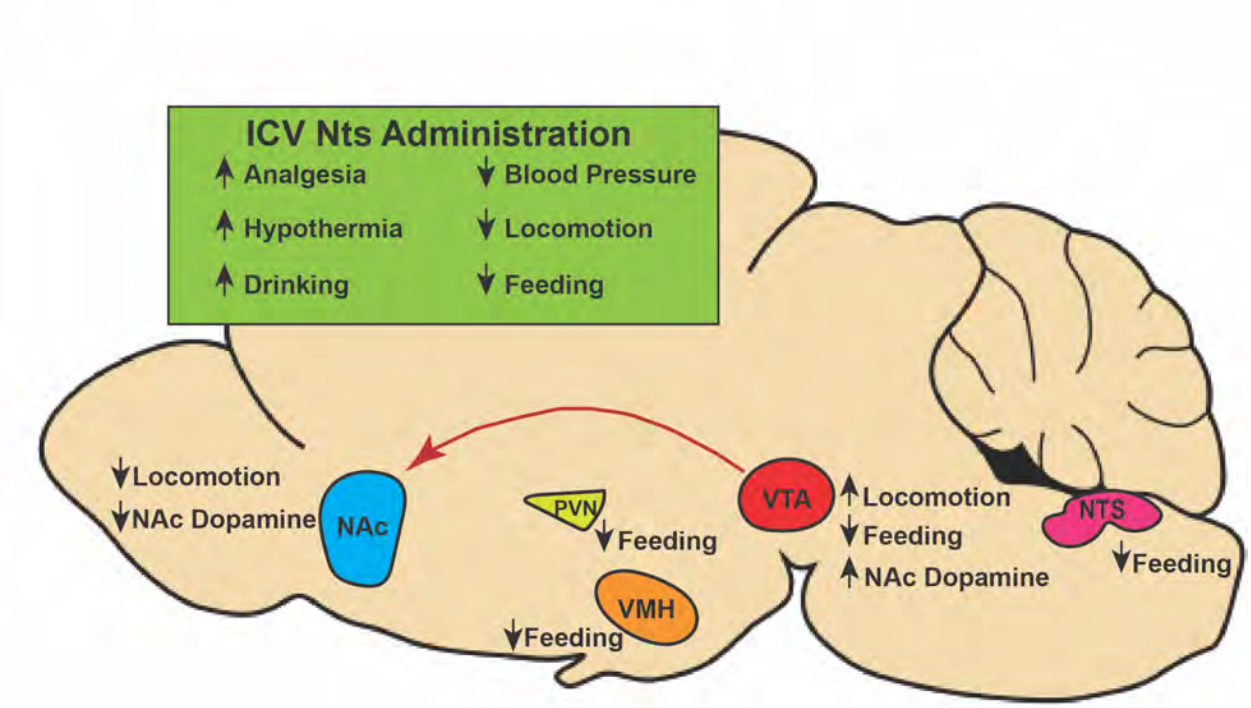


Table 1.4. Brain-wide vs Site-Specific Effects of Nts

Summary of the literature describing how pharmacologic Nts treatments within the whole brain (via ICV Nts injection) or in specific brain regions modifies physiology. – indicates no change in physiology was observed with Nts injection. NR: not reported.

	Non-specific	Site-specific Nts injection				
	ICV Nts injection	NTS	VTA	VMH	PVN	NAc
Analgesia	Increased[41]	NR	NR	NR	NR	Increased[61]
Blood Pressure	Decreased[6,46]	Decreased[52]	NR	NR	NR	NR
Hypothermia	Increased[41,47]	NR	Increased[54]	NR	NR	-[61]
Locomotor activity	Decreased[47,48]	NR	Increased[55,56]	NR	Slight Decrease[60]	Decreased[62]
Drinking	Increased[49]	NR	Decreased[57]	NR	-[60]	NR
Feeding	Decreased[48,50]	Decreased[53]	Decreased[58]	Decreased[59]	Decreased[60]	-[59]
Extracellular DA in the NAc	Increased[51]	NR	Increased[56]	NR	NR	Decreased[63]

Analgesia: Central administration of Nts or Nts agonists suppresses pain, and most evidence supports a primary role for NtsR2 in Nts-mediated analgesia. For example, Nts-mediated analgesia is blunted by the nonspecific NtsR antagonist SR142948A, but not the NtsR1-specific antagonist [9]. Nts-mediated analgesia is largely intact in NtsR1 knockout mice, but mice lacking NtsR2 have impaired thermal nociception [40–42]. Furthermore, NtsR2 selective agonists, including levocabastine, diminish a variety of pain responses in similar fashion to morphine [39], thus spurring interest in developing selective NtsR2 ligands to provide analgesia without addictive properties/dependence [39]. However, NtsR1 may also mediate certain aspects of pain signaling [64], and, hence, non-specific Nts agonists potentially hold promise for treatment of chronic pain.

Blood Pressure: Treatment with Nts causes a sustained decrease in blood pressure [6,46], which is thought to be mediated via NtsR1 [11] and may result from Nts action within the NTS [52]. Systemic treatment of rhesus monkeys with a brain penetrant Nts analog (NT69L) also induced hypotensive effects that precluded escalated dosing [65]. While this vasodepressor response dampened enthusiasm for potential clinical use of systemic Nts agonists, it remains possible that targeting certain NtsR-expressing populations within the brain might be useful to bias for specific Nts effects while bypassing hypotensive side effects. For example, since NtsR2 is not implicated in Nts-mediated hypotension, NtsR2-specific agonists may avoid any undesirable effects on blood pressure [11]. Similarly, the discovery that at least some NtsR1 forms heterodimer complexes with other receptors, such as DA receptor 2 (D2R),

suggests that medical compounds targeting particular heterodimers might be useful for directing treatment to specific NtsR1 populations, while also circumventing the hypotensive effects mediated via hindbrain NtsR1 populations [66].

Body Temperature: Central Nts signaling via NtsR1 causes hypothermia in rodents [67]. Since mice lacking NtsR1 or rodents treated with NtsR2-specific analogs do not exhibit Nts-induced hypothermic responses, NtsR2-specified agents may have promise for use in clinical pain management while at the same time circumventing hypothermic side effects [39,41]. The precise NtsR1 neurons that mediate control of body temperature have yet to be established, but their molecular signature may provide insight into designing approaches for Nts agonists so as to avoid undesired hypothermic side effects. On the other hand, given that Nts-mediated hypothermia is protective in experimental brain injury models, selectively targeting NtsR1 neurons providing this action might be useful in treating stroke or other brain injury.

Locomotor Activity: Nts either promotes or suppresses locomotor activity depending on the site of action. Systemic, intracisternal and ICV Nts [48] or NtsR1 agonist treatment decreases psychostimulant-induced locomotor activity, which is mediated, at least in part, via NtsR1 in the nucleus accumbens (NAc) [68,69]. Conversely, infusion of Nts into the VTA evokes an increase in baseline and psychostimulant-induced locomotor activity [55,56]. The differential response to the administration of Nts within the VTA vs. globally throughout the CNS may be due to the cellular location of NtsRs. For example, intra-VTA Nts directly regulates NtsR1 and DA-

expressing soma, which release DA to the NAc to modify motivated behaviors, including promoting goal-directed movement [28]. In contrast, the NAc contains D2R/NtsR1 hetero-complexes on glutamate terminals [70]; Nts acting at these receptors increases glutamate transmission, which in turn activates postsynaptic medium spiny GABA neurons. Additionally, some striatal GABA neurons may express D2R/NtsR1 complexes, permitting their direct activation. In either case, Nts in the NAc potentiates NAc GABA signaling [63,70,71], which is generally thought to suppress DA release from NAc DA terminals and diminish DA-mediated locomotor activity [70]. NAc Nts may also promote striatopallidal GABA release that facilitates inhibitory modulation of motor thalamus projections to the motor cortex, thereby decreasing locomotor activity [70,72]. While this mechanism has yet to be fully tested, it could be relevant to disease pathogenesis. Indeed, enhancement of striatopallidal GABA transmission is observed in response to NAc Nts administration or treatment with antipsychotics, and could be a common mechanism via which they protect against the psychomotor aspects of schizophrenia [72–74]. Overall, the distinct sites of NtsR1 in the VTA (directly on DA neurons) vs. the NAc (on glutamate terminals or GABA neurons) may explain why Nts in the VTA increases NAc DA release and locomotor activity while Nts in the NAc decreases DA release and locomotor activity. Since systemic Nts agonism suppresses locomotor activity, Nts potentiation of GABAergic signaling in the NAc may override Nts-induced DA release from VTA DA neurons. Thus, use of Nts agonists to target specific brain regions could be useful for treating distinct diseases. For example, NAc Nts agonists may be useful to suppress excessive psychomotor symptoms of schizophrenia,

while targeting Nts signaling to the VTA may enhance voluntary exercise, which would be useful to support weight loss [28,75].

Studies of NtsR1 knockout mice support a role for NtsR1 in regulating normal locomotor activity, although, differences in study design may have masked its importance in some cases. For example, mice lacking NtsR1 have negligible novel environment-induced locomotor activity [41] but exhibit higher baseline and psychostimulant-induced locomotor activity compared to controls when allowed to acclimate to assessment chambers [37,43,44]. Since NtsR1 knockout mice have a mild anxiety phenotype [76], the presentation of a novel environment may represent a mild stress that initially obfuscates the hyperlocomotor phenotype of these mice. NtsR1 knockout mice also exhibit increased psychostimulant-induced striatal DA release [44], which is hypothesized to enhance striatal D2R transmission and reduce striatopallidal GABA release [72]. As mentioned above, striatopallidal GABA may be important for restraint of motor cortex activity [72]. Thus, loss of NtsR1 may reduce striatal GABA release with psychostimulant administration, ultimately contributing to the hyperactive behavior observed in NtsR1-deficient mice. Similarly, psychostimulant-treatment elicits a hyperdopaminergic response in schizophrenia patients, and the degree of DA release correlates with positive symptoms. These parallels have led to the use of NtsR1 knockout mice as an animal model of schizophrenia, particularly for psychomotor measures.

Drug Addiction: Since Nts modulates the mesolimbic DA system that governs motivated intake of natural rewards (food, water), as well as pharmacological rewards (drugs of abuse), Nts may contribute to addiction. To date, Nts has primarily been studied in regulating the intake and effects of psychostimulants, including cocaine, amphetamine and nicotine [70,77–83]. As with other Nts-mediated physiology, the effects of Nts with regard to drug addiction depends upon site of action. Nts administration within the VTA mimics some effects of psychostimulant treatment, including promoting self-administration, hyperactivity and DA release to the NAc, as well as generating a conditioned place preference and locomotor sensitization [56,70,73]. Conversely, Nts in the NAc abrogates amphetamine-induced locomotion as well as "rewarding" VTA electrical self-stimulation [73,84]. At face value, these findings suggest diverging roles for Nts to promote psychostimulant reward via the VTA, or to suppress it via the NAc. However, addiction is a complex and incompletely understood process, often producing different acute and chronic adaptations to neural circuitry that have yet to be disentangled. The role of Nts in addiction is similarly complex, such that Nts-NtsR1 signaling has been contradictorily implicated in promoting drug abuse as well as in attenuating it [70,73,82,83]. Thus, while Nts is well established to engage DA signaling systems that contribute to drug intake, there is currently no consensus view on how Nts modifies the system to regulate drug seeking or addiction. Further work is needed to dissect the acute and chronic roles of Nts in drug seeking and reinstatement, and this line of research holds promise to identify novel therapeutic interventions to treat addiction.

Drinking: Central infusion of Nts or Nts_{8-13} in water-deprived rats evokes significant increases in water consumption above saline-infused controls [49]. There is also data to suggest a site-specific role for physiological Nts in drinking behavior, as ingestion of hypertonic saline upregulates Nts mRNA in a specific population of neurons within the LHA [85]. Since the LHA receives afferents from the medial preoptic area (MPO) and subfornical organ, two brain areas that relay changes in blood osmolality to other regions of the brain [86], it is tempting to speculate that LHA Nts neurons might coordinate sensation of osmotic need with Nts-mediated drinking behavior. Indeed, experimental activation of LHA Nts neurons promotes voracious water intake [87]. Nts has also been shown to amplify the effect of hypertonic saline solution on the firing rate and depolarization of supraoptic magnocellular neurosecretory cells, which act as osmosensors and respond to hyperosmotic extracellular fluid by permitting vasopressin release from the neurohypophysis [88]. Thus, Nts might promote water intake and also facilitate water retention via vasopressin release, the timing of which closely mirrors the osmotic threshold for the sensation of thirst. It remains unclear whether any specific NtsR isoform governs general drinking behavior, but both NtsR1 and NtsR2 have been implicated in mediating ethanol consumption and its effects [11]. Though NtsR1 and NtsR2 contribute to ethanol-mediated ataxia and the hypnotic effects of ethanol, respectively, and while mice deficient in either receptor display increased ethanol consumption, these effects on ingestion of ethanol may have more to do with the interaction of Nts with DA signaling and its involvement in reward-seeking behaviors and less to do with modulation of ingestive behaviors to regulate osmolality [11].

Feeding and Body Weight: Systemic Nts treatment mildly suppresses homeostatic feeding in mice, particularly during the dark phase when they are most hungry and consume the bulk of their daily food. Repeated systemic Nts treatments did not result in significant long-term suppression of feeding [48]; however, this may be due to the fact that the normal weight animals in this study had minimal body fat to lose, and, hence, any weight loss presumably caused a homeostatic counter-response to ensure sufficient energy balance for survival. Nts may exert a stronger anorectic effect in the face of increased motivation. Indeed, in rodents that are hungry, due to either fasting [43,50] or due to having an increased appetitive drive that accompanies obesity [67], central Nts or NtsR1 agonists restrain feeding. Pharmacological data and genetic knockout mouse studies indicate NtsR1 as the principal mediator of the anorectic action of Nts [41,43], and this is further bolstered by the fact that Nts-induced suppression of feeding is absent in mice lacking NtsR1[87]. Studies of different strains of NtsR1 knockout mice, however, have produced differing conclusions about the necessity of NtsR1 for regulation of homeostatic feeding and body weight **(Table 1.3).** Some strains of NtsR1 knockout mice exhibit mild hyperphagia for chow that leads to modestly increased body weight as mice age as well as increased basal body temperature [41,43]. Given the mild stress and hyperlocomotor phenotype of mice lacking NtsR1, it is possible that these mice might eat modestly more food to support their elevated physical activity. By contrast, the commercially available strain of NtsR1 knockout mice from Jackson Labs (originally developed by Deltagen, USA) display opposing consumatory behaviors: they eat less chow than littermate controls, but overconsume palatable (e.g. rewarding) high fat/high sucrose food and exhibit increased sucrose-

preference, both of which promote weight gain [37]. These findings imply that while NtsR1 plays a subtle role in the homeostatic maintenance of food intake [37], it is perhaps more crucial for restraining motivated consumption of tasty, calorically dense foods [87]. Since the obesity epidemic stems, in part, from overconsumption of calorie-dense foods, modulation of the Nts/NtsR1 system may hold promise to restrain food intake and support healthy body weight.

Anorectic leptin signaling also depends, in part, on Nts action via NtsR1, and these systems converge to support weight loss. Both central Nts and leptin reduce fasting-induced re-feeding in control mice, but these signals fail to suppress feeding in mice lacking NtsR1 [43] or in rats pre-treated with reagents to block NtsR1 signaling [89], indicating that Nts/NtsR1 signaling may be required for leptin-mediated anorexia. Acute leptin treatment elevates hypothalamic Nts expression, and the absence of this effect in pair-fed control rats indicates that this expression is specifically induced by leptin (not just weight loss) [90]. However, elevated Nts expression and feeding restraint both diminish after chronic leptin treatment [89], which may replicate the hyperleptinemic state of obese individuals who seemingly no longer respond to the appetite-suppressing effects of the hormone. This fluctuation in Nts gene expression is similar to the change in hypothalamic POMC expression, and reduced expression of these two anorectic neuropeptides may partly explain the acquired “leptin resistance” that occurs in obesity [89]. Overall, the functional overlap of the Nts and leptin systems indicate that they must also overlap anatomically and that there might be site-specified Nts/NtsR1 circuits that regulate feeding and body weight.

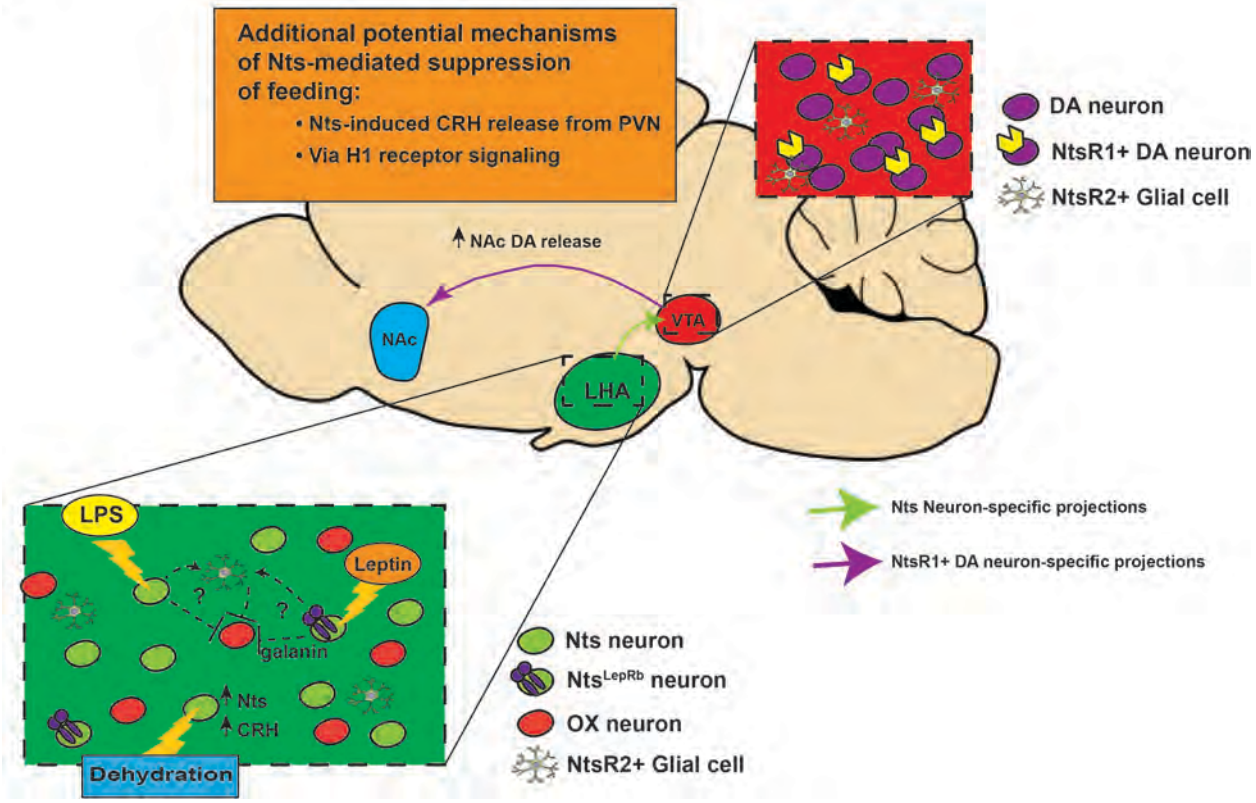
Specific Nts Circuits Implicated In Feeding

Nts injection into select regions of the brain suppresses feeding [53,58–60] **(described in Figure 1.1 and Table 1.4)**. Notably, Nts does not alter feeding if infused into the NAc, where it is known to suppress psychomotor responses [59,62]; thus, Nts suppresses feeding and locomotor activity via distinct circuits, supporting the idea that it may be possible to differentially regulate these actions with application of Nts agonists in a site-specific manner. For example, Nts acts within the paraventricular hypothalamic nucleus (PVN) to suppress feeding [53] and regulate corticotropin-releasing hormone (CRH) expression and release, though it is not clear if CRH is required for the anorexia elicited by Nts at this site [91]. Nts neurons within the dorsomedial hypothalamus project to the PVN, and leptin activation of these neurons may promote endogenous Nts release to the PVN [92]. Given that leptin also induces expression of Nts [90] and CRH [93], and that at least some of its anorectic effect is dependent on Nts and CRH signaling [37,93], it is possible that leptin, Nts and CRH act in concert to suppress feeding at the level of the PVN. Nts may also act within the ventromedial hypothalamic nucleus (VMH) to curb unnecessary food intake, since obese rats, but not their lean counterparts, display a 50% reduction in VMH Nts with fasting [94]; the mechanism by which this occurs, however, is unknown. Nts also suppresses feeding when injected into the NTS [53]. Endogenous Nts may be released from local Nts-expressing NTS neurons; yet, the resident Nts neurons are not regulated by anorectic signals, so it remains unclear if and how they mediate anorectic effects [95,96]. Additionally, Nts may exert its anorectic effect via the histamine signaling pathway, since pharmacological or genetic disruption of the H_1 receptor blunts Nts-mediated

suppression of feeding [97]. These few studies hint at functional sites and mechanisms by which Nts exerts anorexia, but more work is necessary to fully appreciate their contributions.

By comparison, there is a far greater understanding of how Nts acts in the VTA, where it engages the mesolimbic DA system to suppress feeding. The VTA is primarily composed of DA-producing neurons that release DA into the ventral striatum/NAc (the mesolimbic system) or the prefrontal cortex (the mesocortical system). Nts administration specifically into the VTA also induces DA release into the ventral striatum, increases locomotor activity [56] and suppresses food intake in the contexts of either fasting or training that heightens the motivation to consume food [57,58]. These data suggest that Nts may selectively engage the mesolimbic DA circuit, and the motivated behaviors it regulates, more so than the mesocortical DA circuit. Recent anatomical evidence supports this idea, since NtsR1 is expressed on a subset of DA neurons within the VTA that specifically project to the ventral striatum **(Figure 1.2)** [28,37]. Nts acts via NtsR1 to increase the activation of VTA DA neurons and elicit DA release into the NAc [56,98], where DA release is known to regulate both feeding and locomotor activity. Very few VTA DA neurons express NtsR2; hence, NtsR1 is the primary receptor isoform by which Nts directly modifies the activity of VTA DA neurons [28]. However, there are many NtsR2-expressing astrocytes within the VTA, so it is entirely possible that Nts might indirectly alter DA signaling through signaling in astrocytes [28]. Nts acting directly via VTA NtsR1 increases the activity of DA neurons via several mechanisms,

Figure 1.2. Mechanisms of Nts-mediated suppression of feeding.
Nts induces reductions in feeding via multiple mechanisms. Within the VTA, a portion of DA neurons express NtsR and respond to Nts with release of DA into the ventral striatum (e.g. NAc). The LHA, which contains a substantial number of Nts neurons, provides a source of Nts to the VTA. This LHA Nts → VTA circuit likely contributes to the anorectic response elicited by promoting NAc DA release. LHA Nts neurons additionally contribute to reductions in feeding via other mechanisms. LHA Nts neurons have been shown to hyperpolarize OX neurons in response to direct stimulation as well as in response to either leptin or LPS treatment. Though Nts^{LepRb} neurons respond to leptin to hyperpolarize OX neurons, they likely do so through a Nts-independent mechanism as OX neuron do not express NtsR isoforms. Nts^{LepRb} neurons additionally express the neuropeptide Galanin, which has also been demonstrated to hyperpolarize OX neurons. Since glia robustly express NtsR1, it is possible that Nts^{LepRb} neurons act through astrocytes in the LHA to inhibit OX neurons. LPS induces activation of LHA Nts neurons, and subsequent reductions in feeding are likely mediated through similar mechanisms as described for leptin since OX neuronal activity is decreased with LPS administration. Finally, CRH gene expression in LHA Nts neurons correlates with the degree of anorexia that accompanies dehydration and these CRH-expressing Nts neurons are thought to coordinate feeding with hydration status. Outside of the population of LHA Nts neurons, release of anorectic CRH from the PVN has been demonstrated to rely upon Nts action, and ICV Nts-induced decreases in feeding are mediated in part via H_1 receptor signaling.



including attenuating D2R auto-inhibition via NtsR1/D2R hetero-complexes, inhibiting IPSCs induced via D2R and $GABA_B$ receptors and activating a nonselective inward cation current [99]. However, the source of Nts input to the VTA that activates DA neurons and mediates anorectic actions via this circuit has yet to be defined. Rats have Nts-containing soma within the VTA that may release DA locally, but the paucity of Nts soma in mice suggests that VTA-acting Nts must originate from other regions [16,18,22].

Nts neurons within the lateral hypothalamic area (LHA) are a potential source of the Nts that mediates anorectic actions via the VTA, and the many cues that induce anorectic LHA Nts neuron signaling are depicted in **Figure 1.2.** LHA Nts neurons densely project to the VTA and SN [37,75], and the overlap of LHA Nts neurons with known anorectic systems suggests that the LHA Nts → VTA circuit may contribute to Nts-mediated anorexia. Indeed, 15-30% of all LHA Nts neurons co-express the long form of the leptin receptor (LepRb) [100,101], functionally linking the anorectic Nts and leptin systems. While LepRb and Nts are separately expressed in other sites throughout the brain, their expression only overlaps within the LHA, indicating that Nts^{LepRb} neurons are the unique joint mediators of Nts and leptin action. If this is true, then disruption of either leptin or Nts signaling via these neurons should disrupt VTA DA action and physiology. Indeed, loss of leptin signaling via the Nts^{LepRb} neurons mildly increases feeding at early ages, diminishes locomotor activity and disrupts mesolimbic DA signaling, leading to increased adiposity and weight gain [100]. Similarly, the anorectic effects of circulating leptin are abrogated in the face of systemic NtsR1 antagonism or in NtsR1 knockout mice [43,89], and specific leptin-mediated activation

of the LHA Nts^{LepRb} neurons in mice lacking NtsR1 caused them to eat more food, decreased VTA tyrosine hydroxylase expression and increased body weight [37]. Leptin-deficient *ob/ob* mice also have diminished VTA tyrosine hydroxylase and exhibit hyperphagia, both of which are resolved with leptin treatment [100]. This effect of leptin treatment is likely mediated, at least in part, via the leptin-sensitive Nts^{LepRb} neurons that engage VTA NtsR1 neurons. Thus, at least some portion of the anorectic function of leptin and VTA Nts signaling is mediated via LHA Nts^{LepRb} neurons and NtsR1-expressing neurons, presumably those in the VTA. However, given that most Nts neurons in the LHA do not express LepRb, there may be other mechanisms via which LHA Nts neurons coordinate anorectic actions via the VTA or other projection sites. Activating the general population of LHA Nts neurons, the majority of which do not express LepRb, induces Nts release to the VTA as well as locomotor activity and metabolic rate, but suppresses feeding leading to weight loss [75,87]. Since antagonizing NtsR1 or D1R blocks the feeding suppression elicited with LHA Nts neuron activation, it is likely that there is a functional link of LHA Nts neurons with Nts- and mesolimbic-dependent control of feeding behaviors [87].

LHA Nts neurons may suppress feeding via another mechanism: local projections onto neurons expressing Orexin/Hypocretin (OX). OX neurons promote food intake and locomotor activity to support arousal [102], hence, acute inhibition of OX neurons may suppress feeding behavior. Leptin or experimental activation of LHA Nts neurons hyperpolarizes OX neurons, although this occurs independent of Nts signaling [102]. Given that adult OX neurons do not express NtsR1 [37], and that NtsR2 is

primarily expressed by glia [27,28], any Nts released locally into the LHA is unlikely to directly regulate OX neurons. Nts could presumably act via NtsR2-expressing glia to indirectly modify the activity of OX neurons. Alternately, LHA Nts neurons may release other signals that inhibit OX neurons, such as galanin [102]. While Nts released from LHA Nts neurons may not directly suppress the activity of OX neurons, the interconnectivity of these neuronal populations is important for normal regulation of feeding and body weight. For example, mice genetically lacking LepRb in LHA Nts^{LepRb} neurons do not exhibit appropriate regulation of OX neuronal activity in response to fasting, leptin or ghrelin, and, hence, cannot adapt to alter feeding in response to changes in peripheral energy balance [100,101].

LHA Nts neurons are also regulated by other signals that convey energy status and lead to changes in feeding accordingly. For example, treatment with lipopolysaccharide (LPS) leads to the activation of LHA Nts neurons, which in turn inhibit downstream OX neurons [103]. Inflammatory activation of the LHA Nts → OX circuit may serve to suppress OX-mediated arousal, producing lethargy necessary to minimize energy expenditure when mammals are sick. LHA Nts neurons are also activated during dehydration-anorexia, a state in which dehydrated animals cease eating until they have restored serum osmolality via drinking water [85]. In rats, dehydration-anorexia causes increased synthesis of Nts and CRH within the same LHA neurons [85]. These data suggest LHA Nts neurons may coordinate the need for water and food but prioritize water seeking above feeding until normal cellular osmolality is restored. While LHA Nts neurons may be activated by diverse physiologic cues (leptin,

inflammatory cues, dehydration), all of these signals are known to suppress feeding, consistent with the central role of Nts as an anorectic peptide.

Much remains to be understood concerning precisely how Nts neurons control feeding, but these collective data confirm that central Nts signaling is important for the normal physiological processes of feeding and energy balance. It therefore stands to reason that any disruptions of Nts signaling present in disease states will also derange feeding behavior and body weight. Altered Nts signaling is specifically implicated in the pathophysiology of schizophrenia, Parkinson's disease, eating disorders and obesity, and hence, may contribute to the altered feeding and energy balance associated with these diseases.

Nts and Schizophrenia

Hyperactivity of the mesolimbic DA system and resulting elevations in striatal DA contribute to psychosis, a defining feature of schizophrenia [11,71]. Since Nts attenuates DA signaling via actions in the NAc, loss of Nts action via this site might promote a hyperdopaminergic state that contributes to the pathogenesis of schizophrenia. Indeed, Nts is reduced in the cerebrospinal fluid (CSF) of untreated schizophrenic patients, and Nts levels correlate with disease severity, such that individuals with higher drug-free Nts levels have with fewer behavioral deficits [71]. By contrast, treatment with typical and atypical antipsychotic drugs elevates striatal Nts [71] and blunts DA-mediated locomotion similar to that evoked by administration of Nts into the NAc. Nts may act via NtsR1 expressed on either NAc glutamatergic terminals or cell bodies and dendrites of NAc medium spiny GABA neurons [70,71], where Nts action at NtsR1-D2R heterodimers might support GABA release and, consequently, inhibit striatal DA release [71]. Similar to antipsychotics, Nts in the NAc may also increase striatopallidal GABA, which is thought to restore thalamocortical glutamatergic signaling and attenuate psychomotor effects [74]. Thus, Nts and antipsychotics may act similarly at the level of the NAc to suppress excessive DA-signaling and hyperdopaminergic psychosis.

Given that Nts action via NtsR1 may be required for normal DAergic tone, loss of action via NtsR1 might promote development of schizophrenia. NtsR1 knockout mice have therefore been studied as a potential model for this disease. Indeed, NtsR1 knockout mice exhibit excess striatal DA, typical of human schizophrenia, as well as

altered striatal expression of D1R and D2R [44]. A possible explanation for these effects is that NAc NtsR1 is necessary to restrain striatal DA release (via mechanisms discussed earlier). If this is true, then loss of NtsR1 might lead to unchecked striatal DA signaling that promotes psychomotor dysfunction. Yet, NtsR1 knockout mice also lack NtsR1-expression on the VTA DA neurons that release DA to the NAc, which would be expected to result in diminished DA signaling. Given that Nts promotes distinct actions via the NAc and VTA, it is possible that loss of Nts-NtsR1 action also has site-specific effects. Hence, loss of Nts-NtsR1 action via the NAc may be the primary driver of the hyperdopaminergic state characteristic of schizophrenia, whereas loss of NtsR1-driven VTA DA signaling might not be pathogenetic. Such mechanisms have yet to be fully tested but could explain the behavioral disruptions observed in NtsR1 knockout mice that are pathogenomic of schizophrenia, including increased psychostimulant-induced locomotor activity and avolition (e.g. a lack of motivation to do tasks that have an end goal) [11]. Polymorphisms in the NtsR1 gene have also been identified in human individuals with schizophrenia; however, it remains unknown whether these polymorphisms result in altered Nts binding properties or function [71]. Thus, while there is some data linking genetic disruption of Nts signaling with schizophrenia, further work is required to determine whether it indeed contributes to disease onset.

Individuals with schizophrenia have a higher prevalence of obesity and type-2 diabetes compared to the general population, suggesting some pathogenetic overlap with the systems that control body weight [104]. Furthermore, some antipsychotic medications, particularly olanzapine and clozapine [104], promote weight gain, which

can spur patient non-compliance in taking these medications and, as a result, relapse of the psychotic effects. Antipsychotics may contribute to weight gain via a number of mechanisms, some of which may be thwarted by Nts signaling. First, antipsychotics antagonize hypothalamic serotonin 5-HT2C and histamine H_1 receptors, and antagonism of these two receptors induces feeding [105]. In contrast, Nts agonism of histamine H_1 signaling is thought to contribute to its anorectic action [97]. Secondly, antipsychotics increase OX expression in rodents [106], which promotes arousal and feeding that may lead to weight gain. By contrast, Nts neurons inhibit OX neurons, thereby negating their feeding-promoting effects [102]. Thus, while Nts and antipsychotics behave similarly by suppressing DA-dependent locomotion, they exert opposing effects on regulation of food intake. Going forward, it will be important to assess whether Nts agonism may have efficacy for treating the psychomotor and metabolic symptoms of schizophrenia.

Antipsychotic-mediated antagonism of DA signaling and Nts action may mechanistically converge in their ability to modify the motivational salience of food (how much it is “wanted”) but not its hedonic value (how much it is “liked”) [107]. D2R antagonists such as raclopride decrease intra-accumbal DA and effort-related responding for palatable foods, but do not alter consumption of freely available standard chow. Similarly, Nts signaling more effectively suppresses fasting-induced or motivated feeding compared to homeostatic feeding [87]. Thus, D2R antagonism by antipsychotics may dampen the VTA DA-mediated “wanting” of highly pleasurable foods, but are unlikely to alter the “liking” of foods that is regulated via separate circuits. This

is consistent with reports that schizophrenic patients on antipsychotics rate all foods types, including those deemed less-appetitive according to healthy controls, as having high hedonic value [108], and this food “liking” might potentiate their feeding and weight gain. Nts signaling is also required to suppress intake of highly palatable foods [37], and the VTA NtsR1 neurons that project to the NAc may be a common node by which Nts and antipsychotics modify motivated feeding behaviors. It is possible that Nts acting via VTA NtsR1/D2R hetero-receptor complexes might exert NtsR1-mediated anorectic actions while blocking D2R-mediated psychomotor effects, without antagonizing D2R-mediated feeding control. The recent report of bivalent compounds that selectively target these NtsR1-D2R hetero-complexes while biasing for NtsR1-mediated signaling suggests future potential to selectively target this system in schizophrenic patients [109], which would, perhaps, blunt associated psychomotor effects while restraining feeding. Since weight gain is a major reason for medication noncompliance among schizophrenic patients, such drugs could be a useful alternative to stand-alone antipsychotics that produce this and other undesirable side effects.

Nts and Parkinson's Disease

Parkinson's Disease (PD) is a neurodegenerative disorder characterized by the progressive loss of nigrostriatal DA neurons and is associated with symptoms of disordered movement, such as tremor, muscular rigidity, and bradykinesia. Many NtsR1-expressing neurons are found within the SN [30,31], and since approximately half co-express DA [37], there may be an overlapping mechanism for Nts and DA action in PD. This may explain why brains of PD patients have diminished nigrostriatal DA neurons along with decreased Nts binding and NtsR1 mRNA within the SN [110,111]. While PD is typically characterized by the motor impairments that ensue in later stages of the disease, pre-diagnostic PD patients display deviations in body weight [112]. The cause of altered energy balance may differ over the course of the disease. Initial body weight deviations in PD could result from disruptions in the DA-mediated regulation of feeding, as these changes are observable in PD patients prior to the development of motor symptoms [112]. In patients with symptomatic PD, autonomic dysfunction results from alpha-synucleinopathy within the enteric nervous system, which leads to the dysphagia, gastroparesis, constipation, nausea, and mal-absorption that may ultimately contribute to weight loss [112]. During later stages of PD, the rigidity and tremor elevates patients' resting energy expenditure, which may exacerbate weight loss [112]. Curiously, non-medicated PD patients exhibit elevated plasma Nts compared to healthy controls and levodopa-treated PD patients [110,111], and the SN of PD patient brains have higher Nts levels [111]. This elevation in Nts may be compensatory and may reflect an Nts-driven enhancement of midbrain DAergic signaling to offset the diminished signaling resulting from loss of SN DA neurons. Based on this reasoning,

Nts analogs could potentially be used as anti-Parkinsonian drugs to stimulate DA signaling via any remaining NtsR1-expressing SN DA neurons. Indeed, in rodent models of PD, Nts reduces muscular rigidity and tremor [111]. Use of NtsR1 antagonists have also been considered for use as therapeutic agents due to the fact that striatal Nts-NtsR1 signaling decreases striatal DA via suppression of D2R receptors. However, no improvement in motor symptoms was observed in PD patients receiving NtsR1 antagonists, though the study may have been underpowered to detect significant effects [111,113]. While Nts signaling is altered by PD, it is yet unknown whether it contributes to the development of the disease or whether it is simply a consequence of disrupted DA neurons.

Nts and Obesity

Central Nts expression is diminished in obese rodents compared to normal weight controls, suggesting that loss of Nts signaling might contribute to pathogenesis of obesity [94,114–117]. For instance, significant reductions in Nts concentration have been detected within the VMH of obese Zucker rats when fasted, whereas fasting did not diminish VMH Nts in lean counterparts [94]. In addition, Nts levels are specifically decreased within the LHA of rats fed a high-fat diet as well as mice that are obese due to genetic loss of leptin expression, emphasizing the potential overlap of signaling via Nts^{LepRb} neurons for regulating feeding and body weight [94,114–116]. Based on these data, it has been hypothesized that enhancing Nts signaling might support weight loss in obesity. Indeed, 10 days of systemic treatment with brain-penetrant NtsR1 agonists curbs food intake and promotes weight loss in genetically obese rodents, including leptin-deficient *ob/ob* mice [67] and LepRb-deficient Zucker rats [118]. Importantly, the appetite-suppressing effects of systemically-administered Nts agonist PD149163 persisted over the entire treatment period, while the initial hypothermia and suppressive locomotor effects were normalized by the end of the study. Unfortunately, the effect of sustained treatment on blood pressure was not reported [67]. At the least, these data suggest that sustained Nts agonism might support weight loss without long-term side effects on body temperature and metabolism.

Disruption of the DA system is linked with obesity; thus, modulating Nts systems engaging mesolimbic DA circuits might conceivably restore disrupted DA action to support weight loss. The precise nature of the DA system deficit in obesity has been

debated, but one hypothesis suggests that diminished DA signaling promotes weight gain. In support of this, obese rodents exhibit reduced striatal and hypothalamic D2R binding, and obesity-prone rodents exhibit reduced D1R expression, low basal levels of DA within the NAc and diminished DA release to the striatum and prefrontal cortex [119]. Humans harboring the Taq1A polymorphism of the D2R gene also have reduced D2R binding sites [119], and, in general, striatal D2R binding density negatively correlates with BMI in obese persons [119]. Together, these data support a pathogenetic role for a hypo-functioning DA system in obesity. Thus, it is not hard to imagine that Nts agonists administered in the VTA, which have been demonstrated to elicit elevations in striatal DA, might be useful to normalize reduced DA signaling [56,75].

Peripheral sources of Nts may also factor into the pathogenesis of obesity. Under normal circumstances, ingestion of food elevates Nts plasma levels [120], and while the majority of this peripheral pool of Nts does not access the brain, a limited amount may access some brain regions to exert anorectic feedback [15]. However, individuals who are obese due to lifestyle or Prader-Willi syndrome (a genetic disease characterized by severe hyperphagia and childhood obesity) have elevated circulating pro-Nts [5,121]. It is yet unclear whether the pro-Nts in these individuals is processed to the active form; hence, elevated levels of pro-Nts might reflect diminished Nts function. Indeed, variants in the gene for carboxypeptidase E (an enzyme responsible for removal of Lys-Arg dibasic residues in the pro-Nts precursor), are significantly associated with BMI, and mice with nonfunctional carboxypeptidase E have impaired pro-Nts processing and reduced levels of mature Nts compared to controls [122]. Thus, future work will be

required to determine whether low or elevated levels of mature, functional Nts correlate with likelihood of obesity. Persistently elevated circulating Nts levels, however, could represent a compensatory effort to enhance the minimal amount of circulatory Nts that reaches brain structures with a permissive or absent BBB, so as to mediate some anorectic function [121]. Consistent with this idea, Roux-en-Y Gastric Bypass surgery elevates plasma Nts levels relative to sham-operated rats, which contributes to their reduced food intake via an NtsR-dependent mechanism [15]. Peripheral Nts has been shown to increase neuronal activation in several brain regions implicated in the homeostatic regulation of food intake, including the arcuate nucleus, PVN, and NTS [15]. Thus, elevated peripheral Nts in gastric bypass patients may act at these feeding centers to promote reduced feeding. Vagal afferents may additionally be necessary for peripheral Nts to elicit a central anorectic effect [15]. Since morbidly obese individuals also exhibit increased levels of plasma Nts following gastric bypass surgery [13], this may be a beneficial adaptation to enhance some central Nts signaling and reduce appetite.

Nts and Eating Disorders

The sexually dimorphic expression of Nts in the brain suggests that Nts signaling may contribute to the noted differences in feeding regulation between males and females, and, perhaps, to the pathogenesis of female-prevalent eating disorders. Nts/Neuromedin are expressed within the MPO and the anteroventral periventricular nucleus (AVPe), though females have 4 times the number of Nts-expressing cells in the AVPe compared to males [123]. Furthermore, estrogen promotes Nts gene expression in the MPO of female rodents [124,125], and Nts expression levels fluctuate in accordance with plasma estradiol during the estrous cycle [123]. Given that central estradiol suppresses feeding in part via actions in the MPO [126] and that expression of Nts in this brain region is regulated by estradiol [124], the anorectic functions of estradiol and Nts may be mechanistically intertwined. Furthermore, increases in levels of sex steroid hormones that occur during puberty, including estrogen, result in gene expression changes that promote development of eating disorders, such as anorexia nervosa (AN) and bulimia nervosa, in genetically predisposed individuals [127]. This is evidenced by twin studies showing that genetic effects on disordered eating increase throughout puberty, a time period in which ovarian hormones, like estradiol, drive developmental changes [127]. Alterations in gene transcription that result from elevated estradiol during puberty can moderate food intake, and disordered eating, assessed via the Minnesota Eating Behavior Survey (MEBS), correlates with plasma estradiol levels in females [127]. Together these data suggest that dysfunction of estrogen and Nts-mediated signaling may potentiate the development of eating disorders.

There are a number of Nts-influenced mechanisms implicated in appetite suppression that may contribute to development of eating disorders. For example, the parabrachial nucleus (PB) → Central Amygdala (CeA) neural circuit has been elegantly tested and shown to coordinate anorexigenic signals with suppression of food intake [128]. Since some PB Nts neurons send fibers to the CeA, Nts might mediate some of the anorectic function of this circuit [129].–Amygdala dysfunction is specifically implicated in eating disorders, and given the dense Nts inputs to this region and the sizeable population of Nts neurons within the amygdala, altered amygdala Nts signaling could be a contributing factor. Indeed, the amygdala of non-recovered AN patients contains significantly reduced grey matter volume relative to weight-restored AN patients and healthy weight controls [130]. Additionally, estradiol heightens the anorectic response to the satiety cue CCK via a NTS → lateral PB → CeA circuit [131,132]. Since this pathway contains Nts projections and conveys degree of satiety in an estradiol-dependent manner, Nts may contribute to the pathogenesis of eating disorders. Additionally, Nts may exert central anorectic effects in part through Histamine H_1R [97], which is expressed within the amygdala. Consistent with the female prevalence of eating disorders, females have higher histamine H_1R densities compared to males, and females with the restricting subtype of AN have further elevated levels compared to normal weight female controls [133]. Thus, it is conceivable that Nts, which exerts its central anorectic effects in part through Histamine H_1R [97], could elicit a greater degree of anorexia in both females and AN patients through this enhanced central histaminergic system. Additionally, plasma activity levels of prolyl endopeptidase are significantly lower in females with either the restricting or

binging subtypes of AN and in females with bulimia nervosa compared to healthy controls [134]. Prolyl endopeptidases are known to degrade Nts, and their prevalence within the brain make these enzymes likely regulators of Nts levels. Thus, based on the likely low degradation of central Nts by prolyl endopeptidases and the measured increases in CNS histamine H_1R densities in eating disorder patients, one might expect heightened Nts-mediated anorectic action in these individuals.

Nts signaling is well established to regulate the mesolimbic DA system, but this system is hyperactive in AN patients, especially upon viewing images of underweight women [135]. Hyperactivity of the ventral striatum has also been shown to occur in recovered AN patients in response to the taste of chocolate, a highly palatable substance [136]. This heightened salience to both highly palatable food as well as underweight stimuli may explain why these individuals exhibit restraint and avoidance behaviors with regard to food [136]. Aberrant DA signaling in the ventral striatum is likely in these patients, as increased D2/D3 R binding densities are present in the ventral striatum [135], and reduced levels of the DA metabolite Homovanillic acid (HVA) have been measured in the CSF of AN-recovered individuals [135]. Alterations in DA-based reward circuits are not surprising, as AN individuals are essentially addicted to food restriction and exercise [137]. This link between reduced food consumption and excessive exercise has been observed in the activity-based anorexia (ABA) model, in which rats that have a limited time to feed exhibit increases in wheel running activity [137]. This behavior has been attributed to food-anticipatory activity, which is the increased food-seeking behavior and, thus, increased activity that occurs when food

access is limited [137]. Interestingly, when rats subjected to the ABA model are treated with a nonselective DA antagonist, activity levels are reduced, which is similarly observed in *ad lib* fed controls; however, food intake is increased only in the food-restricted rats [138]. *Thus, one could argue that hyperactive Nts signaling to VTA DA neurons, driving DA release in the NAc, might contribute to the pathogenesis of AN.* Given that intra-VTA Nts restrains feeding and increases locomotor activity, behaviors that become maladaptive in AN, altered Nts regulation of VTA DA signaling could explain some of the core behavioral features of restrictive eating disorders.

Loss of Nts action may also promote a hyper-DAergic state that potentiates eating disorders. Mice lacking NtsR1 have a hyperactive DA system along with disrupted feeding and increased DA-mediated locomotor activity [37,44], features similar to human AN. Likewise, administration of the NtsR1 antagonist SR 48692 specifically to the striatum potentiates DA-induced hyperlocomotion and stereotypic behaviors [139], likely due to loss of the suppressive action of Nts on D2R signaling. One can reason, therefore, that striatal Nts is necessary to modulate and restrain the hyperlocomotor and stereotypy behaviors that striatal DA elicits. In this way, loss of Nts signaling within the striatum would be expected to contribute to the hyperlocomotor, stereotypic, and food anticipatory behaviors that are observed in rodent models and mimic core features of human AN. Loss of Nts or NtsR1 signaling may have face validity for human disease, as loss of function variants in multiple genes within the Nts signaling pathway have recently been found to be enriched in individuals with eating

disorders [140]. Going forward it will be important to verify whether loss of function or enhanced signaling via the Nts system contributes to the development of AN.

Specific Aims

Incidence rates of eating disorders, such as anorexia and bulimia nervosa, have increased as has mortality due to all eating disorders [141]. Unfortunately, there are no FDA approved medications to restore body weight. While antidepressant and antipsychotic medications are currently used to treat AN, they do not significantly improve feeding and body mass index compared to placebo [111]. Identification of medications to help restore normal feeding and body weight in individuals suffering with eating disorders has been limited by an incomplete understanding of how the brain regulates feeding. Thus, there is a crucial need to elucidate the neural signals that regulate feeding to direct discovery of interventions to treat eating disorders.

The objective of this dissertation was to examine the role of Neurotensin (Nts) in the development of eating disorders, specifically Anorexia Nervosa (AN). A large body of work describes Nts as an anorectic neuropeptide, hence it is reasonable that Nts signaling might contribute to the feeding suppression that is a core feature of AN. Evaluating the role of Nts in AN, however, has been hindered by the inability to detect Nts neurons that might contribute to energy balance, or to systematically modulate them to reveal their contributions to behavior and body weight. Overcoming this obstacle was a necessary first step to appraising which Nts populations might contribute to AN.

It is also important to understand if Nts signaling is required for proper energy balance. Nts exerts its anorectic actions via binding to the G_q-protein coupled receptor NtsR1, which is widely expressed during development with more restricted expression

in the adult brain [142]. While the specific Nts and NtsR1 neurons that modulate feeding remain to be wholly identified throughout the brain, loss-of-function mutations in Nts and NtsR1 have recently been observed in a subset of female individuals with eating disorders [140]. These data hint that loss of action via Nts-NtsR1 might increase vulnerability to develop AN, and this could be exacerbated by sex and environmental factors known to increase risk for the disorder. Mice null for NtsR1 offer an ideal model to test this possibility, but only male NtsR1 null mice have been studied to date. Given that AN is ~10X more prevalent in females than males, it is possible that a female sex X NtsR1 interaction may occur to contribute to AN, which would have been missed in prior male-only studies.

Alternately, some data suggest that gain of function via the Nts-NtsR1 circuit could contribute to behaviors typical of AN. Notably, NtsR1 is expressed by a subset of VTA DA neurons that modify the motivation to eat and move, and Nts administration into the VTA decreases food consumption [57] while concurrently increasing DA release and locomotor activity [143]. *Since these dual behaviors can potentiate weight loss, enhanced action via this circuit could conceivably contribute to the anorexia and low body weight of AN.* A major source of input to VTA NtsR1 neurons arises from Nts neurons in the LHA, and experimental activation of LHA Nts neurons elicits release of Nts to the VTA, uncouples feeding and energy expenditure, and promotes weight loss [75,87]. Taken together, these data suggest that increased activity of LHA Nts neurons in the established brain could contribute to the development of behaviors associated with AN. Yet, the specific inputs to LHA Nts neurons that might drive endogenous

activation were unknown, and it was unclear if afferent strength to LHA Nts neurons is specifically altered in AN. Given that individuals with eating disorders also display increased fiber density in the LHA (unpublished data, Prevot, Annual Meeting of the Endocrine Society, 2015), it is plausible that enhanced afferent modulation of LHA Nts neurons might contribute to the disorder. In fact, the LHA receives inputs from top-down control centers of the brain that have been implicated in AN, such as the anterior insula [144]. The insula also projects to the central amygdala (CEA), a region that contains neurons that inhibit feeding in response to anorexigenic signals [128,145], and the CEA in turn projects to the LHA [146]. We therefore hypothesized that over-activation of LHA circuits, perhaps via increased afferents from top-down control centers such as the insula and CEA, could mediate increased drive of LHA Nts signaling to alter feeding. However, the prior lack of reliable methods to detect Nts neurons had also prevented determination of their afferents, as needed to evaluate this hypothesis.

Collectively, these data informed my **central hypothesis: Nts neurons in feeding centers are specifically regulated by top-down control centers implicated in AN**, **and altered Nts signaling disrupts feeding and body weight to promote AN-like behaviors.** I explored this hypothesis via the following aims:

Aim 1: Identify populations of Nts neurons predicted to modulate feeding behavior: Since Nts has anorectic effects, I hypothesized that Nts neurons would be enriched within brain centers known to control feeding. I therefore crossed a Nts^{Cre} mouse with a Cre-inducible GFP reporter mouse, in which a loxP-flanked transcriptional

blocking cassette is followed by GFP, to create *Nts^{Cre};GFP* mice. This permitted visualization of Nts neurons and allowed for mapping of their location and density throughout the brain. This revealed the presence of Nts neurons in feeding centers and other brain regions implicated in eating disorders, specifically **Anorexia Nervosa (AN)**. Comparisons of Nts-GFP populations in the *Nts^{Cre};GFP* mouse to *Nts*-ISH from the Allen Brain Atlas additionally revealed a few sites in which GFP was expressed but *Nts*-ISH was lacking, suggesting that Nts may be expressed in a developmentally-restricted manner in these regions. In addition to revealing structures in which Nts may contribute to proper development, this work provides the first comprehensive atlas of Nts neurons in the adult mouse brain and will facilitate future work to define the contributions of distributed Nts populations to energy balance and behavior.

Aim 2: Determine if disrupted Nts signaling via loss of NtsR1 increases risk for development of behaviors similar to those of AN: Since rare damaging variants in NTS and NTSR1 were documented in female individuals with AN, I hypothesized that ***loss of NtsR1*** might confer susceptibility to the disorder. To examine this, I characterized feeding and energy balance in male and **female** mice lacking NtsR1 and littermate controls. I also studied mice exposed to ***adolescent stress*** that is thought to compound genetic susceptibility to develop AN. I predicted that environmental stress during adolescence and being of the female sex further compound the genetic susceptibility imparted by deficiency of NtsR1 to develop AN.

Aim 3: Elucidate the afferents to LHA Nts neurons, and whether such projections are altered by risk factors of AN: Since experimental activation of LHA Nts neurons promotes weight loss behaviors, it is possible that augmented synaptic inputs to LHA Nts neurons could alter energy balance relevant to AN. I therefore used *Nts*^{Cre} mice and a Cre-mediated monosynaptic rabies tracing method to define the specific inputs to LHA Nts neurons in an effort to determine whether regions implicated in the pathogenesis of AN (insula, CEA, etc.) exert top-down control on LHA Nts neurons. My data provide an unbiased map of the afferents to LHA Nts neurons. Additionally, I tested the hypothesis that afferent density is altered in mice prone to developing AN by comparing densities of inputs to LHA Nts neurons in female mice with intact NtsR1, female mice null for NtsR1, and NtsR1-null female mice exposed to an adolescent stress paradigm.

Conclusion

This thesis defines the neurocircuitry and molecular adaptations of the Nts-NtsR1 system that have been implicated in control of energy balance and possibly the development of AN. These data are a vital first step to understanding if, how, and where central manipulation of Nts signaling might contribute to AN and possible mechanisms for medical intervention of this pathway. The Nts system has the potential to be a particularly convenient drug target for metabolic aspects of AN that are yet untreated, as there are numerous Nts populations throughout the brain that regulate energy balance in different capacities, via different receptor and circuit mechanisms, and in different contexts. Thus, in the future, targeting specific Nts neuronal circuits could potentially treat divergent conditions that disrupt weight, including AN.

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CHAPTER 2. Mapping the Populations of Neurotensin Neurons in the Mouse Brain

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Abstract

Neurotensin (Nts) is a neuropeptide implicated in the regulation of many facets of physiology, including cardiovascular tone, pain processing, ingestive behaviors, locomotor drive, sleep, addiction and social behaviors. Yet, there is incomplete understanding about how the various populations of Nts neurons distributed throughout the brain mediate such physiology. This knowledge gap stemmed from the inability to simultaneously identify Nts cell bodies and manipulate them *in vivo*. One means of overcoming this obstacle is to study the progeny of Nts^{Cre} mice crossed onto a Cre-inducible green fluorescent reporter line in which GFP follows a loxP-flanked transcriptional "stop" sequence. The mice derived from this cross, from now on termed Nts^{Cre}*;GFP* mice, permit both visualization and *in vivo* modulation of specific populations of Nts neurons (using Cre-inducible viral and genetic tools) to reveal their function. Here we provide a comprehensive characterization of the distribution and relative densities of the Nts-GFP populations observed throughout the Nts^{Cre}*;GFP* mouse brain, which will pave the way for future work to define their physiologic roles. We also compared the distribution of Nts-GFP neurons with *Nts*-*In Situ* Hybridization (*Nts*-ISH) data from the adult mouse brain, courtesy of the Allen Brain Atlas. By comparing these data sets we can distinguish Nts-GFP populations that may only transiently express Nts during development but not in the mature brain, and hence which populations may not be amenable to Cre-mediated manipulation in adult Nts^{Cre}*;GFP* mice. Hence, this atlas of Nts-GFP neurons will facilitate future studies using the Nts^{Cre}*;GFP* line to describe the physiological functions of individual Nts populations and how modulating them may be useful to treat disease.

Keywords: lateral hypothalamus, parabrachial nucleus, periaqueductal gray, central amygdala, thalamus, nucleus accumbens, preoptic area, olfactory tubercle, galanin

Introduction

The tridecapeptide Neurotensin (Nts) was first identified from the bovine hypothalamus [1], suggesting its potential function as a neuropeptide. Yet, Nts is also produced peripherally by intestinal enteroendocrine N-cells and the adrenal gland, and these sources account for the large pool of circulating Nts [2–4]. Since its discovery, Nts has been implicated in regulating a host of physiologic responses, including feeding, locomotor activity, social behavior, analgesia, sleep, and response to addictive drugs [5–18]. How Nts mediates these effects remains unclear, and this is particularly true when considering whether and to what extent these effects are attributable to the Nts produced within the brain versus the periphery. Moreover, central and peripheral Nts may exert opposing control over some processes, such as those impacting body weight. Peripheral Nts mediates intestinal fat absorption and smooth muscle tone important for moving nutrients through the intestine [1,19]; thus, the high circulating pro-Nts levels observed in obese individuals could be predicted to facilitate the fat absorption that underlies weight gain. Contradictorily, gastric bypass surgery further elevates circulating Nts and the number of Nts-producing intestinal cells, suggesting that Nts signaling may contribute to the pronounced weight loss evoked by these procedures [20,21]. A potential mechanism that reconciles these data is that local increases in intestinal Nts might be sufficient to access the central nervous system (CNS) via vagal afferents, which may support pro-weight loss behaviors. Indeed, some circulating Nts can access blood brain barrier-adjacent regions implicated in suppressing homeostatic feeding [21,22]; however, this Nts does not reach deeper Nts receptor-containing brain regions that suppress motivated feeding, such as the ventral tegmental area (VTA)

[21,23,24]. Intriguingly, Nts administration to the VTA or central activation of specific Nts neurons that project there suppresses feeding and promotes physical activity behaviors that support weight loss [23,25–28]. Given the rapid turnover of circulating Nts [21,29] and its limited penetrance into deep brain structures, it is likely that some Nts-mediated physiology is solely regulated via Nts neurons within the brain and that there are distinct mechanisms by which peripheral and central Nts modify body weight and other physiology. Thus, defining the central sources of Nts is an important first step to understand how Nts mediates a diverse repertoire of physiology, including regulation of feeding and body weight.

Nts also exerts site-specific effects within the brain, hinting that distinct Nts populations coordinate specific behaviors. For instance, infusion of Nts into the periaqueductal gray (PAG), the rostral ventromedial medulla, central amygdala (CEA), posterior hypothalamic nucleus (PH), nucleus accumbens (Acb), or medial preoptic nucleus (MPO) results in decreased pain sensation with no effects on feeding [5,30–32]. Activation of Nts neurons in the MPO also modulates social interaction [33]. By contrast, Nts administered into in the VTA suppresses feeding and promotes locomotor activity that can support weight loss [26,34,35]. Thus, it is imperative to identify and systematically test how each Nts-expressing population in the brain contributes to physiology and behavior, as this information could inform the development of precision-treatments for chronic pain, social anxiety, obesity, or eating disorders.

The technical challenge of identifying Nts neurons, however, has hindered discovery of how they coordinate normal physiology. *In situ* hybridization (ISH) is suitable to identify *Nts*-expressing neurons but can't be used to modulate them *in vivo,* as necessary to reveal their physiologic roles. Antibody-mediated Nts immunoreactivity (Nts-IR) only identifies fibers in the CNS, indicating axons of passage or terminals via which Nts is released. Nts-IR fails to identify cell bodies unless animals are pre-treated with colchicine to disrupt the microtubule network required for anterograde transport of peptides [36,37]. Colchicine-treatment effectively concentrates Nts within soma to permit their detection via Nts-IR and has been used to reveal Nts perikarya within the nucleus of the solitary tract (NTS), parabrachial nucleus (PB), dorsal raphe nucleus (DR), PAG, VTA, paraventricular hypothalamic nucleus (PVH), rostral arcuate nucleus (Arc), lateral hypothalamic area (LHA), CEA, MPO, and bed nucleus of the stria terminalis (BNST) [38–43]. Problematically, colchicine causes neuronal dysfunction that may alter gene expression and it is lethal, prohibiting further studies to define how these Nts populations contribute to normal physiology or disease states.

To overcome the limitations of conventional Nts detection methods, investigators have begun to use *Nts^{Cre};GFP* mice that permit visualization and manipulation of all Nts-expressing neurons using Cre-LoxP technology [23,33]. The fidelity of the *Nts^{Cre};GFP* line has been confirmed using ISH and colchicine-mediated Nts-IR, verifying that the line reliably identifies Nts neurons in known Nts-expressing brain regions including the LHA, MPO, and Acb [33,36]. We subsequently used the *Nts^{Cre};GFP* line to determine which Nts neurons provide afferents to the VTA, highlighting the Nts neurons

anticipated to exert the anorectic or social behaviors mediated via Nts in this region [23,33,36]. During our analysis, we also noted substantial populations of Nts neurons throughout the brain that did not engage the VTA and, hence, were beyond the scope of study. Yet, we reasoned that any substantial population of Nts neurons is a likely contributor to Nts-mediated physiology and that identifying these populations will open the door for future studies to reveal their functions. As a first step toward this goal, we have conducted a brain-wide assessment of the distribution of Nts-GFP neurons throughout the brains of *Nts^{Cre};GFP* mice. Additionally, we compared the distribution of Nts-GFP neurons with *Nts*-ISH data from the adult mouse brain [44]. This comparison is important to identify any Nts-GFP populations that, despite expressing GFP, do not actively express Nts in adulthood. This would occur in cells that transiently expressed Nts during development, resulting in recombination and permanent GFP expression even if these cells do not continue to express Nts (or Cre) during maturity. Our work thus provides a comprehensive "Nts-GFP atlas" that will be useful to identify Nts-containing populations in developing and adult mice. This resource will enable investigators to identify Nts populations of interest so that they may be systematically studied in the future using *Nts^{Cre};GFP* mice to finally reveal how various Nts populations mediate diverse physiology.

Materials and Methods

Animals

Nts^{Cre} mice (The Jackson Laboratory, stock # 017525) were crossed with *Rosa26*$^{EGFP\text{-}L10a}$ mice, in which a transgene containing at loxP-flanked transcriptional blocking cassette precedes GFP [45], to create *Nts*^{Cre}*;GFP* mice, which can be used to visualize all Nts neurons via their Cre-mediated induction of green fluorescent protein (GFP). Male progeny heterozygous for *Nts*^{Cre} and *GFP* were utilized throughout this study and are referred to as *Nts*^{Cre}*;GFP* mice. Mice were bred and housed in a 12hr light/12 hr dark cycle. All procedures were approved by the Institutional Animal Care and Use Committee at Michigan State University in accordance with Association for Assessment and Accreditation of Laboratory Animal Care and National Institutes of Health guidelines. Tail biopsies were taken between 2-3 wk of age and genotyped using standard polymerase chain reaction (PCR). The following primers were used to identify *Nts*^{Cre} mice: common forward, 5' ATA GGC TGC TGA ACC AGG AA; cre reverse, 5' CCA AAA GAC GGC AAT ATG GT; and WT reverse, 5' CAA TCA CAA TCA CAG GTC AAG AA. Primers used to detect the presence of Rosa26$^{EGFP\text{-}L10a}$ include: mutant forward, 5' TCT ACA AAT GTG GTA GAT CCA GGC; WT forward, 5' GAG GGG AGT GTT GCA ATA CC, and common reverse, 5' CAG ATG ACT ACC TAT CCT CCC.

Immunohistochemistry and Immunofluorescence

Mice were anesthetized with intraperitoneal pentobarbital and transcardially perfused, first with 1X phosphate-buffered saline (PBS) and then with 10% formalin

(Fisher Scientific, Pittsburgh, PA). Brains were removed, stored in 10% formalin overnight, and then dehydrated with a 30% sucrose solution. Brains were cut coronally into four series of 30 µm sections using a freezing microtome (Leica, Buffalo Grove, IL). To enhance visualization of Nts-GFP neurons, sections were incubated in primary antibody for GFP (Abcam, chicken, 1:1000; RRID: AB_300798). To examine the CEA additional primary antibodies were used to detect Protein Kinase C-Delta (PKC-δ, BD Biosciences, mouse, 1:1000; RRID: AB_397781). After overnight incubation at room temperature in primary antibodies, brain sections were washed 6 times in PBS. Next, species-specific Alexa-488 conjugated (Jackson ImmunoResearch, 1:200; RRID: AB_2340375) or Alexa-568 conjugated antibodies (LifeTech, 1:200; RRIDs: AB_2534013 and AB_2534017) were applied for 1 hour at room temperature. Sections were finally washed with PBS to remove any non-specific binding and were then mounted onto slides and coverslipped with ProLong Antifade mounting media. Immunolabeled brain sections were analyzed using an Olympus BX53 fluorescence microscope outfitted with FITC and Texas Red filters. Images were collected using Cell Sens software and a Qi-Click 12 Bit cooled camera. Images were subsequently analyzed using Photoshop software (Adobe). Three separate male *Nts^{Cre};GFP* mice were analyzed to both map the location of Nts populations throughout the entire brain and qualitatively assess the density of Nts-GFP populations within each brain region. The relative density rating for Nts-GFP neurons used in this study is as follows: ++++ = Very dense; +++ = Numerous or many; ++ = Moderate; + = Sparse; 0 = none observed. Data were compared between the three brains, and an average rating of Nts-GFP cell density was assigned for each brain structure. Additionally, we compared similar

Bregma level images from *Nts*^{Cre}*;GFP* mice with *Nts*-ISH data obtained from the coronally-sectioned adult mouse brain courtesy of the Allen Brain Atlas [44].

Fluorescence *In Situ* Hybridization (ISH)

ISH was performed to detect mRNA for Nts and Galanin (Gal). Mice were anesthetized with pentobarbital and transcardially perfused with 4% paraformaldehyde. Brains were removed, stored in 4% paraformaldehyde for 24 hours, and then dehydrated in 30% sucrose prior to coronal sectioning as described above. Sections were prepared and treated according to the protocol provided by the Advanced Cell Diagnostics RNAScope Multiplex Fluorescent v2 Reagent Kit (cat. no. 323100). Briefly, sections were dried in a 40 °C oven for 1 hour, incubated in the provided hydrogen peroxide solution for 10 minutes at room temperature, and washed in distilled water. Target Retrieval was performed by incubating samples in 100 °C 1X Target Retrieval Reagent for 10 minutes. Slides were dipped in 100% alcohol and allowed to dry. Protease removal was performed by incubating sections in the Protease III solution provided for 15 minutes at 40 °C, and slides were subsequently washed in distilled water. Probe hybridization was achieved by applying Nts (Mm-Nts, cat. no. 420441) and Gal (Mm-Gal-C2, cat. no. 400961-C2) probes for 2 hours at 40 °C. After probe amplification, fluorophores were applied, with TSA plus fluorescein used to detect Nts probe and TSA plus Cy3 used to detect the Gal probe.

Results

General Observations

We observed many Nts-GFP cells scattered throughout the brain, which are described in **Table 2.1** by their location across the caudal-rostral brain axis and relative density. In many cases Nts-GFP cells were evenly distributed throughout a brain region, but we also observed sites in which Nts-GFP neurons were visibly grouped together in clusters; we refer to the latter as Nts-GFP populations. Figures include representative images from *Nts^{Cre};GFP* mice of the brain areas with the largest density of Nts-GFP cells (those with qualitative density ratings of +++/++++ or ++++, see **Table 2.1**) across the entire caudal-rostral axis of the brain. Each Nts-GFP image was assigned a Bregma level according to the mouse brain atlas [46] to permit identification of Nts-GFP containing brain regions using stereotaxic coordinates, and thus how to target specific Nts-GFP populations for future manipulations. Corresponding images of *Nts*-ISH from adult mice (courtesy of the Allen Brain Atlas [44]) are presented alongside each Nts-GFP image to distinguish whether mature neurons in these regions actively express Nts. This is important because Cre-mediated recombination will occur during whichever stage Nts is expressed in the *Nts^{Cre};GFP* mice, inducing permanent GFP expression. Thus, any developmentally-expressing Nts cells will undergo recombination and remain GFP-labeled throughout the lifespan, and this is independent of whether or not such cells actively express Nts in the mature brain. Comparing *Nts*-ISH and Nts-GFP data from adult mice reveals which Nts-GFP cells expressed Nts developmentally versus during adulthood.

Table 2.1. Relative Density of Nts-GFP Neurons and *Nts*-ISH in the Mouse Brain. Caudal to rostral list of brain regions observed to contain Nts-GFP neurons and the Bregma coordinates at which they were found. The relative density of *Nts*-ISH was assessed in each of these regions from the publicly accessible Allen Brain dataset of coronal *Nts*-ISH images, and their corresponding Bregma coordinates are given. Relative density ratings: ++++ = Very dense; +++ = Numerous or many; ++ = Moderate; + = Sparse.

Abbreviation	Structure	Relative Density of Nts-GFP Cells	Representative Bregma Coordinates of Nts-GFP Cells	Relative Density of *Nts*-ISH Cells (Allen Brain)	Representative Bregma Coordinates of *Nts*-ISH Cells
SolC	Solitary nucleus, commissural part	+/++	-8.12	+	-8.00 to -7.92 and -7.48
Rob	Raphe obscurus nucleus	+	-8.12	0/+	-7.32
Ramb	Retroambiguus nucleus	++	-8.12	+/++	-8.00
Sp5C	Spinal trigeminal tract, caudal part	+++/++++	-8.12 to -7.64	+++/++++	-8.24 to -7.92
Irt	Intermediate reticular nucleus	++	-8.12 to -7.56	+	-8.24 to -7.92
MdD	Medullary reticular nucleus, dorsal part	+/++	-8.12 to -7.56	0/+	-8.00 to -7.32
MdV	Medullary reticular nucleus, ventral part	++	-8.12 to -7.56	0/+	-7.92 to -7.64; -7.32
10N	Dorsal motor nucleus of vagus	+/++	-8.12 to -7.48	++++	-7.92 to -7.48
12N	Hypoglossal nucleus	++++	-7.76	++++	-7.76 to -7.08
Gr	Gracile nucleus	++/+++	-7.76	++/+++	-7.92 to -7.76
IOA/IOB	Inferior olive, subnucleus A and B of the medial nucleus	+++	-7.76	0/+	-7.76
CuR	Cuneate nucleus, rotundus part	+++	-7.76 to -7.64	++/+++	-7.76
SolVL	Solitary nucleus, ventrolateral part	+	-7.76 to -7.64	+	-7.92 and -7.48
CeCv	Central cervical nucleus of the spinal cord	+	-7.76 to -7.64	+	-8.12
SolM	Solitary nucleus, medial part	++	-7.76 to -7.64 and -6.72	+/++	-6.64 to -6.36
SolV/SolIM	Solitary nucleus, ventral part and intermediate part	++	-7.76 and -6.72	+/++	-6.64 to -6.36
Cu/cu	Cuneate nucleus and fasciculus	+++	-7.64	+++	-7.64
SolDL	Solitary nucleus, dorsolateral part	++	-7.64	+	-7.92 and -7.48
SubP	Subpostrema area	+	-7.64	0/+	-7.64
Amb	Ambiguus nucleus	+	-7.64 to -7.20	++/+++	-7.92
SolI	Solitary nucleus, interstitial part	+	-7.64 and -6.36	+/++	-6.48 to -6.36

Table 2.1 (cont'd)

Sp5I	Spinal trigeminal nucleus, interpolar part	+++	-7.56 to -7.48	+	-7.48 to -6.84
Mx	Matrix region of the medulla	+	-7.48	+	-7.48
SolCe	Solitary nucleus, central part	+/++	-7.48	+	-7.48
AP	Area postrema	++/+++	-7.48	++/+++	-7.48
IOC	Inferior olive, subnucleus C of medial nucleus	++/+++	-7.56	0/+	-7.76 to -7.48
IOD	Inferior olive, dorsal nucleus	++/+++	-7.56	0	
IOPr	Inferior olive, principal nucleus	+	-7.32	0	
RPA	Raphe pallidus nucleus	+	-7.32	0	
LRt	Lateral reticular nucleus	0/+	-7.20	0/+	-8.12 and -7.20
RVRG	Rostral ventral respiratory group	+	-7.20	0	
CVL	Caudoventrolateral reticular nucleus	+	-7.20	0	
Ro	Nucleus of Roller	0/+	-7.20 to -7.08	0/+	-7.48 to -6.96
PCRt	Parvicellular reticular nucleus	+/++	-7.20 and -6.84 to -6.72	+	-7.20 and -6.64
SolG	Solitary nucleus, gelatinous part	0/+	-7.08	0/+	-7.32 to -7.20
MVe	Medial vestibular nucleus	+/++	-7.08 to -6.84	+	-6.84 and -5.52
SpVe	Spinal vestibular nucleus	+/++	-7.08 to -6.36	+/++	-6.72 to -6.24
Gi	Gigantocellular reticular nucleus	0/+	-7.08 to -5.68	0/+	-7.20 to -7.08; -5.88
SolDM	Solitary nucleus, dorsomedial part	+	-6.96	+	-7.48
IODM	Inferior olive, dorsomedial cell group	+/++	-6.96	0	
SolL	Solitary nucleus, lateral part	+	-6.96 to -6.84	+	-7.48 and -7.08
5Sol	Trigeminal-solitary transition zone	+	-6.96 and -6.24	+/++	-6.64 to -6.48
Pr	Prepositus nucleus	0/+	-6.96 to -6.00	0/+	-6.00
GiV	Gigantocellular reticular nucleus, ventral part	+	-6.84 to -6.72	0/+	-6.96; -6.64
DPGi	Dorsal paragigantocellular nucleus	0/+	-6.84 to -6.36	0/+	-5.88
LPGi	Lateral paragigantocellular nucleus	0/+	-6.84 to -6.72 and -6.24	0/+	-5.88
IOM	Inferior olive, medial nucleus	++	-6.72	+	-7.08

Table 2.1 (cont'd)

Fve	F cell group of the vestibular complex	+	-6.72	0	
Bo	Botzinger complex	0/+	-6.72	0	
RVL	Rostroventrolateral reticular nucleus	0/+	-6.72	0	
MVeMC	Medial vestibular nucleus, magnocellular part	+	-6.72	+	-6.72 to -5.68
DMSp5	Dorsomedial spinal trigeminal nucleus	+/++	-6.36 and -6.00	++/+++	-5.88 to -5.80
DC	Dorsal cochlear nucleus	++	-6.36 to -6.24 and -5.80 to -5.68	++	-6.36
GiA	Giganotcellular reticular nucleus, alpha part	+	-6.36	0/+	-6.36
IS	Inferior salivatory nucleus	+	-6.24	0	
X	nucleus X	0/+	-6.24	0/+	-6.24
Y	nucleus Y	0/+	-6.24 to -6.12	+	-6.00
PCRtA	Parvicellular reticular nucleus, alpha part	+/++	-6.24 to -6.12	+/++	-6.72 to -6.00
LVe	Lateral vestibular nucleus	0/+	-6.24 to -6.00	0/+	-6.24 to -5.88
DCFu	Dorsal cochlear nucleus, fusiform layer	+++/++++	-6.24 to -6.00	+++/++++	-6.24
RMg	Raphe magnus nucleus	0/+	-6.24 and -5.02 to -4.96	0/+	-6.48 and -6.24
DCDp	Dorsal cochlear nucleus, deep layer	++	-6.12 to -6.00	+	-6.12; -5.88
VeCb	Vestibulocerebellar nucleus	0/+	-6.00	0/+	-6.24
7VM, 7DM, 7DI, 7DL, 7L 7VI	Facial nucleus subnuclei	+++	-6.00	+/++	-6.00
SuVe	Superior vestibular nucleus	++	-6.00 to -5.80	+	-6.00; -5.80 to -5.68
Sp5O	Spinal trigeminal nucleus, oral part	+/++	-6.00 to -5.68	+	-5.80 to -5.68
MVePC	Medial vestibular nucleus, parvicellular part	++/+++	-5.80	++/+++	-6.00 to -5.80
Sge	Supragenual nucleus	0/+	-5.80	0/+	-5.80
7N	Facial nucleus	+++/++++	-5.80 to -5.68	+++/++++	-5.80
6N	Abducens nucleus	+	-5.80 to -5.68	0	
Pa6	Paraabducens nucleus	+	-5.68	0/+	-5.68
6RB	Abducens nucleus, retractor bulbi part	++/+++	-5.68	+++/++++	-5.52
GrC	Granule cell layer of cochlear nuclei	++	-5.68	+	-5.80
P7	Perifacial zone	+/++	-5.68	0	

Table 2.1 (cont'd)

LC	Locus coeruleus	+	-5.68	0	
A5	A5 noradrenaline cells	+++/++++	-5.68	+++/++++	-5.68 to -5.52
Eve	Nucleus of origin of the efferent fibers of the vestibular nerve	+	-5.68	0	
VCA	Ventral cochlear nucleus, anterior part	+++	-5.68 to -5.34	0	
SPO	Superior paraolivary nucleus	+	-5.40 to -5.02	0/+	-5.02
PR5DM	Principal sensory trigeminal nucleus, dorsomedial part	+	-5.34	0	
MPBE	Medial parabrachial nucleus, external part	+/++	-5.34	+/++	-5.20
PnR	Pontine raphe nucleus	+	-5.34	0/+	-5.02
CGA	Central gray, alpha part	+/++	-5.34	+	-5.34
CGB	Central gray, beta part	0/+	-5.34	+	-5.34
LPBD	Lateral parabrachial nucleus, dorsal part	+++/++++	-5.34	0/+	-5.20
LPBE	Lateral parabrachial nucleus, external part	+++/++++	-5.34 to -5.20	+++/++++	-5.40 to -5.20
5Tr	Trigeminal transition zone	0/+	-5.34 to -5.20	0	
DTgC	Dorsal tegmental nucleus, central part	+	-5.34 to -5.20	0	
DTgP	Dorsal tegmental nucleus, pericentral part	+	-5.34 to -5.20	0	
LPBI / LPBV / LPBS	Lateral parabrachial nucleus, internal part/ ventral part/ superior part	+	-5.34 to -4.96	0/+	-5.20 to -5.02
LPBC	Lateral parabrachial nucleus, central part	+++/++++	-5.20	+++/++++	-5.40
LDTgV	Laterodorsal tegmental nucleus, ventral part	+/++	-5.20	++/+++	-5.02
SubCD	Subcoeruleus nucleus, dorsal part	+	-5.20	+	-5.20
5ADi	Motor trigeminal nucleus, anterior digastric part	++	-5.20	0/+	-5.20
CnF	Cuneiform nucleus	+++/++++	-5.20 to -4.96	++/+++	-5.20 to -4.96
KF	Koelliker-fuse nucleus	+++/++++	-5.20 to -4.96	++++	-5.20 to -5.02
Su5	Supratrigeminal nucleus	++/+++	-5.20 and -4.96 to -4.84	+	-5.20 and -4.84
5N	Motor trigeminal nucleus	++	-5.20 to -4.84	++	-5.20
CIC	Central nucleus of the inferior colliculus	0/+	-5.20 to -4.84	0/+	-4.96

Table 2.1 (cont'd)

PR5VL	Principal sensory trigeminal nucleus, ventrolateral part	+++/++++	-5.02	0	
MPB	Medial parabrachial nucleus	+++	-5.02	+++	-5.40
LDTg	Laterodorsal tegmental nucleus	++/+++	-5.02	+++	-5.20
PnC	Pontine reticular nucleus, caudal part	+/++	-5.02	0/+	-5.52 to -5.34; -5.02
Sag	Sagulum nucleus	+++/++++	-5.02	+	-4.84
DMTg	Dorsomedial tegmental area	+/++	-5.02 to -4.96	0/+	-5.02
DCIC	Dorsal cortex of the inferior colliculus	++/+++	-5.02 to -4.96	0/+	-5.40 to -5.34
ECIC	External cortex of the inferior colliculus	+/++	-5.02 to -4.16	0/+	-4.84 to -4.72; -4.48
5TT	Motor trigeminal nucleus, tensor tympani part	+	-4.96	+	-4.96
DRC	Dorsal raphe nucleus, caudal part	++/+++	-4.96	+/++	-5.02
SubCV	Subcoeruleus nucleus, ventral part	+/++	-4.96	++	-5.20
cic	Commissure of inferior colliculus	0/+	-4.96	0	
PR5	Principal sensory trigeminal nucleus	++++	-4.96 to -4.84	0	
DRD	Dorsal raphe nucleus, dorsal part	++++	-4.96 to -4.84	++++	-4.96 to -4.84
Tz	Nucleus of the trapezoid body	+	-4.96 to -4.84	0	
LPAG	Lateral periaqueductal gray	+++/++++	-5.20 to -4.16	+/++	-5.20
VLPAG	Ventrolateral periaqueductal gray	+++/++++	-4.96 to -4.16	+++/++++	-4.96 to -4.84
DMPAG	Dorsomedial periaqueductal gray	+++/++++	-4.96 to -4.04	0	
P5	Peritrigeminal zone	++	-4.84	+	-4.84
DLL	Dorsal nucleus of the lateral lemniscus	++	-4.84	++	-4.96
DRI	Dorsal raphe nucleus, interfascicular part	+++	-4.84 to -4.72	+++/++++	-4.72
DLPAG	Dorsolateral periaqueductal gray	+++/++++	-4.84 to -4.16	0	
DpGi	Deep gray layer of the superior colliculus	+++/++++	-4.84 to -4.16	+	-4.60 to -4.24
DpWh	Deep white layer of the superior colliculus	+++/++++	-4.84 to -4.16	0/+	-4.72 to -4.24
TrLL	Triangular nucleus, lateral lemniscus	+	-4.72	0	
DRV	Dorsal raphe nucleus, ventral part	+++	-4.72	++++	-4.96 to -4.84
CAT	Nucleus of the central acoustic tract	+	-4.72 to -4.60	0	

Table 2.1 (cont'd)

MPL	Medial paralemniscal nucleus	+	-4.72 to -4.48	0	
PDR	Posterodorsal raphe nucleus	++	-4.72 to -4.36	++	-4.48 to -4.36
PrCnF	Precuneiform area	+++/++++	-4.72 to -4.10	++/+++	-4.36 to -4.24
PMnR	Paramedian raphe nucleus	+	-4.72 to -4.04	0/+	-5.02 to -4.84; -4.48 to -4.36
PLV	Perilemniscal nucleus, ventral part	+	-4.60 to -4.48	0	
LL	Lateral lemniscus	+++	-4.60 to -4.48	++/+++	-4.60
Pa4	Paratrochlear nucleus	0/+	-4.60 to -4.36	0/+	-4.84; -4.48 to -4.36
VLL	Ventral nucleus of the lateral lemniscus	+	-4.60 to -4.24	0	
SuG	Superficial gray layer of the superior colliculus	0/+	-4.60; -3.52, and -3.28	0	
DRL	Dorsal raphe nucleus, lateral part	++/+++	-4.48	++	-4.60 to -4.36
SPTg	Subpeduncular tegmental nucleus	++/+++	-4.48	+/++	-4.84
ILL	Intermediate nucleus of the lateral lemniscus	0/+	-4.48 to -4.36	0	
InG	Intermediate gray layer of the superior colliculus	++/+++	-4.48 to -4.01	+	-4.60 to -4.36
InWh	Intermediate white layer of the superior colliculus	++/+++	-4.48 to -4.01	0/+	-4.60 to -4.36
MiTg	Microcellular tegmental nucleus	+++/++++	-4.36	++/+++	-4.60
PBG	Parabigeminal nucleus	+++/++++	-4.36	+	-4.16
PTg	Pedunculotegmental nucleus	++	-4.36 to -4.16	++	-4.60
PnO	Pontine reticular nucleus, oral part	+/++	-4.36 to -4.10	+/++	-4.72 to -4.48
CLi	Caudal linear nucleus of the raphe	0/+	-4.36 to -4.24 and -4.04	0/+	-4.48
3N	Oculomotor nucleus	++/+++	-4.24	0/+	-4.24
Su3C	Supraoculomotor cap	++/+++	-4.24 to -4.16	+/++	-4.24
Su3	Supraoculomotor periaqueductal gray	++/+++	-4.24 to -4.16	+/++	-4.24
3PC	Oculomotor nucleus, parvicellular part	++/+++	-4.24 to -4.16	+	-4.16 to -3.80
STr/S	Subiculum Transition Area/ Subiculum	++++	-4.16 to -3.88	++++	-4.16 to -3.88
MEnt	Medial Entorhinal Cortex	+/++	-4.16	++	-4.60 to -4.48
CEnt	Caudomedial Entorhinal Cortex	++	-4.16	++	-4.60 to -4.48
PRh	Perirhinal Cortex	+/++	-4.16	0	
PnO	Pontine Reticular nucleus, Oral part	+	-4.16	0	

Table 2.1 (cont'd)

rs	Rubrospinal tract	++	-4.16	0/+	-4.16
VIEnt	Ventral Intermediate Entorhinal Cortex	+/++	-4.16 to -3.80	+++	-4.24
DLEnt/LEnt	Dorsolateral/Lateral Entorhinal Cortex	+/++	-4.16 to -2.80	++	-4.60 to -4.16
SubB	Subbrachial nucleus	+++/++++	-4.16 and -3.64	+++/++++	-3.80 to -3.64
MnR	Median raphe nucleus	+/++	-4.10 to -4.01	0/+	-4.48 to -4.36
mRt	Mesencephalic reticular formation	++/+++	-4.10 to -3.64	+/++	-4.72; -4.48 to -4.36
Op	Optic nerve layer of the superior colliculus	++	-4.10 to -3.08	0/+	-4.48 to -4.36
EW	Edinger-westphal nucleus	+	-4.04 to -3.88	0/+	-3.28 to -3.16
mlf	Medial longitudinal fasciculus	+/++	-4.01	+	-3.88
PIF	Parainterfascicular nucleus of the Ventral Tegmental Area	0/+	-3.88	0	
PN	Paranigral nucleus of the Ventral Tegmental Area	0/+	-3.88	0	
RSG/RSD	Retrosplenial Granular/Dysgranular Cortex	++++	-4.04 to -0.58	0	
RRF	Retrorubral field	++	-3.88 and -3.64	++	-4.24
PaR	Pararubral nucleus	+	-3.88 to -3.64	0	
IPR	Interpeduncular nucleus, rostral subnucleus	+	-3.88 to -3.16	0	
mRt	Mesencephalic Reticular Formation	+/++	-3.80 to -3.40	0/+	-3.88 to -3.40
IPDM	Interpeduncular nucleus, dorsomedial subnucleus	0/+	-3.88 to -3.52	0	
DpGi	Deep Gray Layer of the Superior Colliculus	++/+++	-3.8 or -3.40	0	
IPDL	Interpeduncular nucleus, dorsolateral subnucleus	0/+	-3.72 and -3.52	0	
IPDM	Interpeduncular nucleus, dorsomedial subnucleus	0/+	-3.72 and -3.52	0	
IPC	Interpeduncular nucleus, caudal subnucleus	0/+	-3.72 to -3.52	0	
IPI	Interpeduncular nucleus, intermediate subnucleus	0/+	-3.72 to -3.52	0	
IPL	Interpeduncular nucleus, lateral subnucleus	0/+	-3.72 to -3.52	0	
MA3	Medial accessory oculomotor nucleus	+	-3.72 to -3.16	0/+	-3.16

Table 2.1 (cont'd)

DpWh	Deep White Layer of the Superior Colliculus	++	-3.64 to -3.52	0	
DS	Dorsal Subiculum	++++	-3.80 to -2.46	++++	-3.80 to -3.64
VS	Ventral Subiculum	++++	-3.64 to -3.40	++++	-3.64 to -2.92
BIC	nucleus of the Brachium of the Inferior Colliculus	+++/++++	-3.8	+++/++++	-3.8
MGV	Medial Geniculate nucleus, Ventral part	+++	-3.72 to -3.64	0	
InC/InCSh	Interstitial nucleus of Cajal w/ shell region	++	-3.72; -3.28 to -3.16	0	
APir	Amygdalopiriform transition area	++/+++	-3.64	0	
PRh	Perirhinal cortex	0/+	-3.64 and -3.28	0	
Dk	Nucleus of Darkschewitsch	+++	-3.64 to -2.92	+	-3.64 and -3.08
bic	Brachium of the Inferior Colliculus	++++	-3.64 to -3.52	+++/++++	-3.88 to -3.52
DIEnt	Dorsointermedial Entorhinal Cortex	+	-3.4 to -3.28	+/++	-4.04
MGM	Medial Geniculate nucleus, Medial	+++/++++	-3.4 to -3.16	++/+++	-3.4
PoT	Posterior Thalamic nucleus, Triangular	+++/++++	-3.4	+	-3.08
PIL	Posterior Intralaminar Thalamic nucleus	+++/++++	-3.4	++/+++	-3.4
PP	Peripeduncular nucleus	+++/++++	-3.4	++/+++	-3.64
SG	Suprageniculate Thalamic nucleus	+++/++++	-3.40 to -2.92	++/+++	-3.4
PMCo	Posteromedial cortical amygdalar nucleus	0/+	-3.28	0	
csc	Commissure of the superior colliculus	0/+	-3.28	0	
mtg	mammillotegmental tract	+/++	-3.28	++	-3.08
scp	Superior cerebellar peduncle	+	-3.28 to -3.16	0/+	-3.28 to -3.16
LT	Lateral terminal nucleus acc optic tract	++	-3.16	0	
APT	Anterior pretectal nucleus	+/++	-3.16 to -3.08	0	
ZIC	Zona incerta, caudal	++/+++	-3.16 to -3.08	0/+	-2.92
IF	Interfascicular nucleus	+	-3.16 to -3.08	0	
p1Rt	p1 reticular formation	+/++	-3.16 to -2.92	0	
MCPC	Magnocellular nucleus post comm	+/++	-3.08	0	
ML	Medial mammillary nucleus, lateral	+++	-3.08	0	

Table 2.1 (cont'd)

MM	Medial mammillary nucleus, medial	+++/++++	-3.08 to -2.92	++++	-3.08 to -2.92
PBP	Parabrachial pigmented nucleus of the Ventral Tegmental Area	+/++	-3.08 to -2.92	0	
fr	Fasciculus retroflexus	+	-3.08	0	
VTA	Ventral tegmental area	+	-3.08	0	
PLi	Posterior Limitans Thalamic nucleus	++	-3.08 to -2.80	0	
PrEW	Pre-edinger-westphal nucleus	0/+	-2.92	0	
REth	Retroethmoid nucleus	+/++	-2.92	0/+	-2.92
pc	Posterior commissure	0/+	-2.92	0	
LM	Lateral mammillary nucleus	+++/++++	-2.92	0	
rmx	Retromammillary decussation	+/++	-2.92	0	
OPT	Olivary pretectal nucleus	0/+	-2.92	0	
Pir	Piriform Cortex	+	-2.8	+	-2.46 to -2.30, -1.58 to -1.46, 0.50, 0.98 to 1.10
PAG	Periaqueductal Gray	+++/++++	-2.7	+	-3.28 to -3.08
PSTh	Parasubthalamic nucleus	++++	-2.70 to -2.06	++++	-2.46 to -2.30
RML	Retromammillary nucleus, Lateral	++/+++	-2.7	0	
SNC/SNR	Substantia Nigra Compacta/Reticular	+++	-2.7	0/+ (SNC), + (SNR)	-3.28 to -2.92, -2.46
LPMC	LP Thalamic nucleus, Mediocaudal	++/+++	-3.08/ -2.54 to -2.46	0	
PMV	Premammillary nucleus, Ventral	++++	-2.54	+++/++++	-2.54
ZID/ZIV	Zona Incerta, Dorsal/Ventral	++	-2.54 to -2.18	+/++ (ZIV)	-1.94
Py	Pyramidal Cell Hippocampus	++++	-2.54 to -1.58	0/+	
LPLR	LP Thalamic nucleus, Laterorostral	+/++	-2.3	0	
LPMR	LP Thalamic nucleus, Mediorostral	+/++	-2.3	0	
APTD	Anterior Pretectal nucleus, Dorsal	+	-2.30 to -2.18	0	
STh	Subthalamic nucleus	++++	-2.30 to -1.94	++++	-2.46 to -2.30
Te	Terete hypothalamic nucleus	+	-2.3 to -1.82	0	
PR	Prerubral Field	++/+++	-2.3	0	
FF	Fields of Forel	++/+++	-2.3	0	
pv	Paraventricular fiber system	+++	-2.3 and -2.18	+/++	-2.30
ArcLP/ArcMP	Caudal Arcuate Hypothalamic nucleus	++	-2.18	0	
PH	Posterior Hypothalamic nucleus	+++	-2.18	+/++	-2.46 to -2.18

Table 2.1 (cont'd)

BLP	Basolateral Amygdalar nucleus, posterior	++	-2.18	+/++	-2.46
BMP	Basomedial Amygdalar nucleus, posterior	++	-2.18 and -1.58	++	-1.7
BLA	Basolateral Amygdalar nucleus, Anterior	0/+	-1.82 to -1.06	0	
BLV	Basolateral Amygdalar nucleus, Ventral	++/+++	-1.46 to -1.22	++	-1.34 to -1.06
BMA	Basomedial Amygdalar nucleus, Anterior	++/+++	-1.46 to -1.22	++	-1.58 to -1.06
MD, MDL, MDC, MDM	Mediodorsal Thalamic nucleus	++	-2.06, -1.94, -1.58, and -0.70	0	
PF	Parafascicular Thalamic nucleus	++	-2.06	0	
CM	Central Medial Thalamic nucleus	++/+++	-2.06 to -1.94 and -1.70 to -1.58	0	
PVP	Paraventricular Thalamic nucleus, Posterior	+++	-2.06 to -1.94	+	-2.18 to -2.06
PV	Paraventricular Thalamic nucleus	+++	-1.82 to -1.70	0	
Po	Posterior Thalamic nuclear group	++	-2.06 and -0.7	0	
SPF	Subparafascicular Thalamic nucleus	+++	-2.06	0	
PoMn	Posteromedian Thalamic nucleus	++	-2.06	0	
PHD	Posterior Hypothalamic Area, Dorsal	++	-2.06 to -1.82	+	-2.18 to -2.06
VMH	Ventromedial Hypothalamic nucleus	++	-2.06 to -1.94 and -1.46	0	
MePD/MePV	Medial Amygdalar nucleus, posterodorsal and posteroventral	++	-2.06 to -1.94 and -1.46	++ (no MePV)	-2.06 to -1.34
Ast	Amygdalostriatal transition	++	-2.06	++/+++	-1.46
ns	nigrostriatal bundle	+++	-1.94	++/+++	-1.94
DMV	Dorsomedial Hypothalamic nucleus, Ventral	++	-1.94	0	
AHiAL	Amygdalohippocampal Area, anterolateral	++	-1.94	+/++	-2.3
PVH	Paraventricular nucleus of the Hypothalamus	0/+	-1.22	0	
CL	Centrolateral Thalamic nucleus	++	-1.94 to -1.22	0	
PC	Paracentral Thalamic nucleus	++	-1.94 to -1.22	0	

Table 2.1 (cont'd)

DEN/VEN	Dorsal and Ventral Endopiriform nucleus	+/++	-1.94 to -0.10	+/++ (VEN); + (DEN)	-1.22, -0.94 to -0.70 (VEN); 0.02 (DEN)
LHA	Lateral Hypothalamus	++++	-1.82 to -1.70	+++	-1.82
PeF	Perifornical nucleus	+++	-1.82	++	-1.82
CEA	Central Amygdalar nucleus	++++	-1.82 to -1.70	++++	-1.94 to -1.70
ArcD/ArcL	Arcuate hypothalamic nucleus, Dorsal/ Lateral	++/+++	-1.70	0	
Xi	Xiphoid Thalamic nucleus	++	-1.70 to -1.34; -0.88	0/+	-0.82
IMD	Intermediodorsal Thalamic nucleus	++/+++	-1.58 to -1.34	NA	
DM	Dorsomedial Hypothalamic nucleus	+	-1.58	0	
PaXi	Paraxiphoid nucleus of Thalamus	++	-1.46 to -0.58	+	-1.22 to -1.06, -0.82
Arc	Rostral Arcuate Hypothalamic nucleus	++++	-1.34 to -1.22	+/++	-1.46
BMA	Basomedial Amygdalar nucleus, anterior	++/+++	-1.34 to -0.94	0	
PMCo/PLCo/Aco	Cortical Amygdalar nucleus	++/+++	-1.34 to -0.22	+/++	-1.70 to -0.70
STIA	ST, intraamygdalar division	++/+++	-1.34	++/+++	-2.06 to -1.70
CxA	Cortex-Amygdala Transition	++/+++	-1.34 to -0.82	+/++	-0.94
AV	Anteroventral thalamic nucleus	+/++	-1.22; -0.94	0	
RChL	Retrochiasmatic, Lateral	+/++	-1.22; -0.94	0	
Ep/MGP	Entopeduncular nucleus	++/+++	-1.22	0	
CPu	Caudate Putamen	++/+++	-1.22 to -1.94	++/+++ in very ventral most region, 0/+ elsewhere	-1.58 to 1.54
MeAD	Medial Amygdalar nucleus, Anterodorsal	++/+++	-1.06 to -0.82	0	
AHP	Anterior Hypothalamic Area, Posterior	++/+++	-0.94	0	
Rch	Retrochiasmatic Area	++	-0.94	0	
ZI	Zona Incerta	0/+	-0.94 to -0.82	0	
ZIR	Zona Incerta, rostral	0/+	-0.94	0	
Py3 CA3	Pyramidal Field CA3 Hippocampus	+	-0.94	0	
IAD	Interanterodorsal thalamic nucleus	+/++	-0.94	0	
IAM	Interanteromedial thalamic nucleus	+/++	-0.94	0	
MeAV	Medial Amygdalar nucleus, Anteroventral	+++	-0.94 to -0.88	+/++	-1.22 to -0.82

Table 2.1 (cont'd)

IM	Intercalated amygdalar nucleus, main	++/+++	-0.94	0	
SO	Supraoptic nucleus	0/+	-0.94 to -0.82	0	
BAOT	Bed nucleus Access of the Olfactory tract	+/++	-0.94 to -0.82	0	
AA	Anterior amygdalar area	++	-0.94 to -0.10	0	
VCl	Ventral part of the claustrum	0/+	-0.88	0	
AM	Anteromedial thalamic nucleus	0/+	-0.88	0	
AHC	Anterior Hypothalamic Area, Central	++	-0.88 to -0.82	0/+	-0.7
MCPO	Magnocellular preoptic nucleus	+	-0.88 to 0.02	0	
AVDM	Anteroventral thalamic nucleus, dorsomedial	0/+	-0.82	0	
SCh	Suprachiasmatic nucleus	++	-0.82 to -0.58	++	-0.46
SM	nucleus stria medullaris	+	-0.58 to -0.46	0	
PVA	Paraventricular Thalamic nucleus, Anterior	+++/++++	-0.46 to -0.22	+++/++++	-0.46
VA	Ventral Anterior Thalamic nucleus	+	-0.7	0	
AA	Anterior Amygdalar Area	+/++	-0.7	0	
VLH	Ventrolateral hypothalamic nucleus	+/++	-0.7	0	
ESO	Episupraoptic nucleus	+	-0.70 to -0.58	0	
LAH	Lateroanterior hypothalamic nucleus	++	-0.70 to -0.58	++/+++	-0.46
RT	Reticular thalamic nucleus	+	-0.70 to -0.46	0	
PT	Paratenial thalamic nucleus	+	-0.70; -0.34	0	
LOT	nucleus of the Lateral Olfactory tract	+	-0.7 to -0.22	0	
ANS	Accessory neurosecretory nucleus	+	-0.58	0	
AHA	Anterior hypothalamic area, anterior	+/++	-0.58 to -0.34	+++	-0.46
TS	Triangular septal nucleus	0/+	-0.58 to -0.22	0	
vhc	Ventral hippocampal commissure	0/+	-0.58 to -0.22	0	

Table 2.1 (cont'd)

BSTMPL/ BSTMPI	Bed nucleus of the stria terminalis, medial division, posterolateral part / posterointermediate part	+/++	-0.46-0.14	+/++	-0.46 and 0.14
df	Dorsal fornix	0/+	-0.46 to -0.22	0	
Cg	Cingulate Cortex	++++	-0.46 to 1.1	0	
EAC/EAM/EA	Sublenticular extended amygdala	++/+++	-0.34	0	
BSTMPM	Bed nucleus of the stria terminalis, medial division, posteromedial part	0/+	-0.34 to -0.10	0	
HDB	nucleus of the horizontal limb of the diagonal band	+	-0.34 to 0.38	0	
GP	Globus pallidus	0/+	-0.22 to 0.02	0	
BAC	Bed nucleus of the anterior commissure	0/+	-0.18	0	
StHy	Striohypothalamic nucleus	+++/++++	-0.18	++/+++	0.02
MPO	Medial Preoptic nucleus	++++	-0.18 to 0.02	+++/++++	0.14
MPA	Medial Preoptic Area	+++	-0.18 to 0.32	++/+++	0.02 to 0.14
LPO	Lateral Preoptic nucleus	+++	-0.18 to 0.38	++/+++	0.38 to 0.62
A14	A14 Dopamine cells	++	-0.1	0	
f	fornix	0/+	-0.1	0	
BSTLI	Bed nucleus of the stria terminalis, lateral division, intermediate part	++	-0.10	+	0.02
SFi	Septofimbrial nucleus	+	-0.10 to 0.02	0	
BSTLP	Bed nucleus of the stria terminalis, lateral division, posterior part	++	-0.10 to 0.38	++	0.26 to 0.38; 0.62
AVPe	Anteroventral Periventricular nucleus	++++	0.02-0.26	++/+++	0.26
VMPO	Ventromedial Preoptic nucleus	+++/++++	0.02-0.38	+++	0.14
VLPO	Ventrolateral Preoptic nucleus	++/+++	-0.10 to 0.26	+	0.02
BSTLV	Bed nucleus of the stria terminalis, lateral division, ventral part	+/++	0.02 to 0.62	++	0.02 to 0.62
Tu	Olfactory tubercle	+++/++++	0.02-1.98	+++/++++	1.54 to 1.70
BSTLJ	Bed nucleus of the stria terminalis, lateral division, juxtacapsular part	+	0.14	++	0.26

Table 2.1 (cont'd)

BSTMPL	Bed nucleus of the stria terminalis, medial division, posterolateral part	+/++	0.14	+/++	0.14
StA	Strial part of the Preoptic Area	++	0.14	0	
BSTLD	Bed nucleus of the stria terminalis, lateral division, dorsal part	++	0.26-0.38	++++	0.14 to 0.26
BSTMA	Bed nucleus of the stria terminalis, medial deivision, anterior part	++/+++	0.26 to 0.62	++/+++	0.62
SHy	Septohypothalamic nucleus	+++	0.26-0.38	++	0.14 to 0.50
PS	Parastrial nucleus	+++	0.26	++	0.14
BSTMV	Bed nucleus of the stria terminalis, medial division, ventral part	++/+++	0.50 to 0.62	++/+++	0.62
IPAC	Interstitial nucleus of the posterior limb of the anterior commissure	+++	0.5	++/+++	0.14 to 0.38
LSI	Lateral Septal Nucleus, intermediate part	+++	0.74	+++/++++	0.74 to 0.86
LSD/LSV	Lateral Septal Nucleus, dorsal / ventral part	+++	0.74	++/+++ (LSD); +++/++++ (LSV)	1.10 (LSD); 0.86 (LSV)
AcbC	Nucleus Accumbens, Core	*between + and +++	0.74-1.78	0/+	0.98
SIB	Substantia innominata	++/+++	0.74	0	
ICj	Island of Cajella	++++	0.74, 1.42, 1.54, 1.94	+++/++++	0.50 and 0.74
AcbSh	Nucleus Accumbens, Shell	++++	0.98-1.42	++/+++	0.74 and 1.34
LAcbSh	Nucleus Accumbens, lateral Shell	+++/++++	0.98-1.18	+++	0.74
ICjM	Island of Cajella, Major Island	+++	1.18-1.42	0	
DTT	Dorsal Tenia Tecta	++	1.34-1.42 and 1.94	0	
PrL	Prelimbic Cortex	++	1.54-1.70	0	
IL	Infralimbic Cortex	++	1.54-1.70 and 1.98	0	
SHi	Septohippocampal nucleus	+++	1.78-1.94	0	1.7
Nv	Navicular Postolfactory nucleus	++++	1.78	+	1.7
VTT	Ventral Tenia Tecta	++/+++	1.98	0	

Table 2.1 (cont'd)

AOM	Anterior Olfactory Area, Medial Part	++/+++	2.1	0	
MO	Medial Orbital Cortex	+/++	2.1	0	

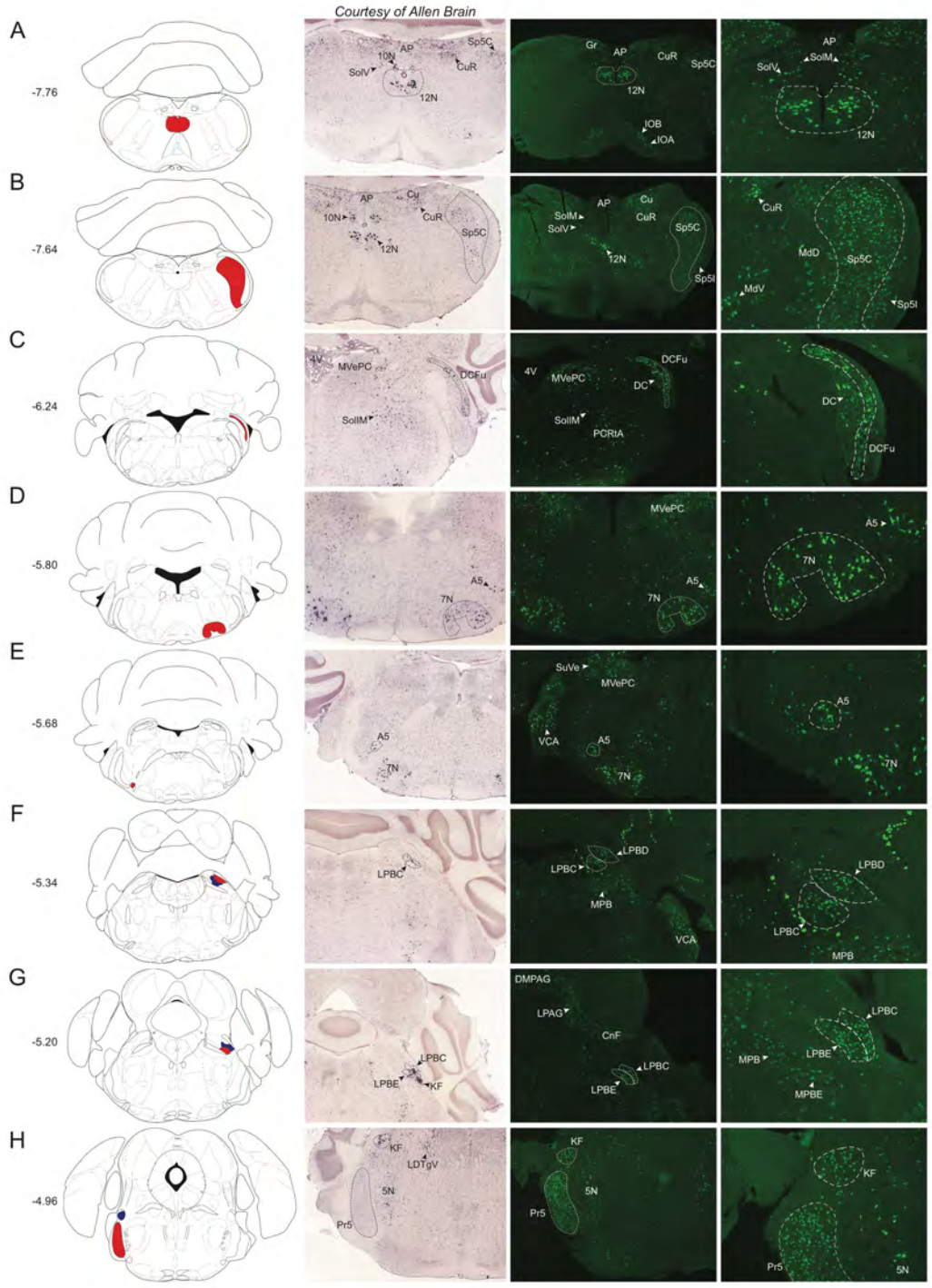
Hindbrain

Starting caudally, the most notable structure containing a dense population of Nts-GFP neurons is the hypoglossal nucleus (12N), which also contains robust *Nts*-ISH (**Figure 2.1A**). Other hindbrain regions containing sizable Nts-GFP populations and dense *Nts*-ISH include the caudal portion of the spinal trigeminal nucleus (Sp5C) (**Figure 2.1B**), the fusiform region of the dorsal cochlear nucleus (DCFu) (**Figure 2.1C**), the facial nucleus (7N) (**Figure 2.1D**), the A5 group of noradrenaline cells (**Figure 2.1D, E**), and the dorsal, external, and central parts of the lateral parabrachial nucleus (LPBD, LPBE, LPBC) (**Figure 2.1F-G**). The Koelliker-fuse nucleus (KF) and the principal sensory trigeminal nucleus (Pr5) contain many Nts-GFP neurons, and while a comparable level of *Nts*-ISH exists in the KF, there is an absence of detectable *Nts*-ISH signal in the Pr5 (**Figure 2.1H**), suggesting that Nts is transiently expressed by Pr5 cells at some stage of development, but not in adult Nts-GFP neurons.

Other hindbrain structures contained more diffuse, but readily identifiable populations of Nts-GFP neurons with qualitative density ratings of ++/+++ or +++, as per **Table 2.1**. Some of these moderately dense populations of Nts-GFP neurons are shown in **Figure 2.1** and include the gracile (Gr) and cuneate nuclei (Cu and CuR), the inferior olivary complex (IOA and IOB), the caudal aspect of the interpolar spinal trigeminal nucleus (SP5I), the area postrema (AP), the parvicellular part of the medial vestibular nucleus (MVePC), the anterior aspect of the ventral cochlear nucleus (VCA), and the medial parabrachial nucleus (MPB and MPBE). We observed other moderately sized Nts-GFP populations (not pictured but described in **Table 2.1**) within the retractor bulbi

part of the adbucens nucleus (6RB), the supratrigeminal nucleus (Su5), and the laterodorsal tegmental nucleus (LDTg). *Nts*-ISH was detected in a similar distribution and density as the Nts-GFP cells within the Gr, Cu, AP, MVePC, 6RB, MPB and LDTg.

Figure 2.1. Nts-GFP and *Nts*-ISH in the Hindbrain. From left to right, each row contains a Bregma-numbered atlas image [46], an image of Nts-ISH at the same Bregma level, courtesy of the Allen Brain Atlas [44], a 4x image of Nts-GFP neurons, and a 10x image of Nts-GFP neurons from the same area. Red and blue shaded areas in the atlas image are outlined in the Nts-GFP images. **A)** Bregma -7.76, **B)** Bregma -7.64, **C)** Bregma -6.24, **D)** Bregma -5.80, **E)** Bregma -5.68, **F)** Bregma -5.34, **G)** Bregma -5.20, **H)** Bregma -4.96.

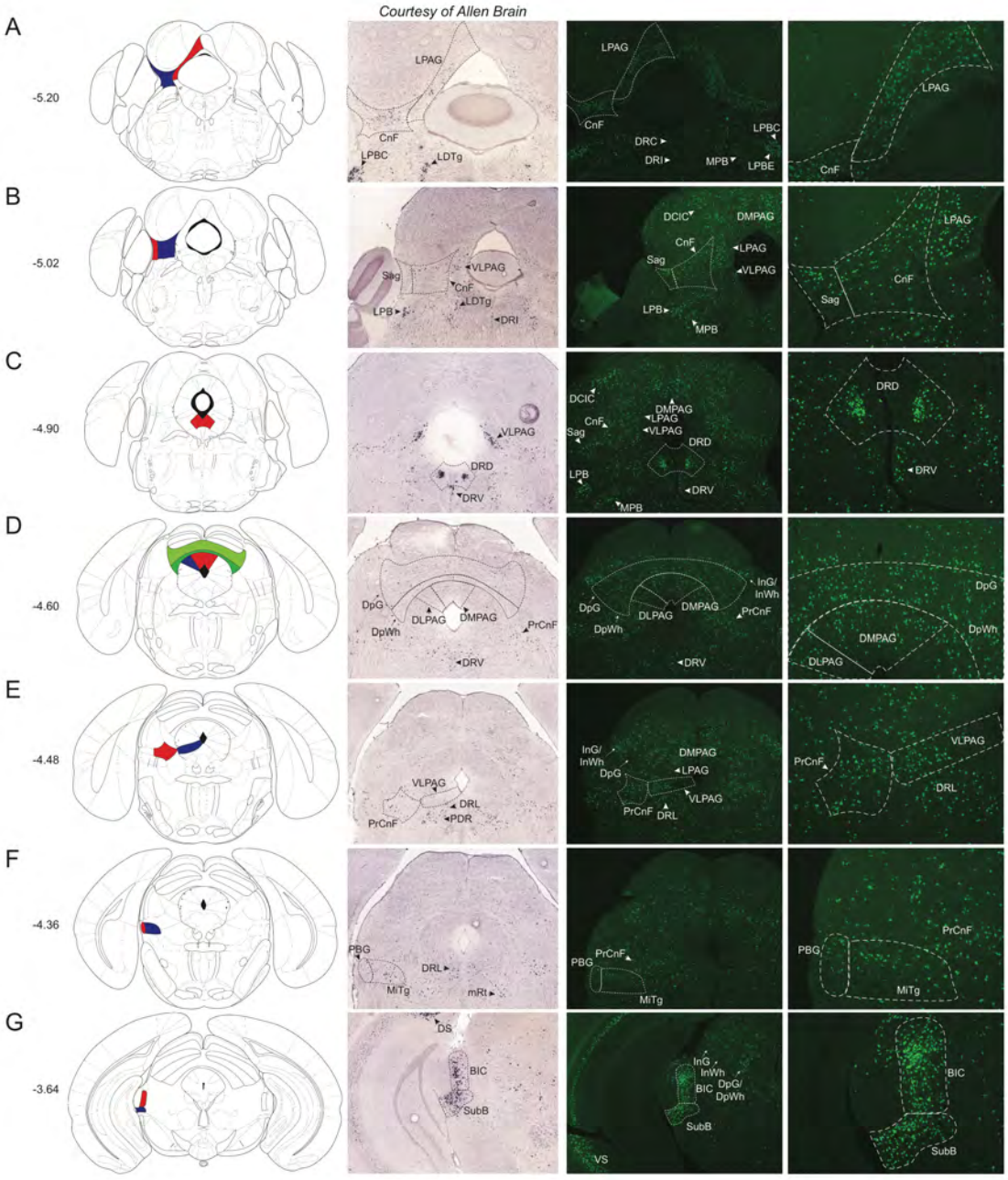


Midbrain

We observed many Nts-GFP neurons evenly scattered throughout the periaqueductal gray (PAG), including within the lateral (LPAG- **Figure 2.2A**), dorso-lateral and dorso–medial (DLPAG/DMPAG - **Figure 2.2D**), and the ventrolateral (VLPAG- **Figure 2.2E**) sub-regions. Interestingly, only the caudal VLPAG exhibited significant *Nts*-ISH (**Figure 2.2B and C**), whereas *Nts*-ISH was absent from the DLPAG and DMPAG (**Figure 2.2D**). This discrepancy between the distributions of Nts-GFP and *Nts*-ISH may signify that Nts is transiently expressed throughout most of the PAG during development, but only in adult neurons of the VLPAG. In contrast, the adjacent cuneiform nucleus (CnF) contains a dense population of Nts-GFP neurons as well as *Nts*-ISH (**Figure 2.2A and 2.2B**). A large, dense cluster of Nts-GFP neurons and *Nts*-ISH was found within the dorsal aspect of the dorsal raphe nucleus (DRD) that lies ventral to the cerebral aqueduct (**Figure 2.2C**). Nts-GFP neurons and corresponding *Nts*-ISH were also found, but more evenly distributed, within the lateral and ventral aspects of the dorsal raphe (DRL and DRV) (**Figure 2.2C-E**). Two regions with particularly dense distributions of Nts-GFP neurons and *Nts*-ISH included the subbrachial nucleus (SubB) and the nucleus of the brachium of the inferior colliculus (BIC) (**Figure 2.2G**). Other midbrain regions with sizable, yet evenly dispersed Nts-GFP neurons and *Nts*-ISH, include the Sagulum (Sag) (**Figure 2.2B**), the deep gray and white layers of the superior colliculus (DpG) (**Figure 2.2D**), the precuneiform area (PrCnF) (**Figure 2.2E**), the parabigeminal nucleus (PBG) (**Figure 2.2F**), and the microcellular tegmental nucleus (MiTg) (**Figure 2.2F**). Midbrain structures with more moderate densities of Nts-GFP neurons (with qualitative density ratings of ++/+++ or

+++) include the dorsal cortex of the inferior colliculus (DCIC), lateral lemniscus (ll), subpeduncular tegmental nucleus (SPTg), located just beneath the decussation of the superior cerebellar peduncle (scp), the intermediate gray and white layers of the superior colliculus (InG / InWh), the oculomotor nucleus (3N) and associated structures, the mesencephalic reticular formation (mRT) and the nucleus of Darkschewitsch (Dk) (**Table 2.1**). At the transition between the midbrain and caudal hypothalamus we also observed scattered Nts-GFP neurons and *Nts*-ISH within the Substantia Nigra Compacta (SNC).

Figure 2.2. Nts-GFP and *Nts*-ISH in the Midbrain. From left to right, each row contains a Bregma-numbered atlas image [46], an image of *Nts*-ISH at the same Bregma level, courtesy of the Allen Brain Atlas [44], a 4x image of Nts-GFP neurons, and a 10x image of Nts-GFP neurons from the same area. Red, blue, and green shaded areas in the atlas image are outlined in the Nts-GFP images. **A)** Bregma -5.20, **B)** Bregma -5.02, **C)** Bregma -4.90, **D)** Bregma -4.60, **E)** Bregma -4.48, **F)** Bregma -4.36, **G)** Bregma -3.64.

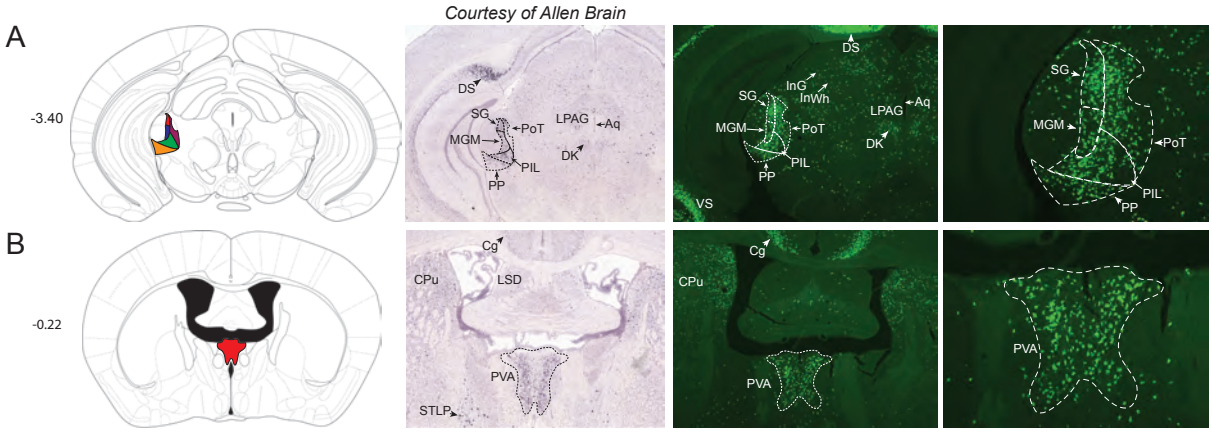


Thalamus

Overall, there were few significant Nts-GFP clusters observed in the thalamus of *Nts^{Cre};GFP* mice compared to other brain areas. Dense populations of Nts-GFP neurons were observed within the medial aspect of the medial geniculate nucleus (MGM), the triangular posterior thalamic nucleus (PoT), the posterior intralaminar thalamic nucleus (PIL), the peripeduncular nucleus (PP), and the suprageniculate thalamic nucleus (SG) (**Figure 2.3A**). Apart from the PoT, these thalamic structures contained ample *Nts*-ISH and, hence, actively express *Nts* in the adult brain. Another concentrated population of Nts-GFP neurons and a similar distribution of *Nts*-ISH was observed within the anterior paraventricular thalamic nucleus (PVA) (**Figure 2.3B**). The density of Nts-GFP neurons increased over the caudal to rostral extent of the PVA, such that the Nts-GFP neurons were most abundant in the rostral aspect. Other thalamic regions contained more modest populations of Nts-GFP neurons, and these areas included the ventral part of the medial geniculate nucleus (MGV), the mediocaudal LP thalamic nucleus (LPMC), the central medial thalamic nucleus (CM), the subparafascicular thalamic nucleus (SPF), and the intermediodorsal thalamic nucleus (IMD) (refer to **Table 2.1**). While the intermediodorsal (IMD) and central medial (CM) thalamic nuclei contain many Nts-GFP neurons, these regions lack comparable *Nts*-ISH**.** Despite reports indicating the presence of Nts-IR fibers within these medial thalamic structures [40,47], the failure to detect Nts-IR soma or *Nts* ISH in these sites together with our data suggests that Nts is only transiently expressed during the development of these thalamic neurons. One notable exception are the visible clusters of Nts-GFP and *Nts*-ISH cells observed in the Xiphoid and Paraxiphoid nuclei of Thalamus (Xi and PaXi);

however, these structures are more closely associated with the hypothalamus. Hence, Nts may play an important role in the development of the thalamus; however, Nts signaling may only be maintained during adulthood in select thalamic cells.

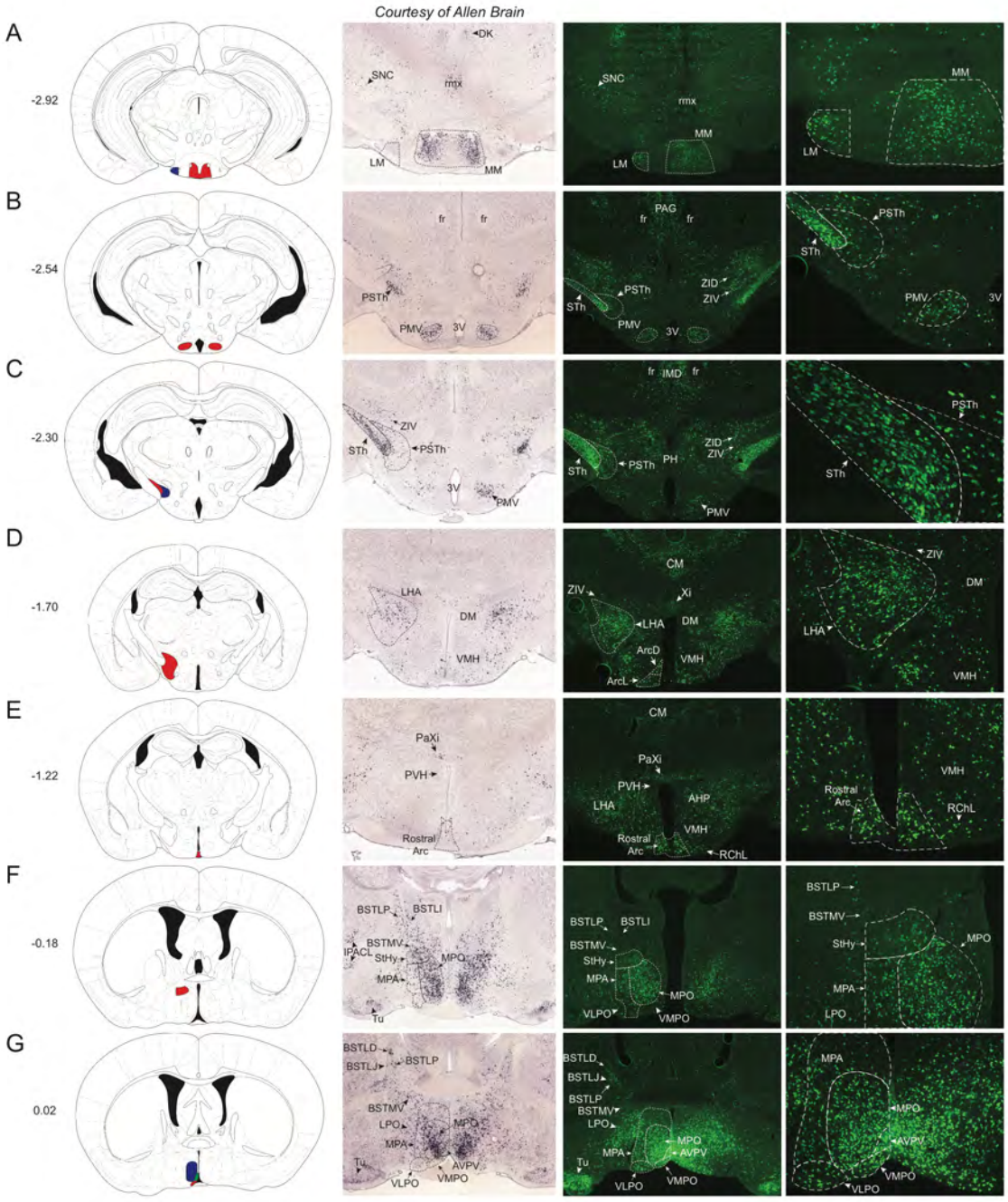
Figure 2.3. Nts-GFP and *Nts*-ISH in the Thalamus. From left to right, each row contains a Bregma-numbered atlas image [46], an image of *Nts*-ISH at the same Bregma level, courtesy of the Allen Brain Atlas [44], a 4x image of Nts-GFP neurons, and a 10x image of Nts-GFP neurons from the same area. Shaded areas in the atlas image are outlined in the Nts-GFP images. **A)** Bregma -3.40 and **B)** Bregma -0.22.



Hypothalamus

Starting at the caudal extent of the hypothalamus, we observed many Nts-GFP neurons in the medial and lateral regions of the mammillary nucleus (MM and LM), yet, comparable *Nts*-ISH was only observed in the MM and was absent from the LM (**Figure 2.4A**). The ventral premammillary nucleus (PMV) contained a distinct cluster of Nts-GFP neurons consistent with *Nts*-ISH data (**Figure 2.4B**). In addition, a densely packed population of Nts-GFP neurons and comparable *Nts*-ISH were apparent within the subthalamic nucleus (STh) (**Figure 2.4C**). The adjacent parasubthalamic nucleus (PSTh) contained more sparsely distributed Nts-GFP and *Nts*-ISH-identified neurons (**Figure 2.4C**). Just above these regions lie the ventral and dorsal portions of the Zona Incerta (ZIV, ZID), which contained sparse Nts-GFP neurons and similar distributions of *Nts*-ISH (**Figure 2.4C**). Moving rostrally, the next large population of Nts-GFP neurons and *Nts*-ISH was found within the lateral hypothalamic area (LHA) (**Figure 2.4D**). Nts-GFP neurons were also noted within the rostral arcuate nucleus (Arc), a region essential for regulating energy balance; however, sparse *Nts*-ISH was observed in this structure (**Figure 2.4E**). Other mediobasal areas that modulate energy balance, such as the ventromedial and dorsomedial hypothalamic nuclei (VMH and DM), contained scattered Nts-GFP neurons but little observable *Nts*-ISH (**Figure 2.4D**). Notably, the paraventricular nucleus of the hypothalamus (PVH) was virtually devoid of Nts-GFP cells and *Nts* ISH (**Figure 2.4E**), which is interesting given the known cellular heterogeneity of this brain area and its importance in energy balance.

Figure 2.4. Nts-GFP and *Nts*-ISH in the Hypothalamus. From left to right, each row contains a Bregma-numbered atlas image [46], an image of *Nts*-ISH at the same Bregma level, courtesy of the Allen Brain Atlas [44], a 4x image of Nts-GFP neurons, and a 10x image of Nts-GFP neurons from the same area. Shaded areas in the atlas image are outlined in the Nts-GFP images. **A)** Bregma -2.92, **B)** Bregma -2.54, **C)** Bregma -2.30, **D)** Bregma -1.70, **E)** Bregma -1.22, **F)** Bregma -0.18 and **G)** Bregma +0.02.



The rostral-medial hypothalamus harbored abundant Nts-GFP neurons, notably within the striohypothalamic nucleus (StHy), the medial preoptic nucleus (MPO), the ventromedial preoptic nucleus (VMPO), and the anteroventral periventricular nucleus (AVPV) (**Figure 2.4F and 2.4G**). Indeed, the sheer density of tightly-packed Nts-GFP cells in the MPO and AVPV made it difficult to resolve individual neurons. The *Nts*-ISH distribution matches that of the Nts-GFP cells within the MPO, but is less pronounced in the StHy, VMPO, and AVPV (**Figure 2.4F and 2.4G**). More modestly-sized populations of Nts-GFP neurons were found within the posterior aspect of the anterior hypothalamic area (AHP), posterior hypothalamus (PH), lateral preoptic nucleus (LPO), ventrolateral preoptic nucleus (VLPO), septohypothalamic nucleus (SHy), and parastrial nucleus (PS) and comparably less *Nts* ISH was present in these regions relative to Nts-GFP cells (**Table 2.1**).

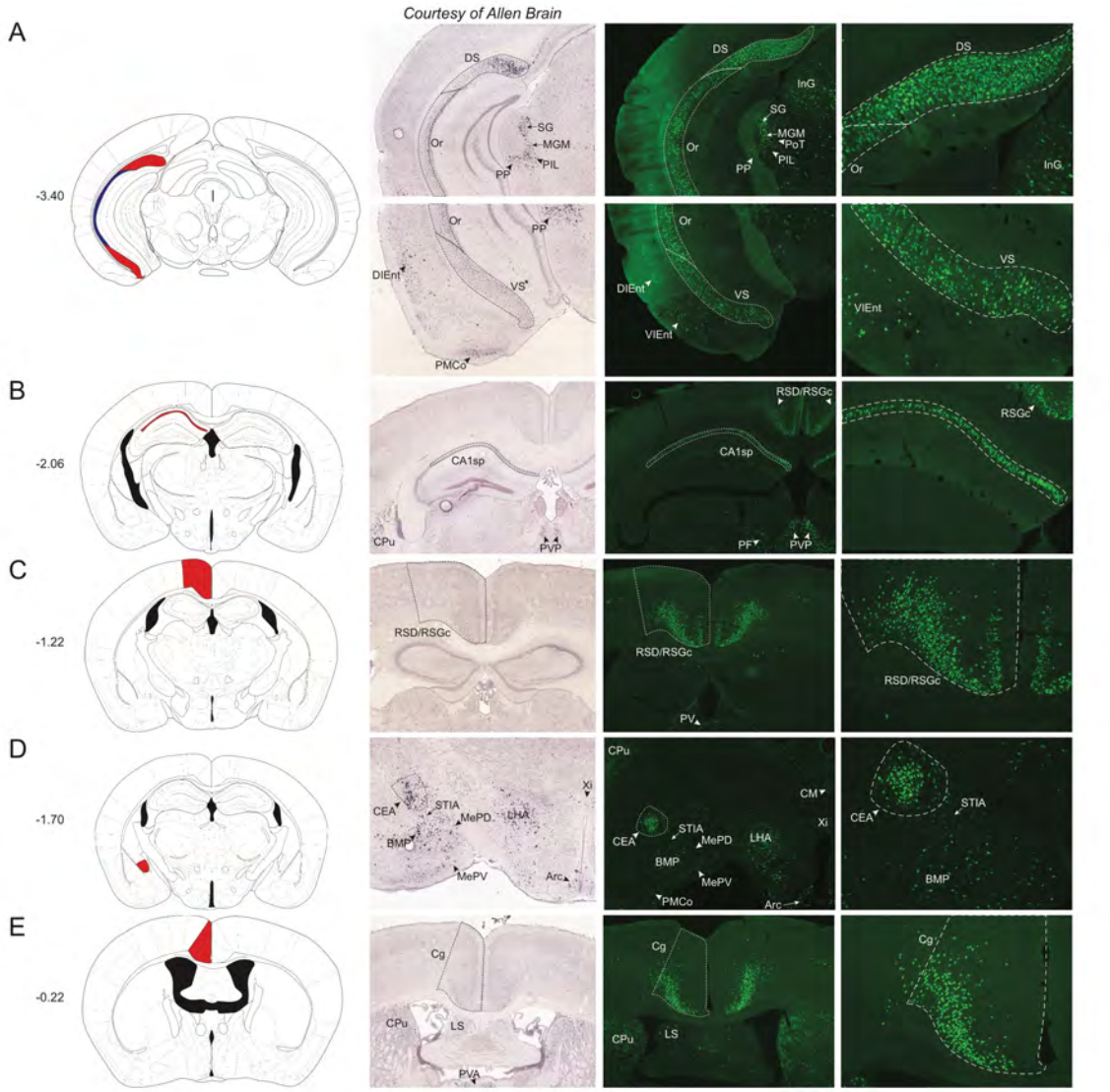
The bed nucleus of the stria terminalis (BNST) complex lies dorsal to the POA and contained a moderate number of Nts-GFP and *Nts*-ISH labeled cells. These cells were mostly scattered throughout the lateral division, including the intermediate (BSTLI), posterior (BSTLP), dorsal (BSTLD) and juxtacapsular (BSTLJ) parts (**Figure 2.4F and 2.4G**). The medial division of the ventral aspect of the BNST (BSTMV) also contained considerable dispersed Nts-GFP and *Nts*-ISH-labeled cells. Intriguingly, the BNST was the rare brain region in which *Nts*-ISH intensity was more robust, and perhaps slightly more abundant, than the corresponding Nts-GFP labeling; this was particularly true for the BSTLD and BSTLI (**Figure 2.4F).**

Cerebral Cortex

Compared to the broad distribution of Nts-GFP neurons throughout the bulk of the hypothalamus, the hippocampus contained more restricted populations of Nts-GFP neurons. Notably, the dorsal and ventral subiculum portions of the hippocampal formation (DS and VS) harbor numerous Nts-GFP neurons (**Figure 2.5A**). *Nts*-ISH is also prominent within the DS, but not the VS (**Figure 2.5A**). The hippocampal CA1 pyramidal cell layer encompasses many Nts-GFP neurons, and these neurons are localized primarily within the caudal aspects of the structure up through the level of the DM (**Figure 2.5B**). Since much less *Nts*-ISH is apparent in the CA1 region from the Allen Brain Atlas, there may be a reduction in the number of mature neurons that continue to express Nts in this region (**Figure 2.5B**). The markedly higher density and distribution of Nts-GFP cells in the VS and CA1 compared to *Nts*-ISH suggests that Nts is expressed developmentally throughout the hippocampus, but expression is not sustained in the VS or CA1 of adult mice.

The distribution of cortical Nts-GFP was also fairly circumscribed, as it was limited to the retrosplenial (RSD and RSGc) and cingulate (Cg) regions (**Figure 2.5B, C and E)**. Sizable populations of Nts-GFP neurons were observed in these regions, but *Nts*-ISH was undetectable (**Figure 2.5B, C and E and Figure 2.3B).** A striking, large population of Nts-GFP neurons was confined within the Cg, but *Nts* ISH was very low and virtually undetectable in this region (**Figure 2.5E and 2.5B**). As with the hippocampus, these data hint that Nts provides a primarily developmental role in the cortex and that it is not an active neuropeptide signal within the adult cortical regions.

Figure 2.5. Nts-GFP and *Nts*-ISH in the Cortex. From left to right, each row contains a Bregma-numbered atlas image [46], an image of *Nts*-ISH at the same Bregma level, courtesy of the Allen Brain Atlas [44], a 4x image of Nts-GFP neurons, and a 10x image of Nts-GFP neurons from the same area. Shaded areas in the atlas image are outlined in the Nts-GFP images. **A)** Bregma -3.40, **B)** Bregma -2.06, **C)** Bregma -1.22, **D)** Bregma -1.70, **E)** Bregma -0.22.

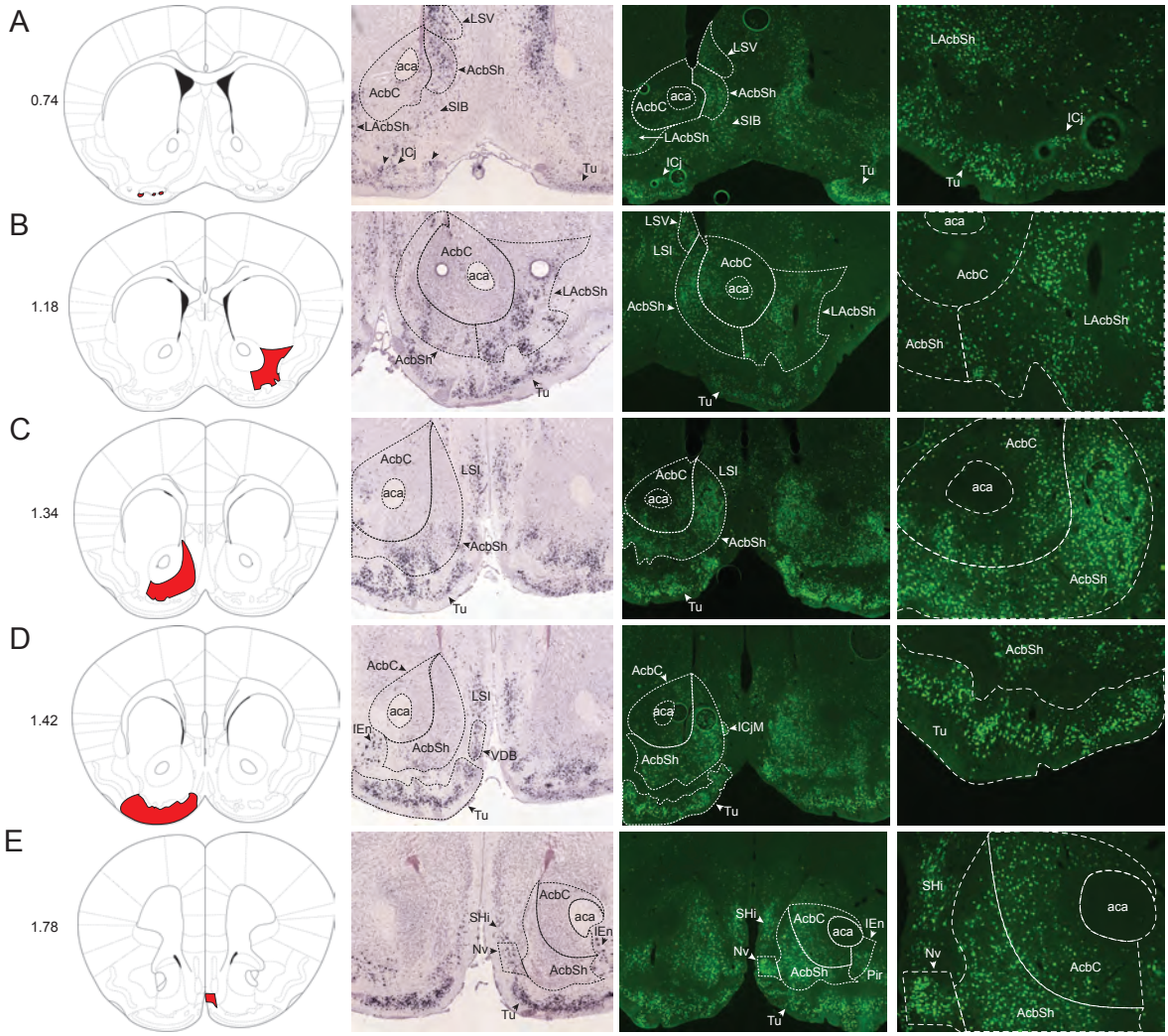


Within the amygdala, only the CEA possessed a significant cluster of Nts-GFP neurons (**Figure 2.5D**). In agreement, intense *Nts*-ISH labeling was observed within the caudal CEA (**Figure 2.5D**). A number of amygdala-associated structures contained more moderate, but still considerable amounts, of Nts neurons (++/+++ -> +++). These structures included the amygdalopiriform transition area (APir), ventral aspect of the basolateral amygdalar nucleus (BLV), anterior aspect of the basomedial amygdalar nucleus (BMA), cortical amygdalar area (COA), intraamygdalar division of the stria terminalis (STIA), cortex-amygdala transition area (CxA), anterodorsal and anteroventral medial amygdalar nucleus (MeAD/MeAV), main intercalated amygdalar nucleus (IM), and sublenticular extended amygdala (EA) (**Table 2.1**). Of these structures, comparable *Nts*-ISH labeling within the Allen Brain Atlas was observed for the BLV, BMA, and STIA, while slightly lower levels of Nts ISH (+/++) was detected within the COA, CxA, and MeAV (**Table 2.1**).

Striatum, Pallidum, and Forebrain

We observed extensive Nts-GFP and *Nts*-ISH in the mouse ventral striatum, which broadly consists of the olfactory tubercle (Tu) and the nucleus accumbens (Acb). Abundant Nts-GFP neurons were found throughout the rostrocaudal extent of the olfactory tubercle (Tu), including within clusters of neurons known as the islands of cajella (ICj) (**Figure 2.6A**). *Nts*-ISH was very intense and mostly similar in distribution to the Nts-GFP neurons throughout the Tu and ICj. The Acb also contained numerous Nts-GFP neurons and *Nts*-ISH that was predominantly located within the medial and lateral shell (AcbSh and LAcbSh) with a more minor population residing in the nucleus accumbens core (AcbC) (**Figure 2.6A-E**). The density of Nts-GFP neurons was greatest at the very medial aspect of the AcbC (+++), whereas this density is much lower within the lateral core (+). Other structures within the striatum and pallidum contained smaller but still considerable densities of Nts-GFP neurons (++/+++ or +++). Notably, these structures include the caudate putamen (CPu), globus pallidus (internal)/entopeduncular nucleus (EP), anterior and ventral aspects of medial portion of the bed nucleus of the stria terminalis (BSTMA/BSTMV), interstitial nucleus of the posterior limb of the anterior commissure (IPAC), lateral septal nucleus (LS), the substantia innominata (SIB), and septohippocampal nucleus (SHi) (**Table 2.1**). Within the CPu, the highest density of Nts-GFP neurons (+++) was found in the caudal aspect of the region spanning between the levels of the rostral Arc and the caudal MPO **(Figure 2.3B and Figure 2.5D, E)**. The CPu, STMA and STMV, IPAC, and LS sub-regions had *Nts*-ISH densities comparable to the observed distribution of Nts-GFP neurons (**Table 2.1**). The navicular postolfactory nucleus (Nv) was the rostral-most

Figure 2.6. Nts-GFP and *Nts*-ISH in the Forebrain. From left to right, each row contains a Bregma-numbered atlas image [46], an image of Nts-ISH at the same Bregma level, courtesy of the Allen Brain Atlas [44], a 4x image of Nts-GFP neurons, and a 10x image of Nts-GFP neurons from the same area. Shaded areas in the atlas image are outlined in the Nts-GFP images. **A)** Bregma +0.74, **B)** Bregma +1.18, **C)** Bregma +1.34, **D)** Bregma +1.42, **E)** Bregma +1.78.



structure with a large Nts-GFP population (**Figure 2.6E**).

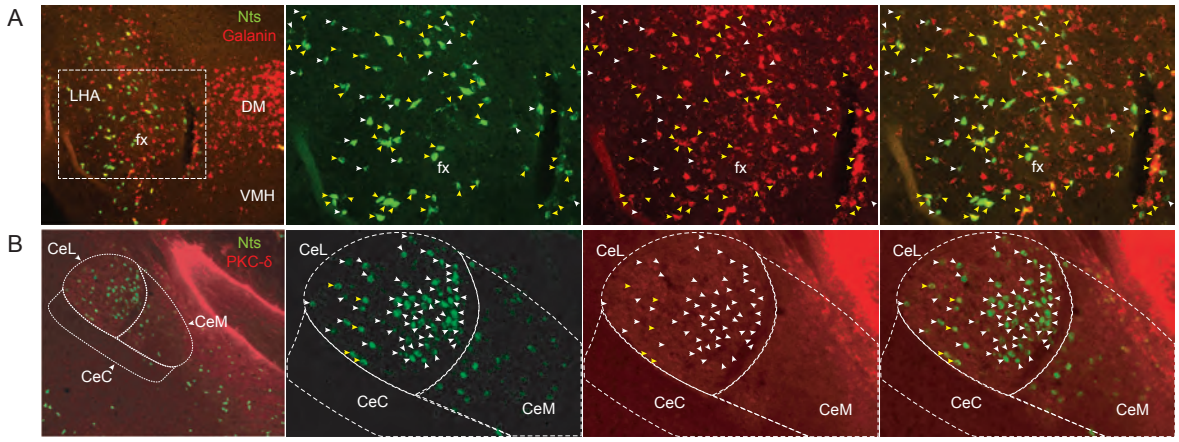
The only notable discrepancy between forebrain Nts-GFP and *Nts*-ISH distributions was in the ventral tenia tecta (VTT) and the medial portion of the anterior olfactory area (AOM), which are olfactory structures contained within the rostral-most aspect of the brain (**Table 1**). While the VTT and AOM contained moderate densities of Nts-GFP neurons, no detectable *Nts*-ISH was present in either structure.

Heterogeneity of Nts Neurons Within Brain Regions

We were struck by the observation of very dense populations of Nts-GFP neurons within the LHA and CEA, regions known to contain multiple molecularly-distinct neuronal populations that exert unique modulation of feeding. We therefore reasoned that Nts-expressing neurons in the LHA and CEA may not be homogeneous and might differ in their expression of other neuropeptides or molecular markers that would provide clues as to their function.

We first tested this hypothesis in the LHA by examining the co-distribution of the neuropeptides Nts and Gal. LHA neurons expressing anorectic Nts are alleged to overlap with the same neuronal population that expresses orexigenic Gal, which was determined by analyzing IR for Nts and Gal in colchicine-treated mice [48]. Yet, subsequent studies showed that LHA Nts and LHA Gal neurons differ in projection targets and physiologic regulation of feeding and behavior, suggesting they may not be a fully overlapping population [23,49]. To examine this possibility, we performed dual ISH for *Nts* and *Gal* (**Figure 2.7A**), thus bypassing the requirement for colchicine treatment, and potentially interrupted anterograde transport that might jeopardize cell health and alter gene expression. Using dual ISH, we observed robust *Gal* throughout the DM, but no *Nts* was found in this structure (**Figure 2.5A**). These findings are consistent with prior ISH [44] and the dearth of Nts-GFP cells in the DM (**Figure 2.4**). By contrast, we noted ample distributions of *Gal*-positive and *Nts*-positive cells within the LHA. While many LHA neurons contained high levels of both *Gal* and *Nts* (**Figure 2.7A, yellow arrows**), many *Gal* neurons did not overlap with Nts neurons. Moreover,

Figure 2.7. Heterogeneity of Nts Neurons Within the LHA and CEA. From left to right, each row contains a 10x image of merged red and green channels, followed by 20X images of green, red, and merged channels. **A)** RNA Scope dual-fluorescent in situ hybridization for Nts (green) and Galanin (Gal, red) in the LHA. Yellow arrows identify neurons expressing Nts and robust levels of Gal. White arrows identify neurons expressing Nts and negligible Gal. **B)** Section of the CEA from an Nts^{Cre};GFP mouse immunostained for GFP (Nts-GFP, green) and PKC-δ (red). Yellow arrows identify the few neurons co-labeled with Nts-GFP and PKC-δ, which lie primarily within the lateral aspect of the CEA (CeL). White arrows identify CEA neurons that contain Nts but no detectable PKC-δ. *LHA= Lateral Hypothalamus, fx=fornix, DM=Dorsomedial Hypothalamic nucleus, VMH=Ventromedial Hypothalamic nucleus, CeL=Central Amygdalar nucleus, lateral , CeC=Central Amygdalar nucleus, central , CeM=Central Amygdalar nucleus, medial.*



we also identified *Nts*-labeled neurons that completely lacked or had negligible *Gal* signal (white arrowheads) (**Figure 2.7A, white arrows**). These data suggest that LHA Nts neurons are heterogeneous and that there are at least two subpopulations of Nts neurons in this structure, one of which robustly co-expresses *Gal* and the other of which does not.

We next examined the CEA, where Nts and protein kinase c-δ (PKC-δ), both of which are implicated in anorexic behavior, have been localized [6,23,50,51]. To investigate whether these purported anorectic proteins overlap spatially, we examined PKC-δ immunoreactivity (IR) within the CEA of *Nts^{Cre};GFP* mice. This analysis revealed a few CEA Nts-GFP cells that also contained PKC-δ-IR (**Figure 2.7B, yellow arrows**), but the majority of CEA Nts-GFP and PKC-δ neurons were largely separate and did not overlap (**Figure 2.7B, white arrows**). Interestingly, most Nts-GFP neurons were found within the medial aspect of the CeL subregion of the CEA, and these did not colocalize with PKC-δ-IR. A small number of Nts-GFP neurons found within the lateral aspect of the CeL, however, did co-express PKC-δIR (**Figure 2.7**). These data corroborate recent literature showing that very little *Nts*-ISH overlaps with PKC-δ within the CeL [52] and further demonstrates that *Nts^{Cre};GFP* mice can be useful to both identify Nts neurons and to define their molecular phenotype.

Discussion

Importance of Mapping Nts Neurons in the Mouse Brain

A major goal of neuroscience is to understand how molecularly- and regionally-specified neuronal populations coordinate behavior and physiology. Because central Nts mediates a diverse array of physiologic responses depending on where it is administered in the brain (analgesia, regulation of body temperature, suppression of feeding, locomotor activity, vasodepressor response), it is likely that regionally-defined populations of Nts neurons coordinate specific functions. Characterizing the roles of these distributed Nts populations requires the ability to identify and then manipulate them *in vivo* to reveal how they mediate behavior and biology. While the use of ISH and colchicine-mediated Nts-IR has been valuable to identify Nts neurons, primarily in rats, these methods don't permit subsequent manipulation of neurons of interest. By contrast, the recombinase-mediated labeling of Nts neurons that occurs in *Nts^{Cre};GFP* mice facilitates Nts neuron detection and permits their manipulation using widely available Cre-Lox tools. Indeed, this approach has already been successful in establishing that Nts neurons in the POA vs. the LHA modulate social and feeding behaviors, respectively (McHenry et al., 2017; Woodworth et al., 2017b). Much, however, remains to be learned about Nts-mediated physiology. Given the differences in Nts expression and brain architecture between rodents [53,54], prior descriptions of Nts-expressing neurons in rats may not translate to *Nts^{Cre};GFP* mice or may not be reliable for guiding function-directed studies. Our work here thus fills a critical gap by providing an "Nts-GFP atlas" that investigators can use to identify Nts populations and then systematically test their function in *Nts^{Cre};GFP* mice.

Important Considerations in Using *Nts^{Cre};GFP* mice to Study Nts Neurons

Nts^{Cre} mice are engineered so that Cre expression is an excellent proxy of which cells are actively expressing Nts. However, as with any knock-in recombinase mouse model, once *Nts^{Cre}* mice are bred onto a Cre-inducible reporter line, Cre expressed at any point during development causes recombination and permanent reporter labeling. Thus, while Cre-inducible expression of reporters like GFP are ideal to permit cell detection, immediate recombination upon Cre expression prevents discrimination of which cells transiently expressed Nts/Cre during development vs. those that actively express them in adult cells. This confound must be considered when examining *Nts^{Cre};GFP* mice. Since Nts expression differs within the neonatal, postnatal, and adult brain of rats [55–57], it is likely that there is also some Nts-dependent ontogeny in the mouse brain. Moreover, because Nts receptors are broadly expressed in the developing rat brain but their expression becomes more circumscribed in maturity [58,59], it is possible that the Nts system exerts different functions during the formation of neural circuits as compared to signaling in the mature brain. Hence, prior to performing any manipulations of Nts-GFP neurons in adult *Nts^{Cre};GFP* mice, it is important to verify whether the cells in question are actively expressing Cre/Nts, or whether they were labeled during development. Only cells actively expressing Cre can be modulated using Cre-Lox methodologies. For this reason, we compared the distribution of Nts-GFP neurons with adult *Nts*-ISH provided by the Allen Brain Institute [44], reasoning that any sites of Nts-GFP neurons that lack *Nts*-ISH represent Nts-GFP populations that transiently expressed Nts and underwent recombination during development but do not actively express Nts in adulthood. We noted several brain

areas with discrepant Nts-GFP and *Nts* ISH profiles (see **Table 2.1**) and have pointed them out in the text. We acknowledge, however, that the Allen Brain *Nts*-ISH may not perfectly represent *Nts* expression, as technical artifacts or probe sensitivity could result in under-detection of *Nts*-expressing neurons. Additionally, the Allen Brain *Nts* ISH data are derived from a single sample, and, thus, caution should be taken when drawing conclusions from differences between Allen Brain data and the *Nts^{Cre};GFP* mouse. Consequently, the absence of *Nts*-ISH in areas with Nts-GFP neurons should not be taken as absolute confirmation of their "developmental" profile or that that they do not express Cre/Nts during maturity. Investigators interested in Nts-GFP neurons in these regions, however, may wish to confirm levels of Cre/Nts expression. This can easily be done by injecting Cre-inducible reagents into adult *Nts^{Cre};GFP* mice at sites of interest, and only neurons actively expressing Nts will express Cre and undergo recombination.

One additional consideration is that we characterized the distribution of Nts-GFP neurons from the brains of adult male *Nts^{Cre};GFP* mice, but it is possible that the distribution and/or relative density of Nts-GFP populations may differ in females. Going forward, investigators should validate the distributions of Nts neurons in areas of interest in both sexes, particularly if they are studying the role of Nts in physiology with known sex differences. For example, loss of function Nts variants have been discovered in individuals with anorexia nervosa, a type of eating disorder that is more prevalent in females than males. It is possible that differences in Nts expression or function might contribute to the development and sex difference of eating disorders, though this has yet to be mechanistically examined [60]. Additionally, males and

females also exhibit differences in pain processing; hence, there may be differences in Nts signaling that underline sexual dimorphism in pain sensing and analgesia [61].

Possible Roles of Nts-GFP Neurons in the Hindbrain

Consistent with the distribution of Nts-IR cell bodies in the hindbrain of rats, we observed populations of mouse Nts-GFP neurons in similar hindbrain regions that are implicated in control of satiety, including the PB/LPB, the NTS, and the AP [38,40,62,63]. Some NTS Nts neurons co-express the anorectic neuropeptide cholecystokinin (CCK) [64], and since activation of these neural CCK projections to the PB suppresses appetite [65,66], it is likely that NTS Nts neurons also contribute to decreased feeding. Additionally, vagal afferents to the NTS convey visceral sensory information, such as gut distention and satiety signals released after ingestion of a meal, that can then be relayed to the PB [65]. It is possible that the dense population of Nts neurons in the LPB may receive such information and also contribute to the Nts-mediated anorectic effect. The Nts-GFP neurons that we observed in the LPB likely correspond to previously reported LPBE Nts cells that project to the CEA [67], although it remains to be determined if this specific circuit modulates feeding.

The location of Nts-GFP neurons in the NTS and PB suggests that they might also contribute to the cardiovascular effects of Nts. Indeed, carotid sinus and aortic nerve afferents terminate in the dorsomedial NTS where a modest number of Nts-GFP perikarya exist (**Table 2.1**) [62]. Direct infusion of Nts into the NTS elicits hypotension and bradycardia, further indicating that this peptide enhances the baroreceptor reflex

[68]. The NTS also relays visceral sensory information from baroreceptors and chemoreceptors [67,68] to the PB and CEA, which in turn modulate cardiovascular changes. Thus, Nts action via the NTS and/or PB could conceivably contribute to the hypotensive effect observed after systemic or hindbrain Nts administration [69].

The distributions of Nts-GFP neurons observed in other hindbrain regions diverge somewhat from prior reports, so it is difficult to speculate on their potential roles. The numerous Nts-GFP neurons observed in the caudal Sp5C, DC and LPB agrees with previous reports of Nts expression in these regions [38,40,43]. However, to our knowledge, we are the first to detect Nts-GFP neurons in the facial nucleus (7N), the A5 noradrenaline cell group (A5), or the PR5. We also observed a dense pocket of Nts-GFP neurons in the 12N of adult mice, and *Nts*-ISH corroborates that this an actively expressing Nts population within the adult mouse brain [44]. These data were somewhat surprising, however, since adult rats do not have detectable 12N Nts neurons [38–42]. In rats, Nts is transiently expressed within the 12N at birth, but expression decreases dramatically between postnatal days 4 and 7 and is sparse or undetectable by adulthood [70]. Yet, the persistence of *Nts*-ISH in the adult mouse 12N suggests that Nts is being actively expressed from this population and may signal via release of Nts.

Possible Roles of Nts-GFP Neurons in the Midbrain

To our knowledge, Nts neurons have not been previously described within the midbrain Sag, PBG, and SubB, and hence the physiologic roles for these neurons remain unknown. In general, midbrain Nts signaling has been implicated in pain processing, locomotor activity, and other motivated behaviors, and the PAG and DRD are thought to play important roles in pain responses. The distribution of Nts-GFP neurons and *Nts*-ISH within the mouse DRD is consistent with previous reports [38,40,43,71]. Since Nts-expression increases in the and DR and MiTg of rats subjected to chronic pain, it is possible that mouse Nts-GFP neurons identified in these regions are also involved in pain processing [72]. We also observed a larger, more uniform distribution of Nts-GFP neurons in the PAG, particularly within the caudal extent, and this skewed caudal distribution is consistent with previous observations of Nts-IR cell bodies lying primarily within the caudal PAG [73]. Excluding the VLPAG, the absent or very minimal amount of *Nts*-ISH signal throughout the majority of the mouse PAG hints at transient Nts expression during development and that Nts may not be expressed in the majority of adult mouse PAG neurons. Only the mouse VLPAG contained numerous Nts-GFP neurons and *Nts*-ISH, and this confined expression may correspond with the moderately dense populations of PAG Nts neurons described in rats [38] and guinea pigs [43]. Since the PAG activates brainstem structures that modulate pain suppression circuits in the spinal cord [73,74], PAG Nts neurons might contribute to the analgesic effects of central Nts injection [30]. For example, PAG Nts neurons in rats densely project to the nucleus raphe magnus (NRM), which is known to induce profound analgesia when stimulated [74], and Nts administration in the NRM

inhibits the tail-flick response [74,75]. Thus, PAG-derived Nts released in the NRM might contribute to antinociception [75]. The Nts-GFP and *Nts* ISH-labeled neurons found in the adjacent CnF might also contribute to Nts-mediated analgesia, since this region is responsive to nociceptive input [76]. While the CnF also provides Nts afferents to the NRM analgesia center [74], the CnF is typically regarded as part of the mesencephalic locomotor region implicated in locomotor control [77]. Since central administration of Nts reduces locomotor activity, CnF Nts may contribute to modulation of ambulatory activity [9]. To date, however, the role of CnF, as well as of DRD and VLPAG, Nts neurons remains to be defined. This could be achieved in the future by use of site-directed genetic tools in Nts^{Cre};GFP mice. Such technologies would permit the manipulation of these distinct midbrain Nts populations, and would thereby reveal their specific contributions to analgesia, locomotor activity, or other aspects of physiology.

Possible Roles of Nts-GFP Neurons in the Thalamus

Nts-expressing neurons have been previously reported within the geniculate nucleus of the mouse, and this is consistent with our finding of a dense population of MGM Nts-GFP neurons [54]; however Nts neurons have not been found in the MGM of rats [54]. Together, these data validate that there are divergent Nts signaling systems between rodent models [36]. Apart from the MGM, the expression of Nts and its role within thalamic nuclei has been virtually unexplored. A dense Nts-GFP neuronal population was present in a grouping of thalamic nuclei, including the MGM, SG, PIL, and PP, and these nuclei comprise a multimodal region designated the caudal

paralaminar nuclei, which receives and integrates diverse auditory, visual, and somatosensory inputs [78]. Thus, Nts-GFP neurons in the caudal paralaminar nuclei may conceivably contribute to processing auditory and visual inputs and relaying this information to the cortex or higher order structures. We identified a substantial population of Nts-GFP neurons in the paraventricular thalamic nucleus (PV), with the highest density noted in the PVA subregion, and this assessment agrees with reports of Nts neurons in the PV of the rat [40,79] and the Japanese monkey [80]. Nts-GFP neurons were also relatively abundant in the posterior paraventricular thalamic nucleus (PVP), which contained a slightly lower density of Nts-GFP neurons than the PVA (**Table 2.1**). In general, many PV Nts neurons project to the BNST, a region implicated in mediating reward behavior [81]. While the precise role of Nts within the PVA has yet to be explored, Nts signaling in the PVP decreases ethanol consumption, as injection of Nts or Nts agonist in the PVP reduces ethanol intake whereas injection of Nts receptor antagonist promotes consumption, and Nts levels correlate inversely with excessive ethanol intake [82,83]. While these data provide some clue as to the function of Nts neurons in the PVP, there has been no indication as to the function of PVA Nts neurons, and future work to modulate this large thalamic Nts-GFP population will reveal to what extent Nts neurons contribute to stress, anxiety and fear-related behaviors, as well as food intake, drug addiction, and other motivated behaviors modulated by the paraventricular thalamus [84].

Possible Roles of Nts-GFP Neurons in the Hypothalamus

Consistent with the initial discovery and isolation of Nts from the hypothalamus, we observed many Nts-GFP neurons distributed throughout hypothalamic subregions that largely, but not wholly, matches the *Nts*-ISH distribution. For example, while we noted many Nts-GFP neurons in the adult mouse LM, the absence of *Nts* ISH in this substructure suggests that LM neurons do not continue to express Nts during adulthood. In addition, while the MM of birds has been detailed to contain Nts neurons [85], rats do not express Nts in the mammillary body [39], The mammillary body of both rats and humans does receive Nts input indirectly from the subiculum, and Nts injection within the mammillary body itself increases avoidance latency of passive avoidance behavior [86–88]. Whatever the source of Nts to the mammillary body (local or via projections), these findings indicate it may contribute to learned behavior; nevertheless, this has yet to be rigorously explored.

Our finding of Nts-GFP neurons in the PMV of mice agrees with reports of PMV Nts in rats [40,89]. Although the specific function of PMV Nts has yet to be tested in either species, central Nts treatment blunts maternal aggression/defense behaviors known to be regulated by the PMV [15,90], hence, PMV Nts neurons might conceivably act locally to curb aggressive behavior. Despite the high density of PMV Nts neurons, they do not overlap with PMV neurons that express the long form of the leptin receptor (LepRb), and, thus, PMV Nts does not directly contribute to leptin-mediated fertility [91,92]. By contrast, some LHA Nts neurons co-express LepRb and contribute to the anorectic function of leptin and regulation of the mesolimbic dopamine system [11,93].

We observed many Nts-GFP neurons in the STh, as has been described in humans [94], but the rat STh is devoid of Nts [39,54]. Hence, *Nts^{Cre};GFP* mice offer an advantageous system in which to study the function of STh neurons and their potential to treat Parkinson's Disease. The STh provides a strong glutamatergic projection to the substantia nigra pars reticulata (SNr) [95], and given the sheer abundance of Nts-GFP neurons within the STh, they are likely to be part of this glutamatergic circuit. The SN contains NtsR1 [93,96,97], and intranigral infusion of Nts elicits local glutamate release [98], so it is certainly possible that STh Nts neurons project to and provide Nts input to the SNr. The function of such a circuit has yet to be explicitly tested, but it is hypothesized that the STh Nts → SNr circuit might reduce excitatory signaling to the motor cortex [95]. This could contribute to the well-characterized role of systemic or central Nts in restraint of ambulatory movement [9,51]. Modulating the STh→ SNr circuit may be beneficial for individuals with Parkinsons Disease, as deep brain stimulation of the STh and, thus, disruption of STh afferents, can improve motor symptoms by reducing inhibitory action on the motor thalamus [99]. Resolving the function of STh Nts neurons has the potential to reveal less physically invasive, ideally pharmacologic, strategies in the effort to improve locomotor deficits in Parkinson's Disease.

The mouse LHA contains a large population of Nts-GFP neurons, which coordinate peripheral energy status and motivated behaviors necessary for energy balance via engaging VTA dopamine neurons and/or LHA Orexin (OX) neurons [11,92,100]. Some LHA Nts neurons directly co-express LepRb, and are important for

leptin- and ghrelin-induced activation of the mesolimbic dopamine signaling [11,92,93]. LHA Nts neurons also respond to and mediate dehydration-anorexia [101,102] and LPS-induced lethargy [103], both of which are states where feeding behavior is suppressed. The LHA is essential for coordinating thirst and drinking behavior, and, indeed, LHA Nts neurons receive afferents from the osmoregulatory subfornical organ (SFO) [104]. Since experimental activation of LHA Nts neurons suppresses feeding but increases water intake and locomotor activity [23], these neurons may differentially modulate both ingestive behaviors necessary for survival. It may also be possible that distinct subpopulations of LHA Nts neurons mediate different ingestive behaviors: feeding suppression or drinking. Indeed, our finding that many, but not all LHA Nts neurons, co-express Gal suggests that there are at least two subsets of LHA Nts neurons. Going forward, it will be important to distinguish the roles of these subpopulations, including the respective signaling contributions of Gal vs. Nts.

While we observed some Nts-GFP neurons in the Arc of male mice, particularly within the rostral aspect, relatively low *Nts*-ISH was observed, and this slight incongruity is consistent with the decrease in Arc Nts noted between infancy and adulthood [105]. These data suggest that Nts may play a greater role in establishing Arc circuits than in Arc signaling within the mature brain. The Arc is important for regulating homeostatic feeding, and Nts neurons may contribute; indeed, Arc Nts is decreased in food-restricted rats [101] as well as in genetically obese rats relative to lean counterparts [106]. In addition, Arc Nts may play a valuable role in the female reproductive axis, which could not be appreciated in our study of male *Nts^{Cre};GFP* mice. Indeed, Arc Nts

neurons co-express estrogen and progesterone receptors, and Arc Nts expression fluctuates in accordance with estrogen level across the rat estrous cycle [107,108]. Since some Arc Nts neurons project to the median eminence [109] and the anterior pituitary [108], they may have roles in modulating release of growth-hormone-releasing hormone (GHRH) [110,111] and prolactin [108,112–114]. Going forward, studies of female and male *Nts^{Cre};GFP* mice may prove useful in defining the sexually dimorphic roles of Nts both inside and outside of the Arc.

Consistent with prior reports, abundant Nts-GFP neurons were found within the MPO and AVPV, two regions of the hypothalamus that are also sexually dimorphic in nature [39–41,43,79,115]. Indeed, Nts and estrogen receptor are co-expressed in both of these structures, and female rats, when compared to males, have twice as many Nts- and estrogen receptor-co-expressing neurons located within the MPO and AVPV [116,117]. In addition, estradiol modulates Nts-expression within these two nuclei [118–120]. There is conflicting data regarding whether Nts signaling contributes to the LH surge [119,121]; however, the discrepancies in plasma LH levels detected between different studies may simply be due to differential effects observed with Nts injected directly into the MPO vs. brain-wide. Interestingly, most MPO Nts neurons project to the VTA [33,36,122], and pharmacologic studies suggest that MPO → VTA projecting Nts neurons promote locomotor activity [123]. Subsequently, manipulating MPO Nts neurons in *Nts^{Cre}* mice refined this understanding, demonstrating that MPO Nts neurons specifically facilitate social approach toward the opposite sex and drive motivated behaviors directed at finding a potential mate [33]. MPO Nts neurons are also

implicated in courtship behaviors in male European starlings [16] and maternal behaviors in rodents [124–126], strongly supporting their role in coordinating rewarding social behaviors.

Possible Roles of Nts-GFP Neurons in the Cerebral Cortex

The hippocampus is vital for learning and memory, and we observed two major populations of Nts-GFP neurons in this region. Indeed, one of the most strikingly abundant populations of Nts-GFP neurons throughout the brain was found in the subiculum region of the hippocampus (DS and VS). Subiculum Nts neurons project to the mammillary body, and this subiculum Nts → mammillary body circuit is implicated in modulating memory and learning within the context of fear, as Nts injection in the mammillary body promotes passive avoidance behavior [86–88]. In addition, subicular efferents to the mammillary body contribute to corticosterone release and stress response following fear memory retrieval [127]. Thus, these findings suggest subiculum → mammillary body Nts projections may be involved in memory and learning in the context of fear. Nts may modulate these behaviors throughout the lifespan, as Nts expression is preserved in the mature rodent subiculum, though levels diminish slightly with age [54,128]. Humans also have a dense population of Nts-positive subiculum pyramidal cells that are no longer apparent after 4 years of age [87,129], which may similarly reflect decreased Nts synthesis in the maturing brain below the detection level. Given these changes in Nts expression, it is possible that Nts is important in both forming Subiculum-based memory and learning circuits as well as for modulating established pathways. Many Nts-GFP neurons were also found within the hippocampal

CA1 region, but the considerably lower *Nts* ISH in the adult brain suggests that Nts may be predominantly expressed during CA1 development. CA1 projections to the subiculum are important for memory retrieval, whereas the CA1 to entorhinal cortex pathway is crucial for memory formation [127]. While it is unknown if CA1 Nts neurons specifically project to the these regions, Nts administration to the entorhinal cortex enhances spatial learning via an Nts Receptor-1-dependent mechanism [130]. A role for Nts in memory is further substantiated by the finding that the Nts Receptor-1 agonist PD149163 reduces memory error and preserves novel object discrimination in rats [131,132]. Going forward it will be important to determine whether the Nts populations of the Subiculum and CA1 contribute to Nts-mediated learning and memory.

Of the cortical regions, only the retrosplenial cortex and cingulate cortex contained very dense populations of Nts-GFP neurons but had no detectable *Nts*-ISH according to our assessment of the Allen Brain Atlas. These regions have high levels of prepro-*Nts*-ISH at birth that drastically diminish and essentially disappear a few weeks later, and this ontogenetic profile was noted across species [71,87,128,133]. Thus, the discrepancies we noted between the Nts-GFP and *Nts*-ISH in mice agree with these data and support that Nts provides mainly a developmental role, perhaps in circuit formation or synaptic guidance, in the retrosplenial and cingulate cortex. Indeed, Nts neurons projecting to the anterior ventral thalamic nucleus (AV) were observed within the retrosplenial cortex of 3-5 day old rats, and the temporally-restricted detection of these Nts neurons suggests a role for Nts in development or synaptogenesis within the retrosplenial cortex [134].

The CEA was the only amygdalar structure with a high density of Nts-GFP neurons (**Figure 2.5D**), primarily within the lateral (CeL) and capsular (CeC) divisions, and this distribution was similar to prior reports [39–41,43,79]. Since both Nts and PKC-δ are found within the CEA and since both have been implicated in anorexic behavior [6,23,50,51], it seemed plausible that they might be found within the same neurons and coordinate anorexia. However, we and others found little overlap of Nts and PKC-δ in the CEA (**Figure 2.7**) [52]. Instead, the Nts-IR found in the caudal CeL subregion of the CEA overlapped with corticotropin-releasing factor (Crf) and somatostatin (Sst), and many of these Nts neurons project to the LPB [52]. These findings do not rule out a role for CEA Nts neurons in mediating anorexia, but do imply that alternate, PKC-δ-negative and independent mechanisms exist via which Nts exerts its anorectic effects, and these remain to be elucidated. Other physiological roles of CEA Nts neurons have been explored. For example, since hypertonic-induced dehydration decreases Nts levels within the CEA, CEA Nts neurons might contribute to osmotic regulation and the modulation of fluid balance [135]. Additionally, CEA Nts neurons have also been demonstrated to project to the central gray, NTS, dorsal vagal complex, LHA, and BNST [81,136–140], and such projections sites provide a clue as to the other potential functions of these neurons. For instance, CEA Nts afferents to the PAG, where Nts has been exhibited to elicit antinociception, are thought to contribute to analgesia, as these PAG-projecting CEA Nts neurons become hyperpolarized in response to neuropeptides that elicit hyperalgesia [30,141]. Moreover, CEA Nts neurons are a candidate source of Nts to the NTS, where Nts decreases blood pressure and heart rate [68,136]. Nts neurons have been identified within projections from the CEA to the LPB, and these

projections are speculated to partake in modulation of the autonomic response to stressful environmental stimuli [142,143]; thus, such LPB-projecting CEA Nts neurons may provide one channel via which Nts exerts its known cardiovascular effects. Hence, CEA Nts neurons potentially contribute to numerous diverse aspects of physiology, and modulation of these neurons in *Nts*^{Cre} mice may be a useful way to define their precise roles.

Possible Roles of Nts-GFP Neurons in the Striatum, Pallidum, and Forebrain

Numerous Nts-GFP neurons were detected throughout the ventral and dorsal extent of the striatum. The ventral striatum consists primarily of the Tu, AcbSh and AcbC, all of which contained substantial Nts-GFP neurons. The high density of Nts-GFP neurons in the Tu is consistent with *Nts*-ISH and Nts-IR data from the mouse, rat and dog [79,144–146]; however, there is as of yet no understanding of how this large population contributes to Nts-mediated physiology. We also observed many Nts-GFP neurons and ample *Nts*-ISH in the AcbSh (**Figure 2.6)**, where Nts has received considerably more study due to its suppression of psychomotor effects [147,148]. Indeed, part of the mechanism by which typical antipsychotics (such as haloperidol) and atypical antipsychotics (such as clozapine) suppress psychomotor responses may be via increasing Nts content within the AcbSh [146,149,150]. Similarly, stimulants such as cocaine, methamphetamine, and amphetamine increase Nts in the AcbSh [150–153]. Antipsychotics and stimulants appear to modulate increased Nts expression via their differential effects on dopamine receptor subtypes (D1 vs D2), and these data highlight the important, yet incompletely understood, link between Nts and dopamine signaling

[154]. The CPu of the dorsal striatum also contained a considerable, yet more modest, density of Nts-GFP neurons as well as *Nts*-ISH (best seen in **Figure 2.3B**) and is another site via which Nts is thought to blunt psychomotor effects. Blockade of D2 receptors with antipsychotics, agonism of D1 receptors, and glutamate signaling via NMDA receptors are all mechanisms that generate increased Nts expression in the CPu, as well as in the ventral striatum [155,156][157,158]. While the effects of Nts administration in the AcbSh have been well characterized and include a decrease in DA release from VTA-projecting DA neurons as well as a concomitant decrease in locomotor activity [147,148], it is not clear if striatal Nts neurons are the endogenous source of the Nts that drives such changes. Thus, the opportunity to systematically activate ventral or dorsal striatal Nts neurons in $Nts^{Cre};GFP$ mice could help to resolve their specific contributions to the regulation of locomotor activity and the psychostimulant response.

A considerable amount of Nts-GFP neurons was detected within the LS as well as the more caudal BNST, and these two areas are implicated in the control of anxiety and social behaviors. BNST Nts neurons are thought to co-express CRF and project to the LPB, though the function of this circuit has yet to be described [159]. Intriguingly, the BNST and LS exhibit opposing regulation of *Nts* expression in postpartum female mice: *Nts* is increased in the BNST but reduced in the LS [125]. As previously mentioned, Nts inversely modulates maternal aggression, and both the LS and BNST display increased activity with Nts ICV injection [15]. Since LS neurons have been implicated in maternal recognition of pups and the BNST in maternal behaviors [160],

alterations in Nts gene expression within these two regions may differentially modulate maternal responses to newborns. Intensity of Nts-IR in both of these regions also correlates with vocal and non-vocal courtship behaviors in European Starlings; thus, Nts neurons within the BNST and LS may contribute to sexually-motivated behaviors [161]. Moreover, the fact that the BNST and LS are modulated by the VTA dopamine system [162,163] gives further credence to the notion that Nts neurons in these sites play a role in the motivation of social and maternal behaviors.

Conclusion

The goal of this work was to provide a descriptive map of Nts neurons throughout the *Nts^{Cre};GFP* mouse brain. We documented numerous brain regions to contain Nts-GFP neurons, some of which were not thought to possess Nts neurons prior to this report, and the Nts neurons at many of these sites are plausible participants in the regulation of various aspects of physiology, spanning from analgesia, locomotor activity, cardiovascular response, social behavior, addiction, learning, memory, and feeding. These findings emphasize the wealth of information yet to be mined about how Nts neurons contribute to biology and behavior. It is our hope that this work will facilitate subsequent studies designed to both understand the roles of these Nts populations in normal physiology and to determine whether these populations may be tractable targets to improve maladaptive behaviors or disease states.

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CHAPTER 3. Neurotensin Receptor-1 Deficiency Increases Risk for Female Mice to Develop Behaviors Similar to Anorexia Nervosa

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Abstract

Background: Anorexia nervosa (AN) has the highest mortality rate of any psychiatric illness but there are no effective medications to improve body weight. Determining the genetic risk factors that interact with sex and stress to promote AN is necessary to identify biological pathways for intervention. Recently, loss-of-function variants in the neurotensin and neurotensin receptor 1 (*NTSR1)* genes were linked to the risk of having AN. We therefore hypothesized that loss of NTSR1 is a genetic risk factor that interacts with environmental risks to increase vulnerability to develop AN. **Methods:** We studied male and female NtsR1 knock-out mice (*NtsR1*^{KOKO}) and mice with intact NtsR1 (*NtsR1*$^{++}$*)* to define how NtsR1-deficiency interacts with environmental risk factors (e.g. adolescent isolation stress and caloric restriction) thought to promote development of AN-like behaviors. **Results**: NtsR1 deficiency promotes low body weight in unstressed male and female mice. Yet, female *NtsR1*^{KOKO} mice exposed to adolescent stress were exclusively vulnerable to developing aphagia, low body weight, and co-morbidities similar to those observed in AN. **Conclusions:** This work shows that NtsR1-deficiency increases vulnerability to develop aberrant behaviors associated with AN. Our findings of a genetic X sex X stress interaction have face validity for AN and are translationally relevant since loss of function variants in the NTSR1 pathway may contribute to development of this disorder. These data support future studies on the precise role of NTSR1 signaling in behavioral and body weight regulation to determine if targeting NTSR1 action could improve outcomes in AN.

Keywords: wheel running, adolescent stress, feeding, aphagia, body weight, sex difference

Introduction

Anorexia Nervosa (AN) is an eating disorder that is ten times more prevalent in females than in males and has the highest mortality rate of any psychiatric illness [1,2]. Individuals with AN relentlessly pursue thinness via restricting food intake and engaging in compensatory behaviors (such as exercise), and AN patients may also exhibit episodes of binge-eating/purging to lose weight [3]. Comorbidities of AN include depression, anxiety [4,5], and obsessive-compulsive disorder (OCD), manifesting as obsessive preoccupations with dieting and body weight and a compulsion to exercise [6]. Yet, there are no FDA-approved medications to treat AN. Potential pharmacotherapies include antidepressant and antipsychotic medications that target mood/anxiety symptoms; however, these do not facilitate body weight restoration [7–10]. Thus, there is a need to identify targetable pathways for therapeutic intervention using animal models; however, the complex interaction of various genetic, environmental, and social risk factors contributing to AN has made it difficult to recapitulate experimentally in mice [5,11–16]. The fact that numerous factors contribute to this disease has resulted in difficulty with defining the precise etiology, making AN challenging to model.

Since 50-70% of the risk of developing AN is heritable [17], identifying genetic risk factors can provide a starting point toward understanding and treating this multifactorial disorder. Indeed, genomic studies have identified candidate loci and genes associated with risk for developing AN and overlapping metabolic and psychiatric disorders [18–27]. Recently, whole exome sequencing of 93 unrelated individuals diagnosed with eating disorders identified rare damaging variants in Neurotensin (*NTS*),

Nts Receptor 1 (*NTSR1*), and related pathway genes that are enriched in individuals with AN [28]. Preliminary assessment of these variants suggested that they might disrupt NTS-NTSR1 signaling, raising the possibility that loss of action via this pathway might contribute to AN.

Importantly, Nts-NtsR1 has been implicated in body weight homeostasis. For example, peripheral Nts is important for intestinal fat absorption [29], while central Nts affects feeding via NtsR1 [30,31]; therefore, both Nts pools could promote low body weight. Furthermore, a subset of ventral tegmental area (VTA) dopamine (DA) neurons co-express NtsR1 and contribute to DA-mediated weight loss behaviors [32–35]. Remarkably, ablating adult VTA NtsR1 neurons causes excessive locomotor activity without sufficient caloric intake leading to low body weight, similar to the maladaptive behaviors and low body weight observed in AN [36]. In addition to the established role for Nts-NtsR1 in the adult brain, essentially all VTA DA neurons express NtsR1 during development, which may contribute to the organization of mesolimbic circuits that govern motivated behaviors [37]. Indeed, germline removal of NtsR1 action, as in male NtsR1 knock-out (*NtsR1*^{KOKO}) mice, increases locomotor activity, decreases chow intake, and alters DA signaling to impact body weight [38]. Collectively, these data support a potential role for developmental disruption of NtsR1 in altering behaviors relevant to AN.

We examined whether NtsR1 deficiency interacts with other risk factors, specifically female sex and exposure to adolescent stress, to increase vulnerability to developing AN-like behaviors [39,40]. We studied *NtsR1*^{KOKO} mice to model the genetic

risk of lacking NtsR1, similar to loss-of-function variants in the NTS-NTSR1 pathway in patients with AN. Male and female *NtsR1*^{KOKO} mice were tested via a translational paradigm that incorporates adolescent social isolation and caloric restriction exposures associated with AN [15]. Indeed, social isolation during adolescence has been previously shown to elicit behavioral measures of depression and anxiety in mice with genetic predisposition to such phenotypes [41], and both depression and anxiety are common comorbidities in AN [4,5]. In addition, intentional dieting or unintentional weight loss during this period often precedes the emergence of AN in humans [15,42,43]. Our data suggest that NtsR1 deficiency increases vulnerability for low body weight in both sexes, but along with adolescent stress, this genetic alteration specifically causes females to develop disordered feeding and heightened exercise-like activity as well as anhedonia- and OCD-like behaviors similar to that observed in AN. Hence, disruption of the Nts-NtsR1 system may confer genetic risk for developing this disorder.

Methods and Materials

Animals

Mice with intact NtsR1 (*NtsR1*$^{++}$*)* and heterozygous NtsR1 knock-out mice (*NtsR1*$^{KO+}$, Stock # 005826) on the C57BL/6 background were purchased from Jackson Laboratories (Bar Harbor, ME) and bred in our colony (12 hr light/12 hr dark cycle with *ad libitum* access to water and standard Teklad 7913 chow diet) unless otherwise specified. Tail biopsies from progeny were genotyped as previously described [35,38] to identify *NtsR1*$^{++}$ and *NtsR1*^{KOKO} mice for studies. All mice were weaned between 3-4 wk, single housed at 5 wk (to invoke isolation stress), and assessed for weekly food intake and body weight until 16 wk. Mouse numbers for the baseline cohort (no adolescent caloric restriction): *NtsR1*$^{++}$ males = 17, *NtsR1*^{KOKO} males = 18, *NtsR1*$^{++}$ females = 16, *NtsR1*^{KOKO} females = 17. Mice were also studied in a translational paradigm involving adolescent caloric restriction (see below): *NtsR1*$^{++}$ males = 18, *NtsR1*^{KOKO} males = 18, *NtsR1*$^{++}$ females = 18, *NtsR1*^{KOKO} females = 19. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Michigan State University in accordance with Association for Assessment and Accreditation of Laboratory Animal Care and National Institutes of Health guidelines.

Translational Paradigm to Assess Interaction of AN Risk Factors

NtsR1^{++} and *NtsR1*^{KOKO} mice of both sexes were first characterized at baseline, and then a separate cohort was tested via the paradigm of Madra and Zeltser [15]. All study mice were single housed at 5 wk (beginning of pubescent period/mid-adolescence)

to not only induce social isolation stress but to also allow for weekly measurements of food intake along with body weight. For the cohort of mice subjected to the adolescent stress model of AN, food intake was measured daily between 6-7 wk (end of pubescent period), then at 7 wk (beginning of late adolescence when sexual maturity is reached) mice were provided 75% of their averaged daily *ad libitum* food intake for the next 11 days (week 7-8.5). These manipulations were performed during the adolescent period since onset of AN is most typical during adolescence, a period when individuals face significant stress [3]. During this period and immediately following (8.5-9.5 wk), body weight and food intake were measured daily, and an aphagic episode was counted if a mouse ate ≤ 0.5 g of food within 24 hr.

Metabolic Phenotyping

Chow and body weights were measured weekly from 5-16 wk. Body composition was assessed at 16 wk using nuclear magnetic resonance (Minispec L550; Bruker, Billerica, MA). Mice were then acclimatized to TSE metabolic cages (PhenoMaster; TSE Systems, Chesterfield, MO) for 2 days, and the subsequent 24 hr of data was used for analysis. Ambulatory activity was measured at 16 wk as breaks of infrared beams along the x-, y-, and z-axis of the cage. Mice were similarly assessed in TSE cages with a running wheel. An ambient temperature of 20 °C to 23 °C was maintained throughout analysis, and airflow rate throughout the chambers was adjusted to maintain an oxygen differential of ~0.3% at resting conditions.

Sucrose Preference and Operant Responding

Mice underwent the two-bottle choice paradigm at 20-29 wk to measure intake of palatable 0.5% sucrose solution versus water, as prevously [44]. This test provides an index of stress-induced anhedonia [45], which is relevant to AN as major depressive disorder is relatively common, and individuals with AN typically display anhedonia in both social and food-related contexts [4,46,47]. Body weight, water, and sucrose were measured at 10:00 AM daily. Mice were then trained and tested on a progressive ratio (PR) reinforcement schedule [35,48]. The PR breakpoint was the number of active port nose pokes performed to earn the last reward at the end of the testing period. Mice were tested until they reached stable PR breakpoint (e.g. rewards earned varied by ≤ 10% over 3 consecutive days), and graphs show the average stable PR breakpoint, which represents motivation to work for sucrose reward. PR breakpoint was also measured after overnight *ad libitum* access to sucrose or fasting. The translational paradigm cohort additionally underwent three consecutive days of 60% caloric restriction followed by two days of *ad libitum* chow refeeding, and PR breakpoints were determined each day. Two mice from that cohort were excluded because they failed to meet training criteria [48] (male *NtsR1*$^{++}$ n=17, female *NtsR1*^{KOKO} n=18.)

Elevated Plus Maze

Anxiety was tested via the Elevated Plus Maze, as per [35]. Maze activity under red light was tracked for 5 minutes with a CCD camera connected to a computer running TopScan automated video tracking software (Clever Sys). One *NtsR1*^{KOKO}

male (n=17) and one *NtsR1*^{KOKO} female (n=18) from the baseline and translational cohorts, respectively, fell off the maze during testing and were excluded from analysis.

Open Field Activity and Grooming

Anxiety-like behavior was additionally assessed using the Open Field Test. Mice were placed in the outside corner of open field chambers (38 x 38 x 35 cm) and monitored for 5 minutes using video tracking software. Since the software does not recognize grooming behavior, a blinded observer viewed recorded videos and scored seconds spent grooming, which provided a measure of OCD-like behavior in mice [49,50].

Marble Burying

Behavioral compulsivity was additionally assessed via the marble burying tests. Plastic cages (45 x 24 x 15 mm) were filled with a 5 cm-thick even layer of bedding. On the bedding surface, 20 glass marbles (1 cm in diameter) were placed in an even arrangement consisting of 5 rows of 4 marbles. Mice were placed in the cages for 30 minutes, after which the number of buried marbles, or those covered at least 2/3rds by bedding, were counted.

Statistics

Student's t-tests and 2-way ANOVAs with post-hoc Tukey tests were calculated using Prism 7 (GraphPad). For all 2-way ANOVAs, multiple comparisons were performed for factors of genotype and sex, unless specified otherwise. Repeated

measures two-way ANOVA with Sidak post-hoc analysis was used when body weight and food intake comparisons were made between *NtsR1*$^{++}$ and *NtsR1*^{KOKO} mice at different points in time. All data represent the mean ± SEM. For all data, *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.

Results

Lacking NtsR1 predisposes for low body weight

First, we examined whether NtsR1 deficiency interacts with environmental-social risk (social isolation) to promote AN-like behaviors **(Figure 3.1A)**. Social isolation was practically necessary to measure feeding of individual mice over time and is biologically relevant to AN, since it promotes development of anxiety- and depressive-like phenotypes in mice and since these disorders are common predecessors to AN [14,51,52]. In this study, male and female *NtsR1*^{KOKO} mice displayed lower body weights compared to *NtsR1*$^{++}$controls, which became significant at 11 wk **(Figure 3.1B,C)**. While no differences in cumulative food intake were determined between *NtsR1*$^{++}$ and *NtsR1*^{KOKO} males **(Figure 3.1D)**, there was a significant main effect of genotype on food intake for females ($p<0.0001$), and *NtsR1*^{KOKO} females demonstrated significantly reduced feeding at 15 wk relative to *NtsR1*$^{++}$ **(Figure 3.1E)**. *NtsR1*$^{++}$ and *NtsR1*^{KOKO} mice of both sexes had comparable ambulatory activity in the absence of a running wheel. Males given a wheel exhibited elevated ambulatory activity **(Figure 3.1F)**, consistent with prior findings [53], but no genotype differences in wheel usage **(Figure 3.2A,B).** Since *NtsR1*^{KOKO} males with wheels had increased energy expenditure compared to control males **(Figure 3.1H, 3.3A,B),** their lower body weight may result from increased voluntary physical activity. By contrast, female mice of both genotypes displayed increased ambulatory activity, ran on wheels at similarly high levels **(Figure 3.1G and 3.2A,B),** and exhibited no difference in energy expenditure **(Figure 3.1I, 3.3E,F),** suggesting that the modest reductions in female *NtsR1*^{KOKO} food

Figure 3.1. Effects of NtsR1 deficiency on energy balance. A) Male and female mice with intact NtsR1 or with whole-body knockout of NtsR1 (*NtsR1*$^{++}$ and *NtsR1*^{KOKO}, respectively) were single housed at 5 wk. Average body weight over time for *NtsR1*$^{++}$ and *NtsR1*^{KOKO} **B)** male and **C)** female mice. Cumulative food intake of *NtsR1*$^{++}$ and *NtsR1*^{KOKO} **D)** males and **E)** females. Average ambulatory activity of **F)** male and **G)** female *NtsR1*$^{++}$ and *NtsR1*^{KOKO} mice over 24 hr when housed with and without a running wheel in TSE metabolic cages. Average energy expenditure of **H)** male and **I)** female *NtsR1*++ and *NtsR1*^{KOKO} mice over 24 hr. **J,K)** Fat mass and **L,M)** lean mass for male and female *NtsR1*$^{++}$ and *NtsR1*^{KOKO} mice at 16 wk. Body weight and feeding data were analyzed via repeated measures two-way ANOVA, with Sidak post-tests. Ambulatory data were assessed by two-way ANOVA with post-hoc Tukey tests. Multiple comparisons were performed for factors of genotype and presence/absence of a wheel. Energy expenditure and body composition data were analyzed via Student's t-tests. All data represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

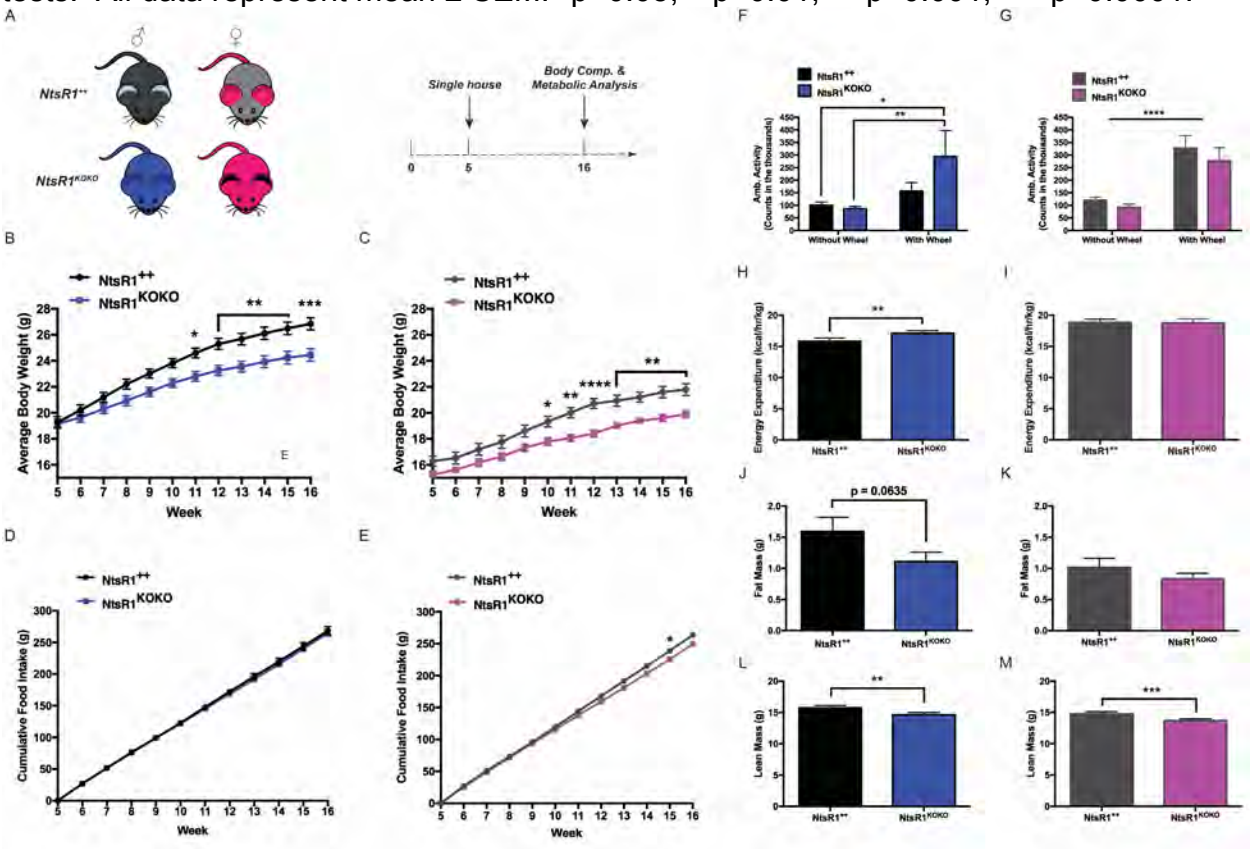


Figure 3.2. NtsR1 deficiency does not alter motivated behaviors that modify body weight. A) Total number of running wheel rotations and **B)** total time spent on the wheel over 24 hr for male and female *NtsR1*$^{++}$ and *NtsR1*^{KOKO} mice. **C)** Percent difference in preference for a 0.5% sucrose solution compared to water. **D)** PR breakpoint for sucrose pellets in *ad libitum* fed mice. All data were analyzed by two-way ANOVA with post-hoc Tukey tests and represent the mean ± SEM. *p<0.05, **p<0.01.

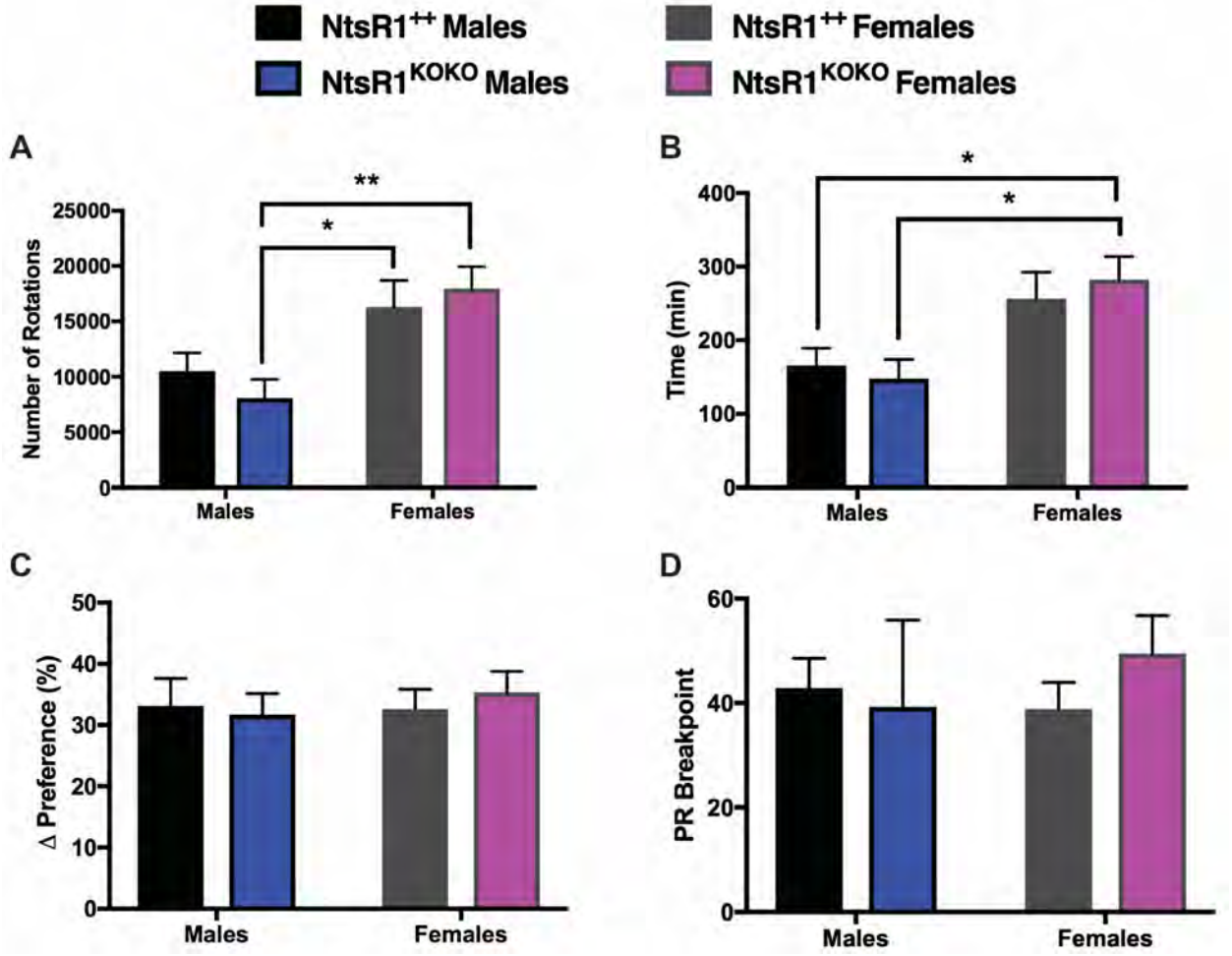
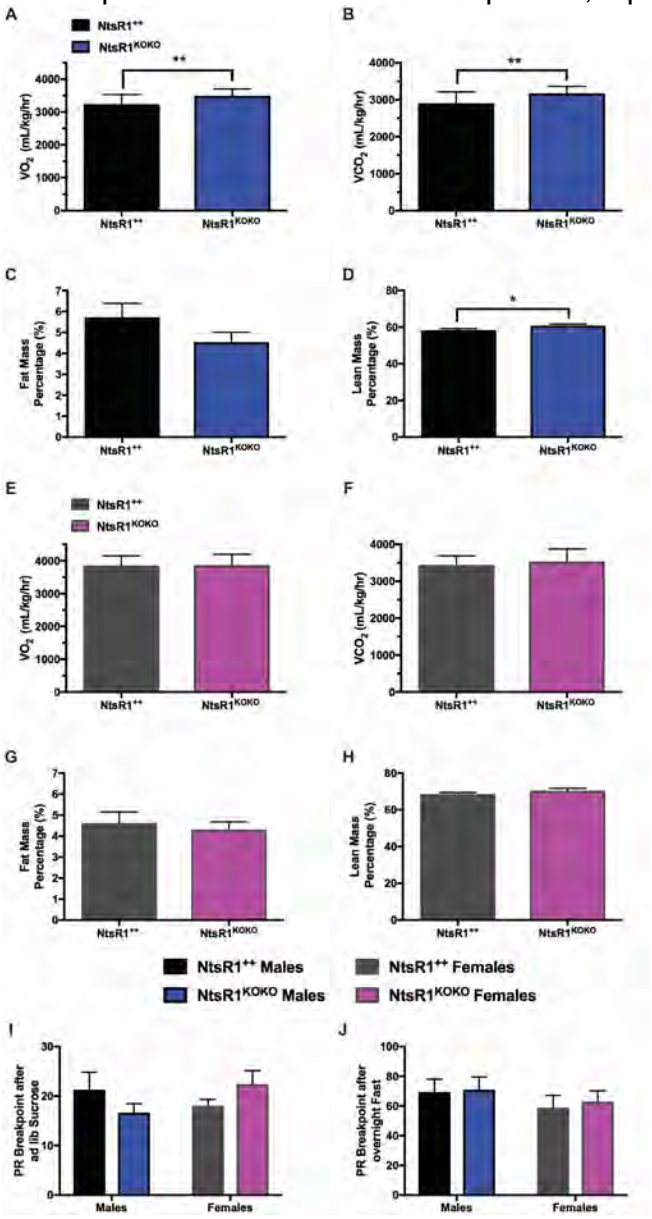


Figure 3.3. Body composition, calorimetry, and operant responding in socially isolated mice lacking NtsR1.
NtsR1^{++} and *NtsR1*^{KOKO} male singly housed from 5 wk of age were analyzed at 16 wk for average rate of **A)** oxygen consumed (VO_2) and **B)** carbon dioxide produced (VCO_2). **C)** Fat mass percentage and **D)** lean mass percentage for *NtsR1*$^{++}$ and *NtsR1*^{KOKO} male mice at 16 wk. Singly housed *NtsR1*$^{++}$ and *NtsR1*^{KOKO} female mice assessed for **E)** oxygen consumed (VO_2) and **F)** carbon dioxide produced (VCO_2), **G)** fat mass percentage and **H)** lean mass percentage at 16 wk. To evaluate motivated responding for palatable reward in the face of altered energy status, mice were either overnight **I)** provided 150 sucrose pellets or **J)** fasted and PR breakpoint was determined the following day. Student's t-tests were used to analyze calorimetry and body composition data. PR responding was assessed via two-way ANOVA with post-hoc Tukey tests. All data represent the mean ± SEM. *p<0.05, **p<0.01.



intake might cause lower body weight. During this time male and female *NtsR1*^{KOKO} mice exhibited trends for lower fat mass and fat percentage **(Figure 3.1J,K. 3.3C,G)**, and significantly lower lean mass relative to controls (**Figure 3.1L,M).** Together these data hint at sex differences underlying the low body weight of NtsR1-null males and females.

Normal anxiety and motivation in NtsR1-deficient mice

We next examined whether lacking NtsR1 in combination with social isolation stress impairs exercise-like activity or motivated feeding behaviors that are altered in AN. Wheel running is a rewarding, voluntary exercise-like activity for rodents [53], and indeed males of both gentoypes ran at comparable levels **(Figure 3.2A,B)**. Female mice tended to run more than males, and female *NtsR1*^{KOKO} mice ran significantly more than *NtsR1*^{KOKO} males **(Figure 3.2A,B)**. Thus, females may be more inclined to exercise-like activity compared to males. We then asked if lacking NtsR1 alters the hedonic value (liking) or the DA-mediated motivation (wanting) for food [54], as anhedonia [55,47] and DA alterations [3,56] have been observed in AN. *NtsR1*$^{++}$ and *NtsR1*^{KOKO} mice of both sexes had similar sucrose preference **(Figure 3.2C)**. They also exhibited similar PR operant responding for sucrose pellets, a measure of DA -mediated reward "wanting" [48], during energy repletion **(Figure 3.2D),** surplus or deficit **(Figure 3.3I, J)**. Hence, lacking NtsR1 alone did not impair hedonic or motivational intake.

NtsR1 deficiency and anxiety

We next evaluated whether lacking NtsR1 might contribute to anxiety disorders co-morbid with AN, including OCD [5]. We found no sex or genotype differences in the percentage of time spent in the open arms of the elevated plus maze or in the center of open field chambers, two measures of anxiety **(Figure 3.4A,C)**. However, females traveled more distance than males in the maze **(Figure 3.4B)** and *NtsR1*$^{++}$ females traveled a greater distance than males in open field chambers **(Figure 3.4D)**. Oddly, *NtsR1*$^{++}$ females also traveled more distance than *NtsR1*^{KOKO} females **(Figure 3.4D)**, which was unanticipated females exhibited equivalently high ambulatory and wheel running activity (**Figure 3.1G and 3.2A,B)**. Anecdotally, we noted that *NtsR1*^{KOKO} females often paused to groom, a stationary behavior that approximates OCD behavior in rodents [49,50] and might explain their decreased distance traveled. Indeed, female *NtsR1*^{KOKO} mice demonstrated a nearly significant increase ($p = 0.0643$) in grooming time relative to female *NtsR1*$^{++}$ controls **(Figure 3.4E,F)**. Thus, lacking NtsR1 might contribute to OCD-like behaviors analogous to those that accompany AN.

NtsR1 Deficiency With Adolescent Stress Promotes Female Vulnerability for Aphagia and Low Body Weight

Next, we modeled the intentional dieting or inadvertent weight loss that often precedes eating disorder development [13,42,43,57] by mildly calorically restricting *NtsR1*$^{++}$ and *NtsR1*^{KOKO} mice between 7 - 8.5 wk [15], but provided *ad libitum* chow for the rest of the study **(Figure 3.5A)**. These male *NtsR1*$^{++}$ and *NtsR1*^{KOKO} mice had similar body weights **(Figure 3.5B)**, including just prior to metabolic phenotyping at 16

Figure 3.4. NtsR1 deficiency predisposes females to compulsive anxiety behavior. **A)** *NtsR1*$^{++}$ and *NtsR1*^{KOKO} mice spend similar time within the open arms of the elevated plus maze. **B)** Distance traveled during the first 5 minutes in the elevated plus maze. **C)** *NtsR1*$^{++}$ and *NtsR1*^{KOKO} male and female mice spent similar percentage of time in the center of open field chambers. **D)** Female *NtsR1*$^{++}$ mice traveled a greater distance in open field chambers relative to males of both genotypes and *NtsR1*^{KOKO} females. Time spent grooming during the open field test for **E)** male and **F)** female *NtsR1*$^{++}$ and *NtsR1*^{KOKO} mice. Elevated plus maze and open field data were analyzed by two-way ANOVA with post-hoc Tukey tests. Grooming data were assessed with Student's t-tests. Graphs depict the mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

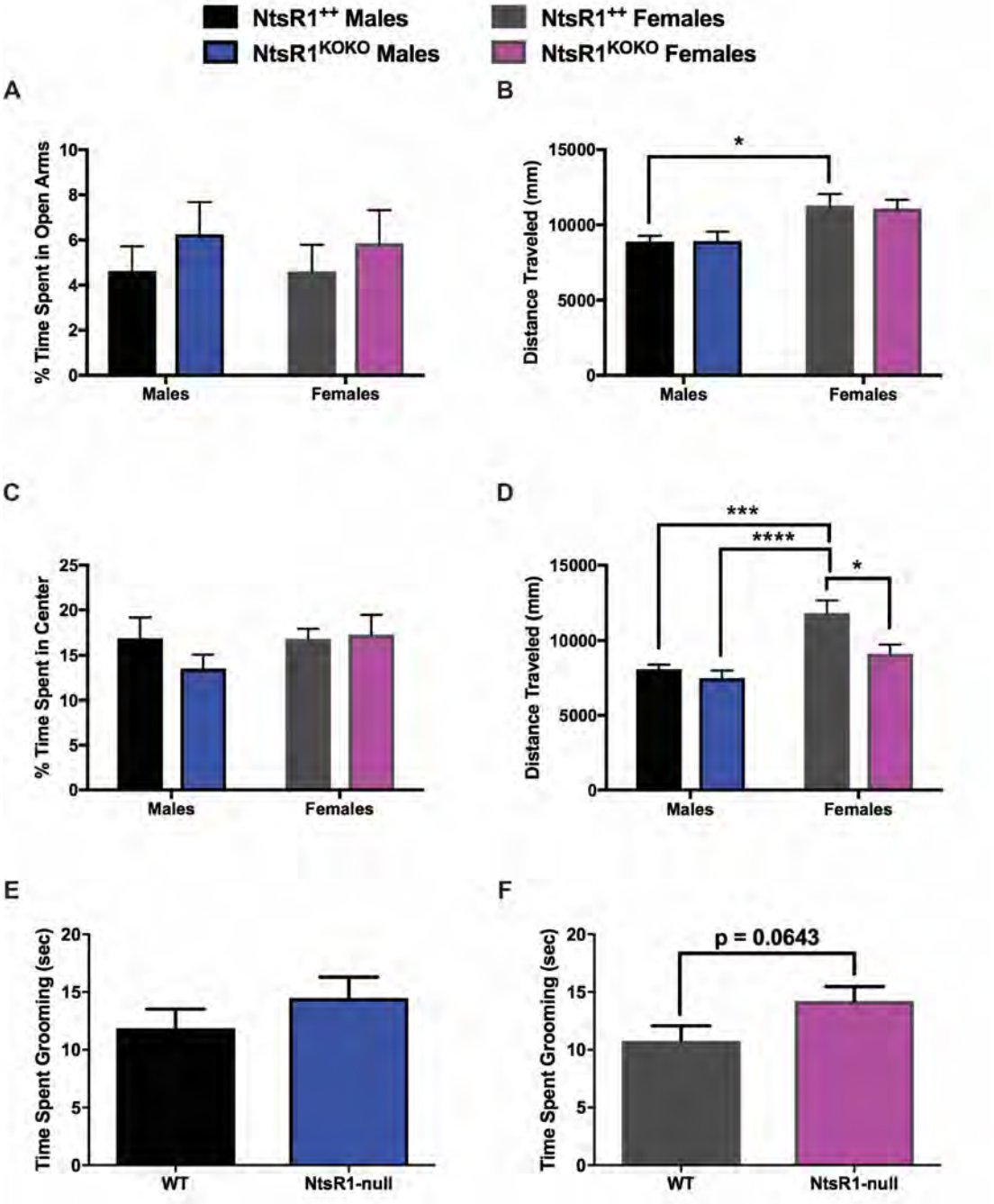
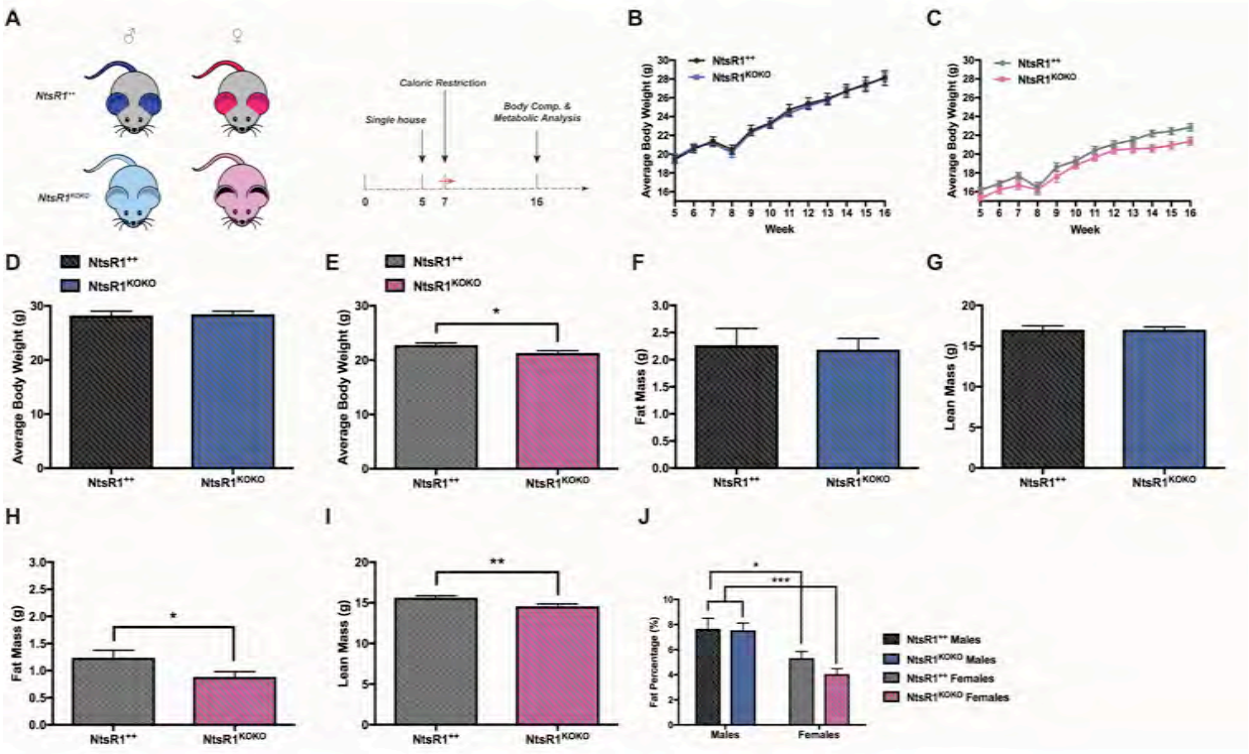


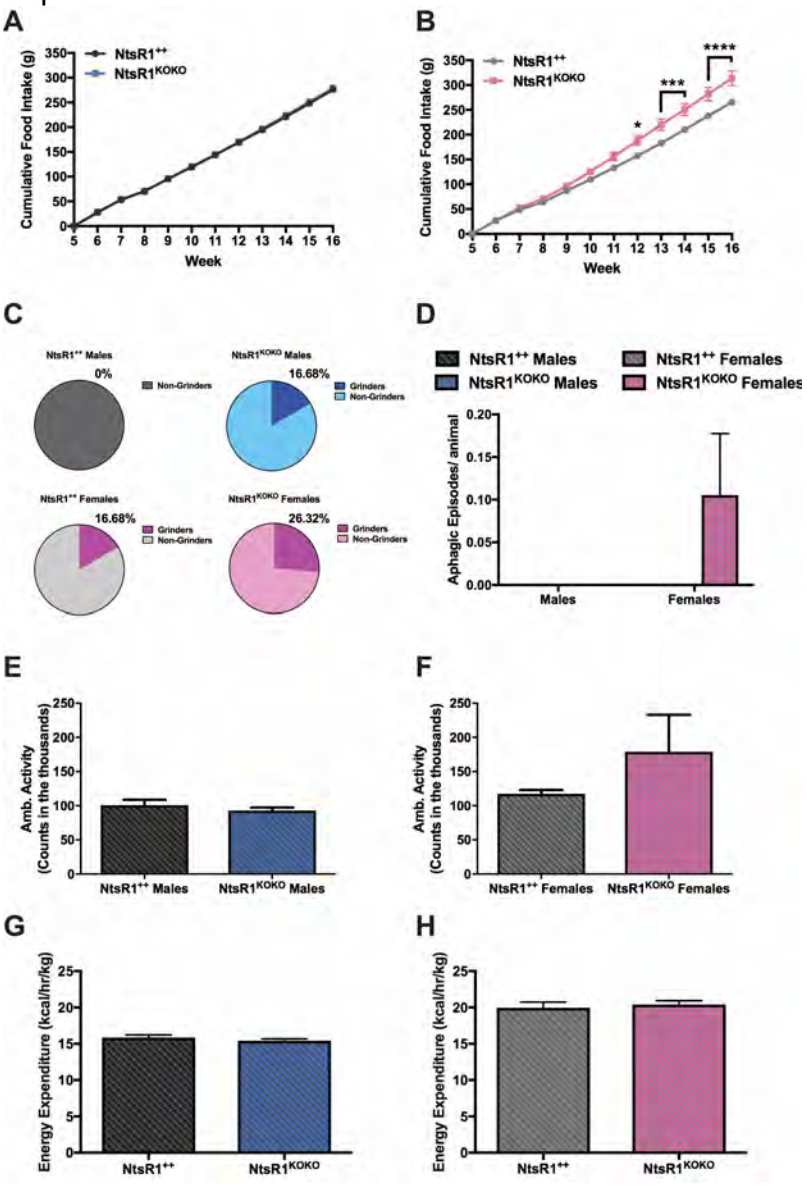
Figure 3.5. NtsR1-null females display altered body composition after adolescent stress exposure. A) Male and female *NtsR1*$^{++}$ and *NtsR1*^{KOKO} mice were subjected to two types of stress during adolescence: single housing beginning at 5 wk of age and restricting daily food consumption to 75% of normal caloric intake between 7 and 8.5 wk. Average weekly body weight for *NtsR1*$^{++}$ and *NtsR1*^{KOKO} **B)** male and **C)** female mice. Average body weights for wildtype and NtsR1-null **D)** male and **E)** female mice at 16 wk of age, when body composition was assessed. Fat mass and lean mass of **F,G)** male and **H,I)** female *NtsR1*$^{++}$ and *NtsR1*^{KOKO} mice at 16 wk. **J)** Fat mass percentage. Weekly body weight data were assessed by repeated measures two-way ANOVA with Sidak post-hoc analysis. Body weight, fat mass, and lean mass measurements taken at 16 wk of age were analyzed via Student's t-tests. Fat mass percentage was evaluated by two-way ANOVA with post-hoc Tukey tests. All data represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.



wk **(Figure 3.5D).** By contrast, *NtsR1*^{KOKO} females had lower body weights compared to controls over the course of the study (main effect of genotype for females, $p<0.0001$) **(Figure 3.5C)** including at 16 wk **(Figure 3.5E).** Thus, male *NtsR1*$^{++}$ and *NtsR1*^{KOKO} mice comparably adapt body weight in response to caloric restriction stress, and, as a result, have similar fat and lean mass **(Figure 3.5F,G).** Caloric restriction reduces body weight similarly in females of both genotypes; yet, afterwards, the *NtsR1*^{KOKO} females fail to gain weight to the same extent as *NtsR1*$^{++}$ mice females and have decreased fat and lean mass **(Figure 3.5H,I).** Interestingly, while non-stressed males and females of both genotypes displayed similar fat mass percentages **(Figure 3.3C,G)**, adolescent stressed males had increased fat mass percentage relative to females **(Figure 3.5J).** These data collectively suggest that NtsR1 deficiency interacts with adolescent social and caloric restriction risk factors to preferentially bias for low body weight in females.

We observed no differences in cumulative food intake between male *NtsR1*$^{++}$ and *NtsR1*^{KOKO} mice **(Figure 3.6A)** but counterintuitively, the lower-weight *NtsR1*^{KOKO} females displayed an apparent increase in food intake compared to controls **(Figure 3.6B)**. However, while weighing food we noted cages with food spillage, a stereotypic/compulsive behavior that occurs if mice gnaw at food but don't consume it [58–60]. No food spillage was observed within male *NtsR1*$^{++}$ mice, but female *NtsR1*^{KOKO} mice had the highest occurrence of spillage amongst the groups **(Figure 3.6C)**. Mice with visible food spillage were excluded from analysis of cumulative food intake, but we could not visibly detect all food spillage events. It is therefore conceivable that the elevated food intake of female adolescent-stressed *NtsR1*^{KOKO}

Figure 3.6. NtsR1-null females exposed to adolescent stress are specifically vulnerable to altered feeding behavior. Weekly cumulative food intake for adolescent isolation and caloric-stress-exposed *NtsR1*$^{++}$ and *NtsR1*^{KOKO} **A)** male and **B)** female mice. **C)** Percentage of mice with overt food spillage that had to be excluded from weekly cumulative food intake measurements. **D)** Average number of aphagic episodes (≤ 0.5 g chow eaten within 24 hr) between wk 7 and 9.5 per group. Ambulatory activity over 24 hr in TSE metabolic cages for *NtsR1*$^{++}$ and *NtsR1*^{KOKO} **E)** males and **F)** females. Average energy expenditure for **G)** male and **H)** female *NtsR1*++ and *NtsR1*^{KOKO} mice over 24 hr. Feeding data were assessed by repeated measures two-way ANOVA with Sidak post-hoc analysis. Ambulatory activity and energy expenditure data were analyzed via Student's t-tests. Data depicting aphagic events was evaluated by two-way ANOVA with post-hoc Tukey tests. Except for graphs exhibiting number of mice demonstrating food spillage, all data represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.



mice reflects increased compulsive-like food spillage rather than genuine increased food consumption. We also examined whether NtsR1 deficiency increases vulnerability to develop the core feature of AN, episodes of self-restricted feeding [15]. Remarkably, the only animals that displayed aphagic episodes were the female *NtsR1*^{KOKO} mice (**Figure 3.6D**). Hence, lacking NtsR1 confers genetic risk that interacts with female sex and adolescent stress to increase vulnerability to disordered AN-like feeding behaviors. Activity or energy expenditure changes could contribute to altered body weight, but male *NtsR1*$^{++}$ and *NtsR1*^{KOKO} mice had comparable ambulatory activity, energy expenditure, VO_2 and VCO_2, which is consistent with their similar body weight and composition (**Figure 3.6E,G and 3.7A,B**). These measures were also similar between adolescent stressed female *NtsR1*$^{++}$ and *NtsR1*^{KOKO} mice (**Figure 3.6F,H and 3.7C,D**), despite their differences in weight and body composition. Thus, although females are more susceptible than males to stress-induced metabolic disruption (**Figure 3.7E,F)**, our data suggest that energy expenditure is not responsible for the low body weight of female *NtsR1*^{KOKO} mice.

NtsR1 deficiency with adolescent stress modifies motivated behaviors in females.

We hypothesized that lacking NtsR1 in combination with adolescent isolation and caloric restriction risk factors might potentiate co-morbid behaviors observed in AN, including excessive exercise, anhedonia, and altered DA signaling. When *NtsR1*$^{++}$ and *NtsR1*^{KOKO} males were provided with a running wheel they exhibited comparable ambulatory activity, wheel rotations, and time spent on wheels **(Figure 3.8A,C,D)**. Adolescent-stressed *NtsR1*^{KOKO} females with running wheels displayed augmented

Figure 3.7. Energy expenditure in mice exposed to adolescent isolation and caloric restriction stress. Assessment of *NtsR1*$^{++}$ and *NtsR1*^{KOKO} mice previously exposed to adolescent social isolation and caloric restriction stress at 16 wk. Average rate of **A)** oxygen consumed (VO_2) and **B)** carbon dioxide produced (VCO_2) in males. **C)** VO_2 and **D)** VCO_2 in female mice. Comparison of energy expenditure of **E)** male and **F)** female *NtsR1*$^{++}$ and *NtsR1*^{KOKO} mice at baseline vs. those subjected to adolescent stress. Calorimetry data were analyzed with Student's t-tests whereas energy expenditure comparisons were performed via two-way ANOVA with post-hoc Tukey tests. Multiple comparisons were performed for factors of genotype and stress exposure during adolescence. Graphs depict mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.

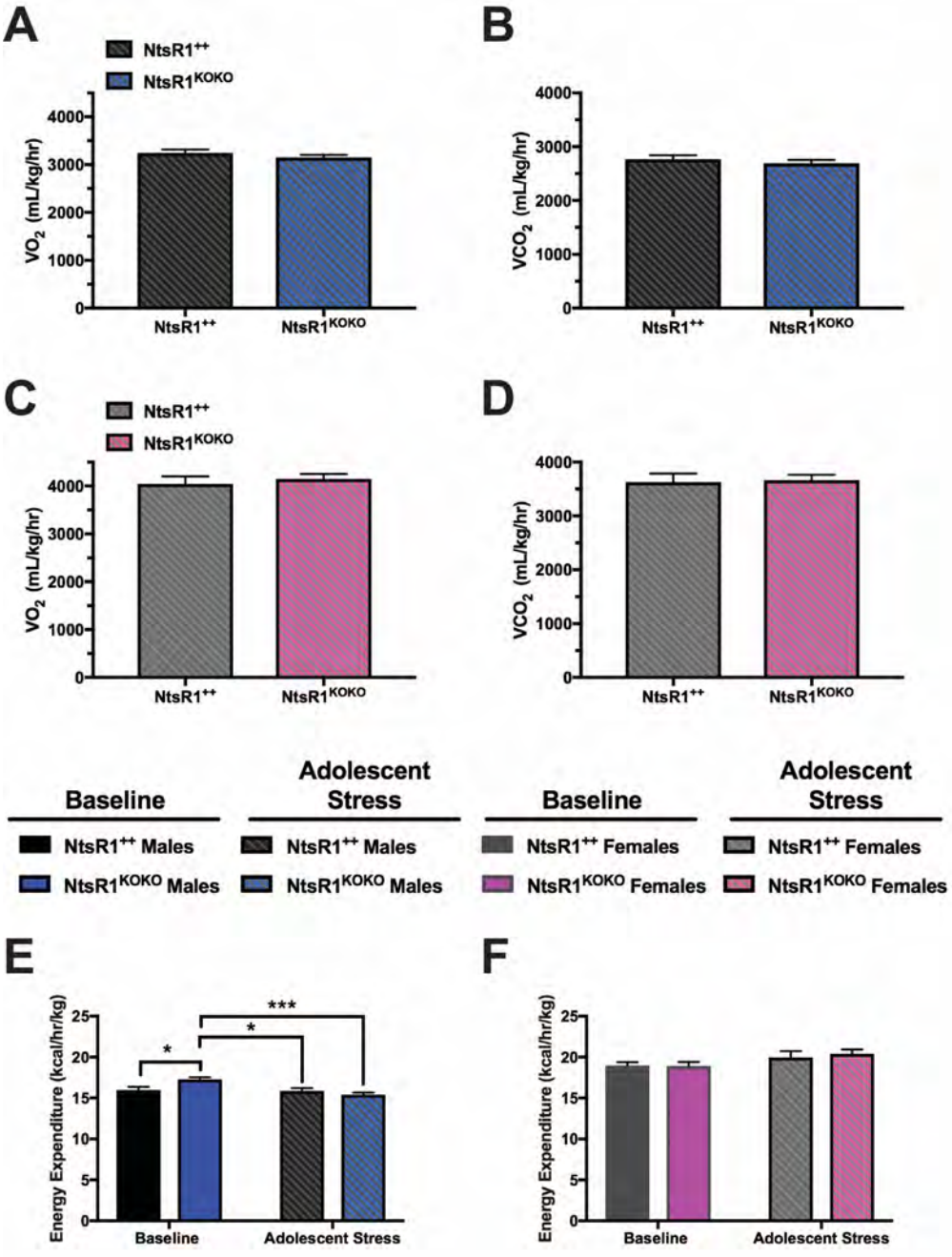
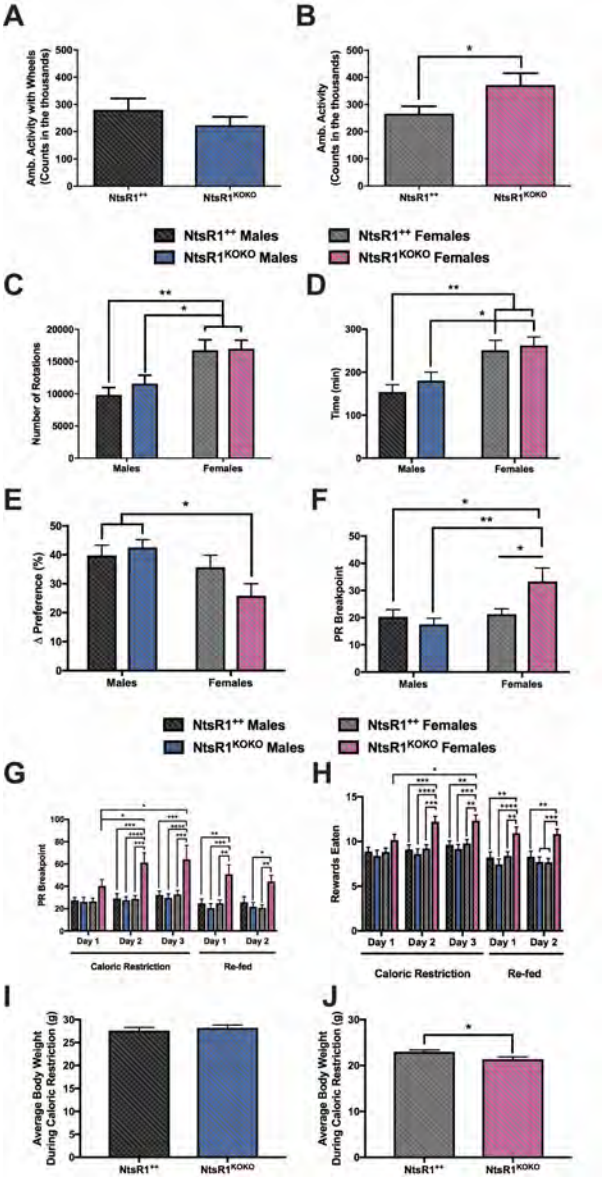


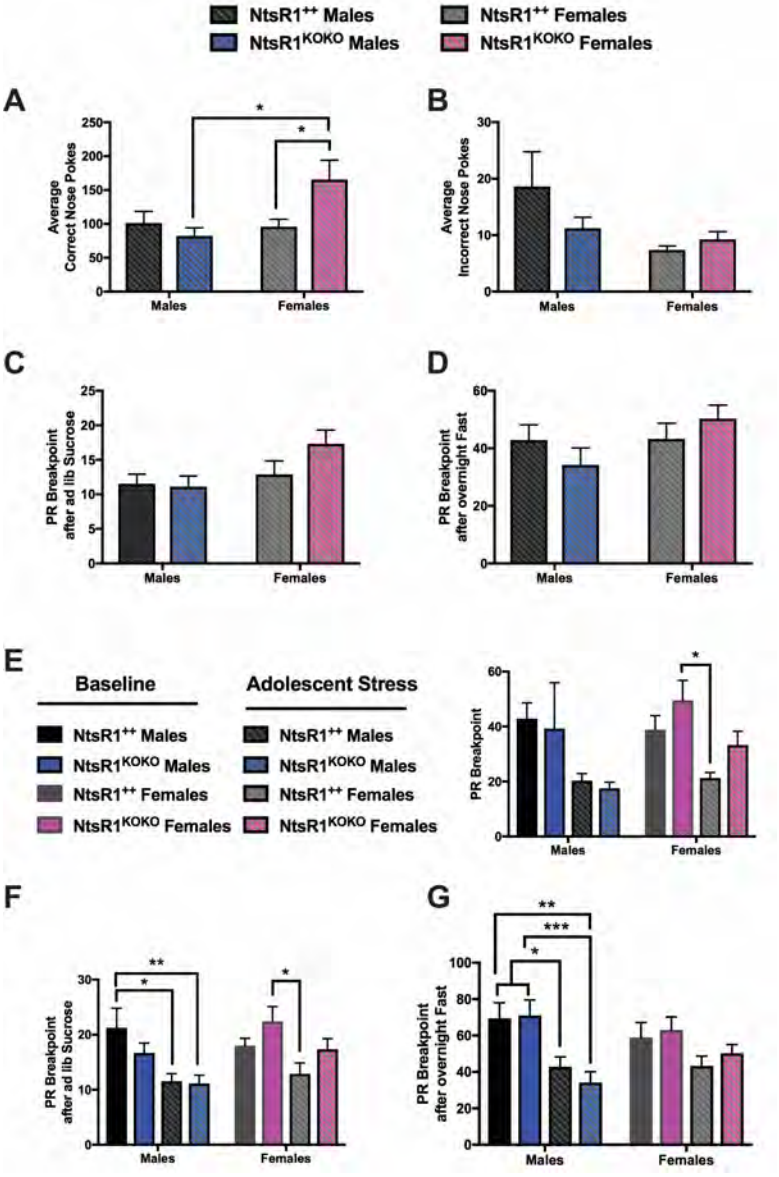
Figure 3.8. NtsR1 deficiency and adolescent stress interact in females to modify motivated behaviors that contribute to energy balance. Differences in 24 hr ambulatory activity between adolescent isolation and caloric-stress-exposed *NtsR1*$^{++}$ and *NtsR1*^{KOKO} **A)** males and **B)** females while they had access to a running wheel. **C)** Number of wheel rotations and **D)** time spent on the wheel for male and female *NtsR1*$^{++}$ and *NtsR1*^{KOKO} mice. **E)** Difference in percent preference between a 0.5% sucrose solution and water. **F)** PR breakpoint for responding for sucrose pellets in *ad libitum* fed mice. Daily measures of **G)** PR breakpoint and **H)** number of sucrose rewards eaten during 3 days of 60% caloric restriction followed by 2 days of *ad libitum* re-feeding. Average body weights of *NtsR1*$^{++}$ and *NtsR1*^{KOKO} **I)** male and **J)** female mice during the 3 days of caloric restriction. Ambulatory activity and body weight data were analyzed with Student's t-tests. Wheel usage, sucrose preference, and PR breakpoint comparisons were assessed via two-way ANOVA with post-hoc Tukey tests. All data represent the mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.



ambulatory activity relative to *NtsR1*$^{++}$ females **(Figure 3.8B)** on top of the high levels of wheel usage by females **(Figure 3.8C,D)**. Adolescent stress did not alter hedonic sucrose intake in males **(Figure 3.8E)** but female *NtsR1*^{KOKO} mice had significantly reduced sucrose preference compared to female *NtsR1*$^{++}$ controls **(Figure 3.8E)**. Moreover, *NtsR1*$^{++}$ females had comparable sucrose preference to males, indicating that the anhedonia of *NtsR1*^{KOKO} mice was not due to stress alone. Thus, genetic loss of NtsR1 and exposure to adolescent stress interact to exacerbate exercise-like activity and elicit sucrose anhedonia, consistent with the reduced hedonic response to sucrose exhibited by persons with AN [61].

In contrast to the similar sucrose "wanting" amongst all non-stressed mice **(Figure 3.2D)**, the adolescent-stressed *NtsR1*^{KOKO} females exclusively demonstrated a higher PR breakpoint and were willing to work more to attain a greater number of sucrose rewards **(Figure 3.8F)**. This augmented responding was not due to nonspecific, hyperlocomotor nose-poking **(Figure 3.9A,B).** Large, acute deviations in energy status (sucrose overfeeding or overnight fasting) did not alter PR breakpoints between genotypes of adolescent stressed mice **(Figure 3.9C,D)**. However, in a more mild, progressive model of energy deficiency (3 days of 60% caloric restriction followed by 2 days of re-feeding), adolescent-stressed *NtsR1*^{KOKO} female mice exhibited enhanced PR responding for sucrose rewards **(Figure 3.8G)** and increased number of rewards eaten **(Figure 3.8H)** relative to males and female controls. The three-day caloric restriction had no genotype-effect on male body weight **(Figure 3.8I),** whereas *NtsR1*^{KOKO} females weighed less than *NtsR1*$^{++}$ females **(Figure 3.8J).** Interestingly,

Figure 3.9. PR responding of mice exposed to adolescent isolation and caloric restriction stress. PR responding for palatable sucrose reward was determined for mice exposed to caloric restriction stress during adolescence. To determine if nose poking of NtsR1-null females was truly motivational and not just a consequence of compulsive behavior, the average number of nose pokes performed in the **A)** active port (correct) and **B)** inactive port (incorrect) was calculated from the test days during which PR breakpoint was achieved. To evaluate motivated responding for palatable reward in the face of altered energy status, mice were either overnight **C)** provided 150 sucrose pellets (e.g. sucrose overfeeding) or **D)** fasted and the PR breakpoint was determined the following day. Additional comparisons of PR breakpoint **E)** in *ad libitum* fed mice, **F)** after sucrose overfeeding, and **G)** after overnight fast were performed between non-stressed and adolescent-stressed *NtsR1*$^{++}$ and *NtsR1*^{KOKO} mice. All data were analyzed via two-way ANOVA with post-hoc Tukey tests. For **E-G**, multiple comparisons were performed for factors of genotype and stress exposure during adolescence. Data represent the mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.

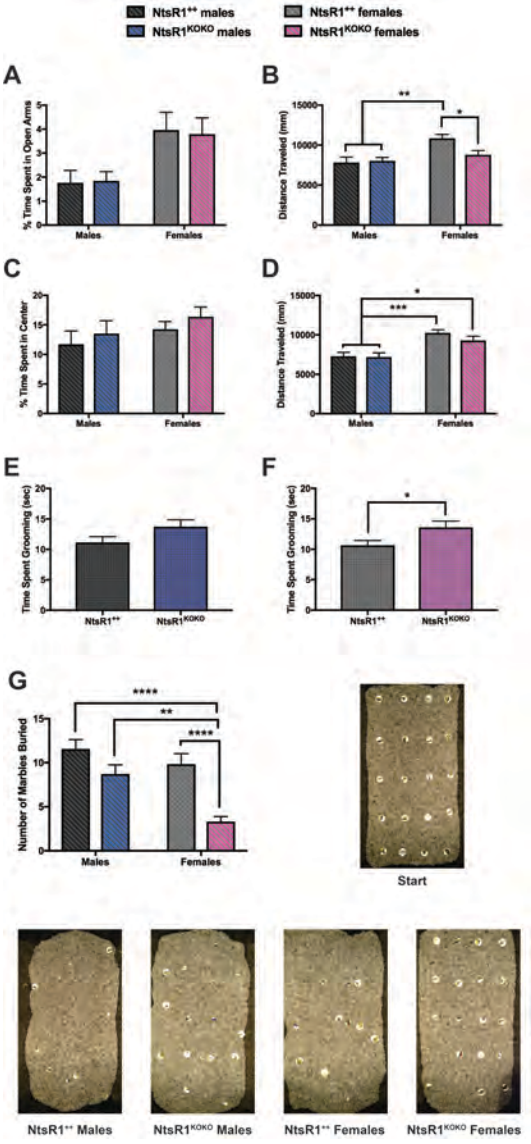


adolescent stress appears to blunt operant responding for sucrose in male mice, but less so in females **(Figure 3.9E-G)**. Altogether, these data suggest that females lacking NtsR1 have heightened motivation for food rewards in the face of adolescent stress exposure, which is suggestive of altered DA circuitry.

NtsR1 deficiency and adolescent stress promotes maladaptive behaviors in females

We assessed if NtsR1-null mice exposed to adolescent stress were vulnerable to anxiety, OCD, and lack of self-care behaviors that are co-morbid in AN. We found no genotype-differences in the elevated plus maze or open field tests of anxiety **(Figure 3.10A,C)**. As with the non-stressed cohort, *NtsR1*$^{++}$ females traveled more distance in the maze relative to males, and *NtsR1*^{KOKO} females also traveled then *NtsR1*++ females **(Figure 3.10B)**. Mice of both genotypes traveled more distance in the open field chambers compared to males **(Figure 3.10D)**. Time spent grooming was assessed for adolescent-stressed males and females, and, similar to the baseline cohort **(Figure 3.4E,F)**, female NtsR1^{KOKO} mice trended toward increased grooming behavior. When grooming data were combined for both the baseline and adolescent-stressed cohorts, female NtsR1^{KOKO} mice, in general, displayed increased compulsive grooming behaviors relative to wildtype controls **(Figure 3.10F)**, whereas no difference was observed between males **(Figure 3.10E)**. Thus, while NtsR1^{KOKO} females display a small increase in compulsive grooming behavior, the difference is real. We also assessed marble burying, a measure of OCD and/or disheveled-like behaviors that indicate lack of self-care. Despite the general hyperlocomotor and hyper-operant

Figure 3.10. NtsR1-null females exposed to adolescent stress develop neglect and inattention to self-care behaviors. A) Time spent within the open arms of the elevated plus maze by male and female *NtsR1*$^{++}$ and *NtsR1*^{KOKO} mice exposed to adolescent social isolation and caloric restriction stress. **B)** Female *NtsR1*$^{++}$ mice traveled a greater distance relative to males of both genotypes and *NtsR1*^{KOKO} females while in the elevated plus maze. **C)** Percentage time *NtsR1*$^{++}$ and *NtsR1*^{KOKO} male and female mice spent in the center of the open field chamber. **D)** Female mice traveled more than males during the open field assay. Combined time spent grooming for all study **E)** males and **F)** females during the open field test. **G)** Marble-burying behavior was significantly lower in *NtsR1*^{KOKO} females relative to males and *NtsR1*$^{++}$ females. Representative images of marble arrangement at the start of the test (Right of graph) and marble displacement at the end of the 30 min test for each group (beneath the graph). Grooming data was analyzed with student's t-tests. All other data were assessed via two-way ANOVA with post-hoc Tukey tests. All data represent the mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.



responding of adolescent-stressed *NtsR1*^{KOKO} females, they buried remarkably fewer marbles compared to female controls and males of either genotype **(Figure 3.10G)**. Taken together, these data suggest that lacking NtsR1 along with adolescent stress does not specifically compound anxiety but does promote disheveled behavior similar to the reduced sense of self-preservation displayed by individuals with AN.

Discussion

Translational Significance

We evaluated how NtsR1-deficiency interacts with environmental risk factors to promote the development of AN-like behaviors in mice. Our study is translationally relevant, since damaging mutations in *NTS-NTSR1* gene pathways were recently identified as a commonly affected biological pathway in patients with AN [28]. Moreover, one individual with an *NTSR1* mutation had a daughter and granddaughter with AN who inherited it [28]. This is significant because it suggests that genetic disruption of NTS-NTSR1 signaling might be a heritable risk factor that could predispose individuals to develop AN. In agreement, we found that mice lacking NtsR1 have lower body weight than controls. Moreover, female mice lacking NtsR1 and exposed to adolescent stress were specifically vulnerable to developing aphagia, low body weight, and co-morbidities similar to those observed in AN. Together, these animal model and human findings support that disruption of the Nts-NtsR1 poses genetic risk to develop AN, and further investigation of this system may suggest treatments to improve outcomes.

Strengths and Limitations Using *NtsR1^{KOKO}* Mice to Model Genetic Risk for AN

Constitutive gene deletion models (as is the case in *NtsR1^{KOKO}* mice) have been criticized for causing developmental disruptions rather than reflecting normal gene function in the adult. We maintain, however, that the developmental, whole-body NtsR1-deficiency of *NtsR1^{KOKO}* mice most accurately models what occurs in individuals with loss of function *NTS-NTSR1* variants, which presumably cause developmental alterations throughout the brain and periphery. As such, *NtsR1^{KOKO}* mice have face

validity for understanding if loss of NTS-NTSR1 signaling contributes to AN, particularly for the subset of individuals with loss of function NTS-NTSR1 mutations. While not everyone with AN harbors mutation in *NTS* or *NTSR1*, disruptions upstream of this pathway may also diminish its signaling; hence, our data is applicable to understanding how direct or indirect loss of NTS-NTSR1 function may confer risk for developing AN.

While there is a rich literature implicating central Nts-NtsR1 in feeding and body weight, we cannot rule out that genetic loss of Nts-NtsR1 may impede intestinal lipid absorption [29] or disrupt vagal regulation of the hindbrain [62] to promote leanness. Peripherally-produced, circulating Nts has a short half-life [63,64], and is unlikely to reach midbrain NtsR1-DA neurons that coordinate motivated behaviors disrupted in AN (e.g. rewarding wheel running, sucrose wanting, and liking). Hence, we postulate that disruption of central Nts-NtsR1 signaling underlies at least some aspects of AN, but it will be important to define the contributions of central vs. peripheral Nts-NtsR1 actions.

Strengths and Limitations of the Sequenced Adolescent Stress Model

The risk of developing AN is multifactorial, resulting from a combination of genetic, sex, and stress risks [5,65]. Developmental timing is also a factor: co-morbid anxiety disorders usually precede the development of disordered eating behaviors [5,66,52,67], while negative energy states from dieting or stress/illness-related episodes typically occur just prior to the development of the eating disorder [42,43,68]. Even though studying AN risk is challenging in humans, it can be ideally tested using mice, which allow for manipulation of genes and environmental factors with great temporal

precision [12,15,69,70]. Indeed, a time-dependent presentation of AN risk factors to genetically prone-mice showed that the brain-derived neurotrophic factor (BDNF) Val66Met gene variant interacts with adolescent stress to promote development of aphagic episodes, similar to the self-imposed food restriction that is a core feature of AN. [15]. Using this multivariate paradigm, we found that female *NtsR1*^{KOKO} mice were uniquely vulnerable to developing self-imposed aphagia during the adolescent stress period, though it was less severe compared to BDNF Val66Met mice. Only a portion of *NtsR1*^{KOKO} females or BDNF Val66Met mice exhibited aphagic episodes, consistent with fact that not all individuals with high risk for eating disorders develop full-blown disease [2]. Thus, NtsR1-deficiency interacting with adolescent stress has face validity for promoting AN, as it recapitulates the sex- and temporal-specificity characteristic of the disorder [71].

Female *NtsR1*^{KOKO} mice exposed to the adolescent stress paradigm also exhibited maladaptive behaviors associated with AN, including excessive exercise-like activity, anhedonia, and OCD-like behaviors. Only the *NtsR1*^{KOKO} females exposed to adolescent stress further heightened ambulatory activity, and it was not due to general hyperactivity (supported by their similar inactive port nose-poking in operant conditioning and reduced marble burying activity compared to other groups.) Rather, the wheel-running and enhanced ambulatory activity of female *NtsR1*^{KOKO} mice reflects increased voluntary activity, much like the drive for individuals with AN to engage in excessive exercise. Voluntary locomotor activity is also linked with food fragmentation behaviors [58]; indeed, female *NtsR1*^{KOKO} mice with increased activity were more prone

to food spillage, a stereotyped behavior analogous to the OCD commonly accompanying AN. Strikingly, only the adolescent-stressed female *NtsR1*^{KOKO} mice demonstrated anhedonia known to accompany AN. None of these behaviors were reported in BDNF Val66Met mice exposed to adolescent stress; however, neither BDNF mutants nor *NtsR1*^{KOKO} females exhibited adolescent stress-elevated anxiety typical of AN. Although though the multivariate-risk model does not produce all aspects of the human disorder, such inconsistencies do not diminish its usefulness to assess AN development or the importance of either genetic risk factor. Indeed, even the activity-based anorexia (ABA) model, the most widely accepted animal model of AN [69,70] fails to recapitulate all features of the disorder and cannot be used to evaluate co-morbidities.

Potential Role of DA Signaling

The incidence of both restricted and enhanced food intake of *NtsR1*^{KOKO} females is not incompatible with AN. A previous report showed that *NtsR1*^{KOKO} males ate less chow than control mice but consumed more palatable high-fat, high-sucrose diet [38], in line with our findings that loss of NtsR1 can promote extremes in feeding behavior. Similarly, individuals with the binge/purge-subtype of AN typically restrain food intake but periodically engage in binge-eating episodes that consist of excessive intake of energy-dense foods [28]. In fact, individuals with AN often transition between subtypes and disorders [72]. Thus, the extremes in eating behavior observed in *NtsR1*^{KOKO} mice are representative of the dynamic continuum of eating disorders.

At first pass, the heightened motivation of *NtsR1*^{KOKO} female mice to work for sucrose rewards (a DA-dependent behavior) might seem inconsistent with the anorexia of AN. Nonetheless, elevated DA has also been demonstrated in the ABA model of AN, and inhibiting DA signaling decreased activity, increased food intake, and minimized weight loss that improved survival [73,74]. Moreover, individuals with AN exhibit heightened reward response in a DA-dependent reward conditioning task [75], enhanced activity in brain areas targeted by DA [76], increased D2/D3 receptor density [77] and functional polymorphisms in D2 receptors are associated with AN [78]. Similar to these indications of hyper-DAergic signaling, *NtsR1*^{KOKO} mice have higher extracellular striatal DA and an amplified psychostimulant response [79]. Hence, developmental disruption of NtsR1 signaling may promote a hyper-DAergic phenotype that can elicit hyperactivity and compulsivity, known co-morbidities of AN. While one could argue that reduced marble burying signifies diminished compulsive behavior in mice, previously characterized DA transporter knockout mice are compulsive, hyperactive, hyper-DAergic and demonstrate reduced marble burying, similar to *NtsR1*^{KOKO} females [80]. Meanwhile, the diminished sucrose preference of *NtsR1*^{KOKO} females is consistent with the reduced “liking” for sucrose and high-calorie foods exhibited by restricting subtype AN patients [47,81,82]. Indeed, enhanced food “wanting” can occur without “liking”, even in hyperDAergic animals, as these are regulated via distinct circuitry [83,84]. Our data are thus consistent with prior work that heightened response to rewarding stimuli, such as palatable food and exercise, along with anhedonia are signature features of AN.

Conclusion

Overall, we show that NtsR1-deficiency is a genetic risk factor that, when interacting with risk factors of being female and exposure to adolescent stress, promotes aberrant feeding, excessive locomotor, and compulsive anxiety-like behaviors that are analogous to symptomatology classically associated with AN. While much work remains to be done, these findings suggest that restoring signaling via the Nts-NtsR1 system may be useful to improve outcomes in AN.

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CHAPTER 4. Neural Inputs to Lateral Hypothalamic Area Neurotensin Neurons in Female Mice

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Abstract

Many neurons within the lateral hypothalamic area (LHA) contain the anorectic neuropeptide, neurotensin (Nts). We hypothesized that vulnerability to develop AN might be linked with increased afferent input to anorectic LHA Nts neurons. We therefore explored whether genetic and environmental risk factors that promote vulnerability to develop AN alter afferent input to LHA Nts neurons. We used genetically modified monosynaptic rabies virus tracing to gain an unbiased survey of afferents to LHA Nts neurons. To examine genetic susceptibility, we labeled inputs of female mice with intact NtsR1 (*NtsR1*$^{++}$) or null for NtsR1 (*NtsR1*^{KOKO}), based on the discovery of variants in the Nts→ NtsR1 pathway in individuals with AN [1]. To examine whether the interaction of genetic and environmental risk factors might exacerbate circuit adaptations, we also studied *NtsR1*^{KOKO} mice exposed to sequenced adolescent stress relevant to AN (social isolation and mild, brief caloric restriction). In general, we observed relative increases in the densities of direct inputs to LHA Nts neurons as a result of lacking NtsR1 or in combination with exposure to adolescent stress. Many of these inputs were identified in structures that are either implicated in anorectic circuits or are altered in individuals with AN. Our results support the idea that augmentations to Nts circuitry may be partly responsible for the altered feeding, activity, and overall energy balance observed in AN.

Key Words: lateral hypothalamic area, rabies virus screen, neural plasticity, anorexia nervosa, adolescent stress, feeding.

Introduction

Anorexia Nervosa (AN) is a devastating disease in which individuals self-impose food restriction and engage in excessive exercise to maintain low body weight [2]. While genetic, biological, sex, and environmental factors are known to predispose development of AN [3,4], the malnutrition and starvation that accompany the disorder can cause adaptations to brain circuitry that exacerbate behavioral disturbances associated with AN and, thus, further advance disease progression [2,5,6]. In human studies it can be difficult to disentangle which brain circuitry alterations promoted the development of AN vs. alterations that resulted from malnutrition [6,7]. However, animal models can be used to systematically test how risk factors for AN alter brain circuitry, and hence to discriminate causative neural changes from effects.

Unpublished data has shown that increased fiber densities are present in the lateral hypothalamic area (LHA) of individuals with AN *(Prevot et al., unpublished, Annual Meeting of the Endocrine Society, 2015).* Hence, enhanced afferent modulation of LHA neurons that modulate feeding and/or movement behaviors might contribute to development of the disorder. The nature of the enhanced inputs (inhibitory or excitatory) and their targets within the LHA, however, are unknown. While there are many neuropeptide-distinct neuronal populations within the LHA, most are orexigenic, and the only anorectic neuropeptide-defined population are neurotensin (Nts) neurons [8–10]. It is therefore conceivable that altered fiber density to the anorectic LHA Nts neurons could contribute to the development of self-restricted feeding and excessive physical activity observed in AN. Indeed, chemogenetic activation of LHA Nts neurons

in adult male mice promotes a state of energy deficit in which mice exhibit increased locomotor activity and energy expenditure that is accompanied by suppressed food intake [11]. Activation of LHA Nts neurons also restrained motivated responding for palatable sucrose rewards, specifically when mice were in a fasted state [11]. This is pertinent to AN, as persons with this disorder are able to restrain food intake despite being in a state of severe energy deficit and even find such restrictive behavior to be rewarding [12]. Furthermore, loss of function variants in Nts and its receptor, Neurotensin Receptor-1 (NtsR1), have recently been described in individuals with AN, suggesting that genetic disruption of Nts → NtsR1 signaling may confer heritable risk for developing the disorder [1]. We therefore hypothesized that vulnerability to develop AN might be linked with increased afferent input to LHA Nts neurons. Moreover, we reasoned that LHA Nts neurons might receive increased top-down modulation by other circuits implicated in AN, such as those mediating taste-and reward-processing [7,13]

Here we used genetically-modified monosynaptic rabies virus tracing to gain an unbiased survey of afferents to LHA Nts neurons, and this method has also been valuable to identify inputs that are particularly malleable to environmental risks [14]. We examined the neural inputs to LHA Nts neurons in female mice since AN is 10X more common in females than males. To examine genetic susceptibility, we labeled inputs of mice with either intact NtsR1 (*NtsR1*$^{++}$) or null for NtsR1 (*NtsR1*^{KOKO}), based on the loss of function variants in the Nts→ NtsR1 pathway that were recently linked with AN [1]. To examine whether the interaction of genetic and environmental risk factors might exacerbate circuit adaptations, we also studied *NtsR1*^{KOKO} mice exposed to sequenced

adolescent stress relevant to AN (social isolation and mild, brief caloric restriction) [15]. We invoked stress during adolescence because it is a period of time in which a surge in pubertal steroid hormones and increased stress provokes development of disordered eating in genetically predisposed individuals by moderating genetic influences [2,16]. Social isolation of mice, especially during adolescence, is stressful and can promote development of behaviors analogous to those associated with anxiety and depressive disorders [15,17,18]. Mild caloric restriction stress during adolescence promotes development of disordered eating in mice, including aphagia [15], and is translatable to the human disorder, as dieting or unintentional weight loss both precede the development of AN [15,19,20]. In general, we observed increases in the densities of direct inputs to LHA Nts neurons as a result of lacking NtsR1 and as a result of a combination of this genetic deficiency with exposure to adolescent stress. Many of these inputs were identified in structures that are either implicated in anorectic circuits or are altered in individuals with AN. Our results support the idea that increased inputs to LHA Nts neurons results from risk factors of AN and that adaptations to Nts/NtsR1 circuitry may be partly responsible for the altered feeding, activity, and overall energy balance observed in AN.

Methods

Animals

Mice were bred in a 12 hr light/12 hr dark cycle and raised with ad libitum access to standard chow diet (Teklad 7913), unless specified otherwise. Only female mice were studied. All protocols involving mice were approved by the Michigan State University Institutional Animal Care and Use Committee (IACUC), in accordance with Association for Assessment and Accreditation of Laboratory Animal Care and National Institutes of Health guidelines.

Nts^{Cre} mice [10] (Jackson stock # 017525) were bred onto a C57/BL6 line for 7 generations to obtain fully backcrossed mice. Some *Nts*^{Cre} mice were crossed with *Rosa26*$^{EGFP\text{-}L10a}$ mice [21] to create *Nts*^{Cre}*;GFP* reporter mice in which all Nts neurons were identifiable by GFP expression. Only progeny heterozygous for the *Nts*^{Cre} allele were used. In addition, wildtype and *NtsR1*^{KOKO} mice (*Ntsr1*^{tm1Dgen}*;* Jackson stock # 005826) on a C57/BL6 background were bred to create heterozygous mice. These mice were then bred with *Nts*^{Cre} and *Nts*^{Cre}*;GFP* reporter mice to create *Nts*^{Cre}*;NtsR1*$^{++}$ and *Nts*^{Cre}*;NtsR1*^{KOKO} animals for tracing studies. Standard PCR was performed from DNA isolated from tail biopsies, and genotyping was carried out with the following primers: *Nts*^{Cre}: common forward: 5’ ATA GGC TGC TGA ACC AGG AA, cre reverse: 5’ CCA AAA GAC GGC AAT ATG GT, and WT reverse: 5’ CAA TCA CAA TCA CAG GTC AAG AA. *Rosa26*$^{EGFP\text{-}L10a}$: mutant forward: 5’ TCT ACA AAT GTG GTA GAT CCA GGC, WT forward: 5’ GAG GGG AGT GTT GCA ATA CC, and common reverse: 5’ CAG ATG

ACT ACC TAT CCT CCC. *NtsR1*^{KO}: Forward: CTC TAA TGT GCC ACA GCT CAG AGA G, common: CAG CAA CCT GGA CGT GAA CAC TGA C, Reverse: CCA AGC GGC TTC GGC CAG TAA CGT T.

To determine if adolescent stress interacts with lack of NtsR1, three *Nts*^{Cre}*; NtsR1*^{KOKO} female mice were subjected to a multivariate risk model of AN [15]. This involved single housing mice at 5 weeks of age, measuring daily food intake during week 6, and restricting daily caloric intake to 75% of average food intake for 11 days starting at 7 weeks of age. Mice were then fed *ad libitum* and single housed for the duration of the study.

Stereotaxic Surgery and Viral Injections

The dual vectors for genetically mediated monosynaptic rabies tracing (rAAV8/hsyn-TVA-RabiesB19G and EnvA-ΔG-Rabies-mCherry) were graciously provided by the Michigan Diabetes Research Center at the University of Michigan, led by Martin G. Myers, Jr. *Nts*^{Cre} mice with intact NtsR1 (*NtsR1*$^{++}$), deficient in NtsR1 (*NtsR1*^{KOKO}), and both deficient in NtsR1 and exposed to the adolescent stress paradigm (adolescent-stressed *NtsR1*^{KOKO}) were anesthetized with isolfluorane/O_2 and positioned in a stereotaxic frame. Holes were drilled in the skull to allow for access of a guide cannula with stylet (Plastics One, Roanoke, VA). The guide cannula was lowered into the targeted region of the LHA to the following coordinates, in reference to Bregma: A/P -1.34, M/L ± 1.00, and D/V -5.20. The stylet was removed, and 200 µL of rAAV8/hsyn-TVA-RabiesB19G was injected bilaterally into the LHA. Bilateral injections

were performed to increase chance for obtaining a well-targeted hemisphere. To prevent backflow of virus, the injector and cannula were left in the injection site for an additional 5 minutes, after which the cannula was removed. The skull access sites were filled with bone wax, and Vet Bond surgical adhesive was used to close the surgical incision site. Three weeks later (to allow for sufficient expression of TVA and Rabies B19G) an identical surgery was performed to inject 150 µL of EnvA-ΔG-Rabies-mCherry into the same site. Mice were perfused 11 days later.

Perfusions and Immunohistochemistry

Intraperitoneal sodium pentobarbital was used to anesthetize mice, which were subsequently transcardially perfused first with 1X phosphate-buffered saline (PBS) followed by 10% formalin. Brains were post-fixed in 10% formalin for 24 hours then dehydrated in 30% sucrose. A freezing microtome (Leica) was used to slice brains into 30 µm coronal sections, which were divided into 4 representative series that were stored in 1X PBS with 1% formalin. Immunofluorescence was performed as previously described [10]. To enhance visualization of GFP-expressing Nts neurons, sections from *Nts^{Cre};GFP reporter* brains were incubated in primary antibody for GFP (Abcam, chicken, 1:1000; RRID: AB_300798). Additionally, all brains were incubated in primary antibody to detect mCherry (Clontech, rabbit, 1:1000; RRID: AB_10013483). Secondary antibodies applied to detect anti-GFP and anti-mCherry included species-specific Alexa-488 conjugated (Jackson, ImmunoResearch; 1:200; RRID: AB_2340375) or Alexa-568 conjugated antibodies (LifeTech, 1:200; RRID: AB_2534017). Images were captured with an Olympus BX53 fluorescence microscope and were visualized with Cell Sense

software. Brains from three well-targeted mice were assessed for each group (*NtsR1*$^{++}$, *NtsR1*^{KOKO}, and adolescent-stressed *NtsR1*^{KOKO}). Images were qualitatively analyzed to assess the relative number of mCherry-labeled cell bodies in each brain region using the following rating scale: 0 = no inputs, + = few inputs, ++ = moderate inputs, +++ = numerous/many inputs, ++++ = very dense inputs. Per group, average ratings of inputs from each structure were determined from the three mice. Since bilateral injections were performed, some mice assessed were bilaterally targeted, whereas others had only one well-targeted hit site. In bilaterally-targeted cases, assessments were performed from the better-targeted side, which was deemed to be the side with greater input densities from areas with known projections, such as the paraventricular hypothalamus, as well as the side with greater colocalization of GFP-Nts neurons with mCherry (infected Nts neurons). Images shown are representative of the group.

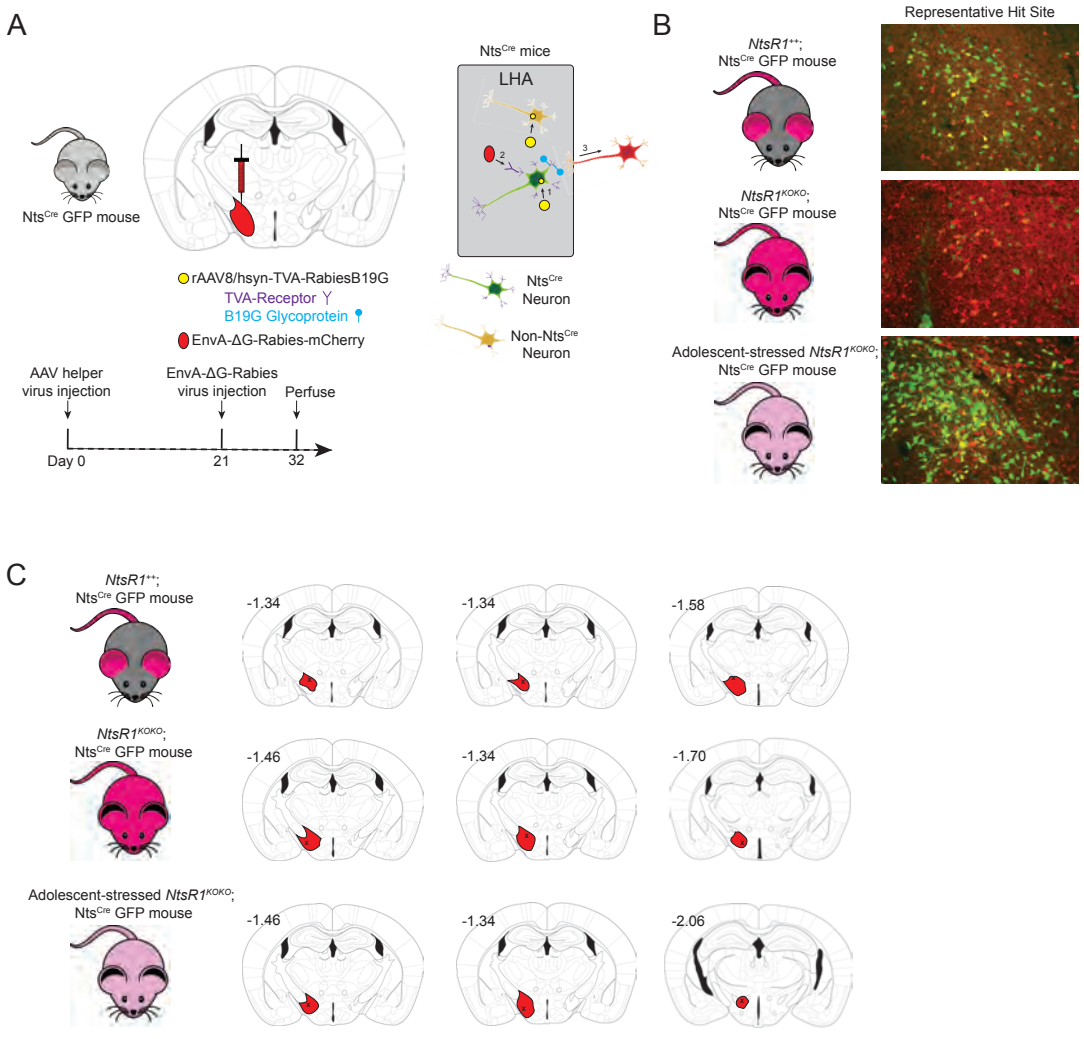
Results

Tracing Method Used to Define Afferents to LHA Nts Neurons

We used a 2-vector, genetically-modified rabies tracing system to obtain a comprehensive understanding of the direct inputs to LHA Nts neurons, as well as how these circuits may be altered as a result of risk factors associated with AN. First, mice were injected in the LHA with an adeno-associated virus (AAV) inducing Cre-dependent expression of the TVA receptor (which specifically binds the avian EnvA glycoprotein) and the rabies B19G glycoprotein; these proteins are requisite for infection and trans-synaptic spread of a modified rabies virus and will *only* be expressed in Cre-expressing LHA Nts neurons. **(Figure 4.1A)**. Three weeks later, mice were injected into the same site with an EnvA-pseudotyped, G glycoprotein-deleted, and mCherry-expressing rabies virus. This system has features to ensure selective identification of the monosynaptic inputs to LHA Nts neurons. First, the rabies virus was pseudotyped with the glycoprotein EnvA that specifically binds to TVA receptors; *since mouse cells do not endogenously express TVA, the only cells that can be infected by this rabies virus are the LHA Nts neurons made to express TVA.* Second, the rabies virus lacks B19G required for retrograde spread; *only the Cre-containing LHA Nts neurons express B19G, so the rabies virus will only spread to the presynaptic inputs of LHA Nts neurons.* Importantly, these labeled input neurons will express the rabies virus and mCherry, but because they lack B19G, the *rabies virus cannot spread further.* Thus, only infected LHA Nts^{Cre} neurons and their presynaptic inputs will express mCherry and can be identified.

Figure 4.1. Confirmation of LHA targeting in *Nts*^{Cre} mice injected with rabies-based sequence of viruses for monosynaptic input tracing.

A) Schematic of rabies-mediated monosynaptic tracing. 1) *Nts*^{Cre} mice, at least two of which were GFP-reporter mice, were injected in the LHA (red) with an AAV allowing for cre-inducible expression of TVA Receptor and B19G glycoprotein. 2) Three weeks later, a G-deleted, mCherry+ rabies virus with Cre-inducible expression of EnvA was injected into the same LHA site. 3) Eleven days were allowed for spread of modified rabies virus to cell bodies of direct presynaptic inputs. **B)** Representative hit sites of each group of *Nts*^{Cre} female mice analyzed. The three groups included *NtsR1*$^{++}$ females, *NtsR1*^{KOKO} females, and *NtsR1*^{KOKO} females exposed to a multivariate risk model of AN during adolescence. Three mice were assessed for each group, at least two of which were *Nts*^{Cre}*;GFP* reporter mice. Each microscopy image is a representative image of a mouse with validated LHA-targeting within each group. **C)** Bregma-labeled mouse atlas images illustrate the LHA-targeting of the three mice used for analysis within each group. The X marks the area where the tract mark was identified within the LHA (red).



First, we verified that mice had correctly targeted injections to the LHA. **Figure 4.1C** shows the sites of the injection tip (X) from each mouse, all of which were localized within the red-shaded confines of the LHA spanning from Bregma -2.06 to -1.34. Since at least 2 of the 3 mice analyzed for each group were *Nts^{Cre};GFP* mice, we also validated that the injection sites within these mice were within the large population of GFP-positive Nts neurons [8]. Moreover, we observed numerous GFP neurons co-labeled with mCherry, confirming that we successfully induced the tracing system in some primary Nts^{Cre} neurons **(Figure 4.1B)**. Certain GFP-labeled Nts^{Cre} neurons lack mCherry, indicating that they were not infected with both vectors, so we failed to label afferants from all LHA Nts neurons. Yet, the many co-labeled cells suggested robust transduction in LHA Nts neurons sufficient to permit a relative assessment of their global afferents, which will solely express mCherry (mCherry+). We therefore assessed the entire brains of these three well-targeted mice from each group (*NtsR1^{++}*, *NtsR1^{KOKO}*, and *NtsR1^{KOKO}* exposed to caloric restriction stress during adolescence) to characterize the relative densities of mCherry+ neurons in each brain region **(Table 4.1)**.

Common Afferents to LHA Nts Neurons Observed in All Three Groups

A number of structures that have been implicated in AN and energy balance provided similarly dense inputs to LHA Nts neurons, irrespective of stress status during adolescence and/or absence of NtsR1. Regions with very dense projections (+++/++++ → ++++) to LHA Nts neurons included the paraventricular hypothalamic nucleus (PVH) **(Figure 4.2B)** and supraoptic nucleus (SO) **(Figure 4.2C)**. The posteromedial division

Table 4.1. Brain regions providing inputs to LHA Nts neurons and their relative input densities in wildtype, *NtsR1*^{KOKO}, and adolescent-stressed *NtsR1*^{KOKO} females.

Table lists brain regions observed to have mCherry+ neurons, which directly project to LHA Nts neurons. The relative densities of mCherry+ identified afferents was qualitatively assessed in three well-targeted *NtsR1*$^{++}$, *NtsR1*^{KOKO}, and adolescent stress-exposed *NtsR1*^{KOKO} females. The rating scale used to evaluate input densities ranged from 0 to ++++, with 0 = no inputs, + = few inputs, ++ = moderate inputs, +++ = numerous/many inputs, ++++ = very dense inputs. The table shows the average input density per group for each brain region.

Structure	Female WT		Female KO		Female Adolescent-Stressed KO	
	Density	Bregma	Density	Bregma	Density	Bregma
Nucleus of the solitary tract (NTS)	0	N/A	0/+	-7.64 to -6.72	+	-7.76, -7.48
Vestibulocerebellar nucleus/Superior vestibular nucleus (VeCb/SuVe)	+	-6.00	0/+	-5.88	+	-5.80
Dorsal raphe nucleus, caudal part (DRC)	+	-4.96	+	-5.20	+	-4.96
Dorsal raphe nucleus, interfascicular part (DRI)	+/++	-4.84 to -4.36	+++	-4.72 to -4.48	++/+++	-4.84 to -4.24
Dorsal raphe nucleus, dorsal part (DRD)	++	-4.36	++	-4.72 to -4.48	+++	-4.72 to -4.48
Dorsal raphe nucleus, ventral part (DRV)	+/++	-4.48 to -4.24	+++	-4.72 to -4.36	++/+++	-4.84 to -4.36
Posterodorsal raphe nucleus (PDR)	+	-4.36	+/++	-4.84 to -4.36	+	-4.84 to -4.36
Dorsal raphe nucleus (DR)	+	-4.16 to -3.80	++/+++	-4.24 to -4.16	+	-4.24
Laterodorsal tegmental nucleus (LDTg)	+	-4.72	+/++	-5.02 to -4.84	++	-5.02 to -4.84
Laterodorsal tegmental nucleus (LDTgV)	+	-4.72	+/++	-4.90 to -4.84	+/++	-5.02 to -4.72
Dorsal raphe nucleus, lateral part (DRL)	++	-4.36	+/++	-4.72 to -4.36	++/+++	-4.72
Medial parabrachial nucleus (MPB)	0/+	-5.20 to -4.72	+/++	-5.02 to -4.84	+	-5.02 to -4.60
Lateral parabrachial nucleus (LPB)	+/++	-5.34 to -4.60	++/+++	-5.20 to -4.84	++/+++	-5.34 to -4.84
Cuneiform nucleus (CnF)	0/+	-5.34 to -4.84	0/+	-4.96 to -4.84	0/+	-5.02 to -4.84
Dorsal tegmental nucleus, pericentral part (DTgP)	++/+++	-4.96	+	-5.02 to -4.84	+++/++++	-5.02 to -4.84

Table 4.1 (cont'd)

Dorsal tegmental nucleus (DTg)	+++/++++	-4.96	+++	-4.90 to -4.72	+++	-4.84
Ventrolateral periaqueductal gray (VLPAG)	+	-4.48, -4.16	+	-4.60 to -4.24	+/++	-4.96 to -4.16
Dorsomedial periaqueductal gray (DMPAG)	+	-4.60 to -3.88	+	-4.48 to -4.04	+	-4.96 to -3.64
Dorsal cortex of the inferior colliculus (DCIC)	0/+	-5.68 to -4.96	0/+	-5.20 to -4.84	0/+	-5.34 to -4.96
Central nucleus of the inferior colliculus (CIC)	0/+	-5.34	0/+	-4.84	0/+	-5.02
External cortex of the inferior colliculus (ECIC)	0/+	-5.34 to -4.16	+	-4.48 to -4.36	+	-4.96 to -3.88
Raphe magnus nucleus (RMg)	++	-4.84 to -4.72, -3.88	+/++	-4.72 to -4.20	++/+++	-4.84, -4.36 to -3.80
Ventral tegmental nucleus (VTg)	+++	-4.72 to -4.60	++/+++	-4.72 to -4.60	+++/++++	-4.72 to -4.60
Pontine reticular nucleus, oral part (PnO)	0/+	-4.72	0/+	-4.72, -4.48 to -4.36	0/+	-4.84 to -4.72
Anterior tegmental nucleus (ATg)	++	-4.36	++/+++	-4.48 to -4.36	+++	-4.54 to -4.36
Deep layers of the superior colliculus (DpG/DpWh)	0/+	-4.48, -4.24, -4.04, -3.80 to -3.16	+	-4.36 to -3.08	+	-4.48 to -2.80
Pedunculotegmental nucleus (PTg)	+	-4.48 to -3.88	+/++	-4.60 to -4.24	+/++	-4.48 to -3.80
Intermediate layers of the superior colliculus (InG/InWh)	+	-4.84 to -3.40	+/++	-4.48 to -3.68	+	-4.48 to -3.28
Lateral periaqueductal gray (LPAG)	+	-4.04 to -3.88	+	-4.16 to -3.28	+	-4.48 to -3.28
Subpeduncular tegmental nucleus (SPTg)	+	-4.48 to -4.36	+/++	-4.84 to -4.60	+	-4.48 to -4.16
Median raphe nucleus (MnR)	+	-4.60 to -3.28	+/++	-4.72 to -4.04	+/++	-4.72, -4.04
Precuneiform nucleus (PrCnF)	0/+	-4.72 to -4.16	0/+	-4.60 to -4.20	+	-4.24 to -3.80
Caudal linear nucleus of the raphe (CLi)	+/++	-3.88	+/++	-4.36 to -4.04	++	-4.36 to -4.16
Retrorubral field (RRF)	+	-4.24. to -4.04, -3.40 to -3.28	+/++	-4.16, -3.68	++	-4.04 to -3.40
Retrorubral nucleus (RR)	0/+	-4.36	+	-4.36 to -4.04	++	-4.04
Subiculum (Sub)	0/+	-4.16	0/+	-4.24 to -3.64	0/+	-4.48, -4.16, -3.88 to -3.52
Dorsolateral periaqueductal gray (DLPAG)	0/+	-3.40	+	-4.16 to -4.04	+	-4.04 to -3.64

Table 4.1 (cont'd)

Edinger-Westphal nucleus (EW)	++	-4.04, -3.40	+++	-4.16 to -3.64	+++	-4.04 to -3.64
Interpeduncular nucleus/ Interfascicular nucleus (IP/IF)	+	-3.40, -3.16, -2.80	+/++	-3.80 to -3.68, -3.28 to -3.16	++	-3.64, -3.08
Pararubral nucleus (PaR)	+/++	-3.40	+	-3.68 to -3.52	+	-3.80
Ventral tegmental area (VTA)	+/++	-3.16 to -2.80	++/+++	-3.40 to -3.08	++/+++	-3.40 to -2.80
Red nucleus (RPC/RMC)	0/+	-3.40, -3.16	+	-3.28 to -3.16	+	-3.64 to -3.08
Rostral linear nucleus (RLi)	+	-3.16	++	-3.16	+/++	-3.64
nucleus of Darkschewitsch (DK)	+	-3.40	+	-3.64 to -3.16	+	-3.28
Peripeduncular nucleus (PP)	+	-3.40 to -2.92	+	-3.52	+	-3.64 to -3.40
Posterior intralaminar thalamic nucleus (PIL)	+	-3.40 to -2.70	+/++	-3.40 to -2.70	+/++	-3.40
Pregeniculate nucleus (PG)	+	-3.40	+	-3.40, -2.92	+	-3.40 to -3.08
Posterior thalamic nucleus group, triangular part (PoT)	+	-3.40 to -2.92	+/++	-3.40 to -2.70	+	-3.40 to -2.80
Pre-Edinger-westphal nucleus (PrEW)	++	-3.28	+++/++++	-3.52 to -2.92	+++/++++	-3.64 to -3.28
Mesencephalic reticular formation (mRt)	+	-4.36 to -3.80, -3.40	+	-4.72 to -3.28	+	-4.48 to -3.52
Zona Incerta (ZI)	+	-146 to -1.34	++/+++	-1.34 to -1.22	++	-1.58 to -1.22
Zona Incerta, caudal part (ZIC)	+	-3.40 to -2.92	+/++	-3.40 to -3.16	+/++	-3.40
Zona Incerta, ventral part (ZIV)	+	-3.08 to -1.58	++	-3.16 to -1.58	++/+++	-1.46
Zona Incerta, dorsal part (ZID)	+/++	-3.16 to -1.70	++	-3.16 to -1.58	++/+++	-1.46
Retromamillary nucleus (RM)	+	-3.08	++/+++	-3.16	++/+++	-3.28 to -2.80
Periaqueductal grapy (PAG)	+	-2.54 to -2.46	+/++	-2.54, -2.18	0/+	-2.92, -2.54
Prosomere 1 reticular formation (p1Rt)	+	-2.92, -2.54	+	-3.16 to -2.46	+	-2.92 to -2.80, -2.46
Anterior pretectal nucleus (APT)	0/+	-3.16 to -3.08, -2.62	+	-3.08 to -2.80	0/+	-2.92 to -2.54
Prerubral field (PR)	+	-3.28 to -3.16	+/++	-3.40 to -3.16	+/++	-3.28 to -2.92
Parasubthalamic nucleus (PSTh)	+++/++++	-2.70 to -2.30, -2.06 to -1.82	++/+++	-2.70	+++/++++	-2.70 to -2.62, -2.30
Subthalamic nucleus (STh)	0/+	-2.70	+	-2.80	0/+	-2.92
Retroethmoid nucleus (REth)	+	-3.40 to -2.70	+/++	-3.40 to -3.16	+/++	-3.52 to -3.16
Medial mamillary nucleus, median part (MnM)	++	-2.92 to -2.80	+++	-2.80	+/++	-2.92

Table 4.1 (cont'd)

Retromamillary nucleus, medial part (RMM)	++/+++	-2.80 to -2.70	++/+++	-3.16 to -2.70, -2.30	+++	-2.92 to -2.62
Retromamillary nucleus, lateral part (RML)	++	-2.80	++/+++	-3.08	++/+++	-2.92 to -2.80
Posterior hypothalamic nucleus (PH)	++	-2.92 to -2.46, -2.06	++/+++	-3.16 to -2.70, -2.30	++/+++	-2.92, -2.30
Prosomere 1 periaqueductal gray (p1PAG)	+	-2.62 to -2.46	++	-2.46, -2.18	+/++	-2.62 to -2.46, -1.94
Perirhinal cortex/Ectorhinal cortex (PRh/ Ect)	0	N/A	0/+	-2.80 to -2.70	0/+	-2.80, -2.30
Medial habenular nucleus (MHb)	0	N/A	0/+	-2.06 to -0.82	0/+	-1.70, -1.22
Lateral habenular nucleus (LHb)	+/++	-1.22 to -1.06	+/++	-1.10	+	-2.06, -1.70 to -1.58, -1.06 to -0.94
Parafascicular thalamic nucleus (PF)	+	-2.46, -2.18 to -2.06	+/++	-2.70, -2.06	++	-2.18 to -1.58
Premamillary nucleus, ventral part (PMV)	+++/++++	-2.70 to -2.30	+++/++++	-2.38	+++	-2.46 to -2.30
Lateral Hypothalamic Area (LHA)	+++/++++	-2.46 to -1.70	+++/++++	-2.06 to -1.70, -1.46 to -1.34	+++/++++	-2.30 to -0.94
Arcuate nucleus (Arc)	+++	-2.18 to -2.06	+++	-1.34	++++	-2.80 to -0.70
Dorsomedial hypothalamic nucleus (DM)	+/++	-2.46 to-2.18, -1.70 to -1.46	++	-2.30, -1.94 to -1.34	++	-1.94 to -1.46
Ventromedial hypothalamic nucleus (VMH)	++	-2.18 to -1.06	++	-1.94, -1.46 to -1.06	+++	-1.94 to -1.22
Central amygdala (CEA)	+	-2.18 to -0.82	++	-2.06, -1.58, -1.34	+/++	-1.82, -1.46 to -1.22
Insula (Ins)	0/+	-2.18, -0.40, 0.20 to 0.62, 1.42	+	-1.22, -0.22, 1.78 to 2.10	+/++	-2.62
Paraventricular hypothalamic nucleus (PVH)	++++	-1.70 to -1.58, -1.22, -0.82	++++	-1.22 to -0.94	++++	-1.22 to -0.70
Somatosensory Cortex (S)	0/+	-2.30, -1.82, -1.06 to 0.20, 0.62, 1.34 to 1.54	+	-0.58 to 0.74, 1.42 to 1.98	+	-0.82 to 1.94
Retrosplenial cortex (RSD/RSG)	0/+	-2.62 to -0.82	0/+		0/+	-1.22
Anterior Hypothalamus (AHP/AHC)	+	-1.22 to -0.82	+	-1.22 to -0.94	+/++	-1.22
Retrochiasmatic area, lateral part (RChL)	+/++	-1.22 to -0.94	+/++	-1.06	+/++	-1.22
Retrochiasmatic area (RCh)	+/++	-1.06	+/++	-1.06 to -0.94	+	-1.06, -0.94
Supraoptic nucleus (SO)	+++/++++	-1.06 to -0.70	+++/++++	-1.22 to -1.06, -0.70 to -0.46	+++/++++	-1.22 to -0.70, -0.46

Table 4.1 (cont'd)

Intertitial nucleus of the posterior limb of the anterior commissure (IPAC)	++	-0.94 to -0.70, -0.22	++/+++	0.2 to 0.32	++/+++	0.26 to 0.50
Anterodorsal thalamic nucleus (AD)	0/+	-0.58	0/+	-0.82 to -0.58	0/+	-1.34
Bed nucleus of the stria terminalis (BNST)						
Bed nucleus of the stria terminalis, medial division, posterolateral part (BSTMPL)	++/+++	-0.10	++	-0.46 to -0.22	++/+++	-0.04 to 0.02
Bed nucleus of the stria terminalis, medial division, posteromedial part (BSTMPM)	++/+++	-0.22	+++	-0.10	+++	-0.10 to 0.02
Bed nucleus of the stria terminalis, medial division, posterointermediate part (BSTMPI)	++/+++	-0.22	+++	-0.10	+++	-0.10 to 0.02
Bed nucleus of the stria terminalis, lateral division, posterior part (BSTLP)	++	0.08 to 0.20	+++/++++	-0.16 to -0.10, -0.50	+++/++++	-0.46 to 0.38
Bed nucleus of the stria terminalis, lateral divison, intermediate part (BSTLI)	+++	-0.16	+++	-0.22 to -0.10	+++/++++	-0.46
Bed nucleus of the stria terminalis, lateral division, juxtacapsular part (BSTLJ)	++	-0.10 to 0.08	++	0.14	+++/++++	-0.22 to 0.14
Bed nucleus of the stria terminalis, lateral division, dorsal part (BSTLD)	++	-0.10 to 0.08	+++	0.02 to 0.26	+++/++++	-0.22 to 0.26
Bed nucleus of the stria terminalis, lateral division, ventral part (BSTLV)	0/+	0.5 to 0.62	+/++	0.20 to 1.34	+/++	0.38 to 0.62
Bed nucleus of the stria terminalis, medial division, anterior and anterolateral part (BSTMA/BSTMAL)	+/++	-0.22, 0.14, 0.62	+++	0.26, 0.02	+/++	0.02 to 0.50
Bed nucleus of the stria terminalis, medial division, ventral part (BSTMV)	+/++	-0.16, 0.26 to 0.62	+/++	0.20 to 1.34	+/++	0.02 to 0.50

Table 4.1 (cont'd)

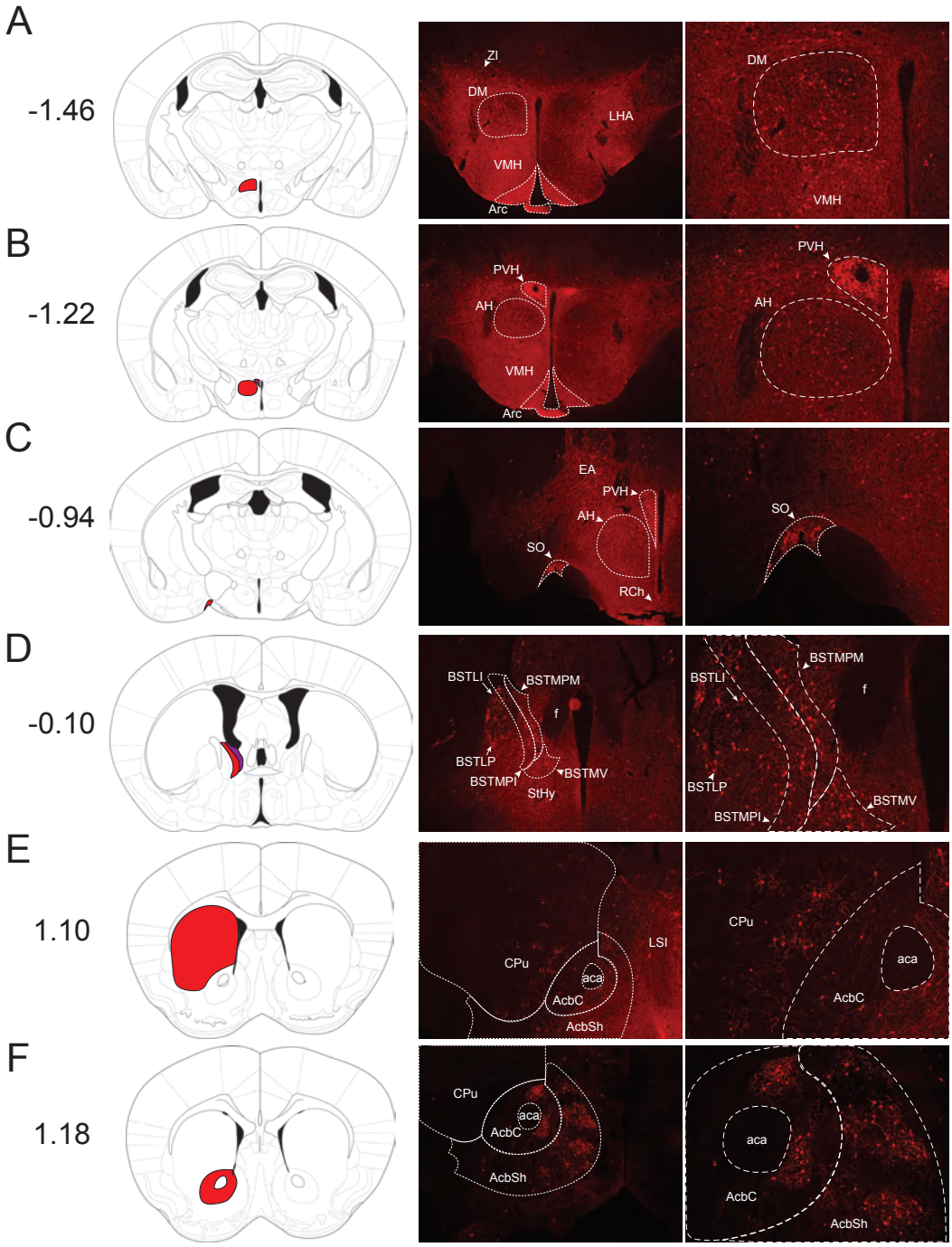
Extension of the amygdala (EAC/EAM)	+	-1.22 to -0.82	++	-0.70	++	-0.70
Caudate putamen (CPu)	+	0.14 to 0.26, 0.62 to 1.94	+/++	-0.22 to 0.32	+/++	-0.16 to 1.94
Globus pallidus (GP)	+	0.08	++	-0.10	+/++	-0.22 to 0.02
Septofimbrial nucleus (SFi)	0/+	-0.46, 0.14	0/+	-0.16	0/+	-0.58, -0.16 to 0.14
ventral hippocampal commissure (vhc)	0/+	-0.70 to -0.64	0/+		0/+	-0.58
Subfornical organ (SFO)	+	-0.58, -0.10	+	-0.46	0/+	-0.82 to -0.46
Paraventricular thalamic nucleus (PV)	+	-1.82 (just PV?)	0/+	-0.82	+	-0.82 to -0.22
Lateral preoptic nucleus (LPO)	+	0.14 to 0.38, 0.62	++/+++	-0.10 to 0.32	++/+++	-0.46 to 0.62
Medial preoptic area (MPA)	+/++	0.62	++	0.26	++/+++	-0.22 to 0.62
Striohypothalamic nucleus (StHy)	+	-0.10	++/+++	-0.16 to -0.10	++/+++	-0.10
Lateral septal nucleus, dorsal part (LSD)	0/+	-0.22 to 0.08, 0.86 to 1.34	0/+	-0.46 to 0.50	+	0.14 to 1.34
Lateral septal nucleus, intermediate part (LSI)	0/+	0.02 to 0.50, 1.04 to 1.34	+	0.32, 1.1	+/++	-0.10 to 1.34
Lateral septal nucleus, ventral part (LSV)	+	0.20 to 0.26, 0.86	++	1.34 to 1.42	++/+++	0.38, 0.98 to 1.1
Medial preoptic nucleus (MPO)	++	-0.58 to -0.22, 0.14 to 0.22	++/+++	-0.10	+++	-0.10 to 0.14
Ventromedial preoptic nucleus (VMPO)	+/++	0.62	++/+++	0.26 to 0.50	+++	-0.10 to 0.26
Ventrolateral preoptic nucleus (VLPO)	++	0.26	++/+++	0.26 to 0.32	+++	0.08 to 0.26
Cingulate cortex (Cg)	0/+	-0.22 to -0.10, 0.62	0/+		0/+	0.14 to 1.10
Anteroventral periventricular nucleus (AVPV)	+/++	0.26 to 0.62	++/+++	0.26 to 0.50	+++	0.14 to 0.62
Nucleus of the horizonta limb of the diagonal band (HDB)	+	0.14 to 0.62	++	0.26 to 0.38	+++	0.14 to 0.38
Septohypothalamic nucleus (SHy)	++	-0.10 to 0.38	+	0.5	+/++	0.38
Vascular organ of the lamina terminalis (VOLT)	++	0.5	++/+++	0.5	+++	0.50 to 0.62
Median preoptic nucleus (MnPO)	0/+	0.02, 0.14, 0.62	++/+++	0.62	++	0.08 to 0.62
Ventral pallidum (VP)	0/+	-0.94 to -0.82	++	-0.16	++	0.5
Nucleus accumbens, shell (AcbSh)	++	0.62	+++	1.18 to 1.98	+++	0.98 to 2.10

Table 4.1 (cont'd)

Nucleus accumbens, core (AcbC)	++/+++	1.18	++/+++	0.74 to 1.34, 1.70 to 1.94	+++	0.74 to 2.10
Motor cortex	0/+	-0.16 to 0.02, 0.62, 1.34 to 1.98	+	-0.82, 0.14, 0.50, 1.70 to 2.10	+	1.34 to 2.10
Nucleus of the vertical limb of the diagonal band (VDB)	0/+	0.74	+	0.86, 1.34	+	0.86 to 1.18
Infralimbic cortex/Dorsal peduncular cortex (IL/DP)	0/+	1.1, 1.42, 1.70 to 1.98	0/+	1.42, 1.70	+	1.94
Orbital cortex	0/+	1.98	+	1.98 to 2.10	+	1.94 to 2.10

Figure 4.2. Structures Implicated in AN with similar density inputs to LHA Nts neurons, regardless of risk factor.

Representative images of structures involved in AN that send similar density inputs to LHA Nts neurons, regardless of risk factor. From left to right, each row contains a bregma-numbered mouse atlas image [22], a 4x representative image from a wildtype mouse of a structure containing monosynaptic inputs to LHA Nts neurons, and a 10x image of inputs from the same structure. **A)** Bregma -1.46, **B)** Bregma -1.22, **C)** Bregma -0.94, **D)** Bregma -0.10, **E)** Bregma 1.10, **F)** Bregma 1.18.

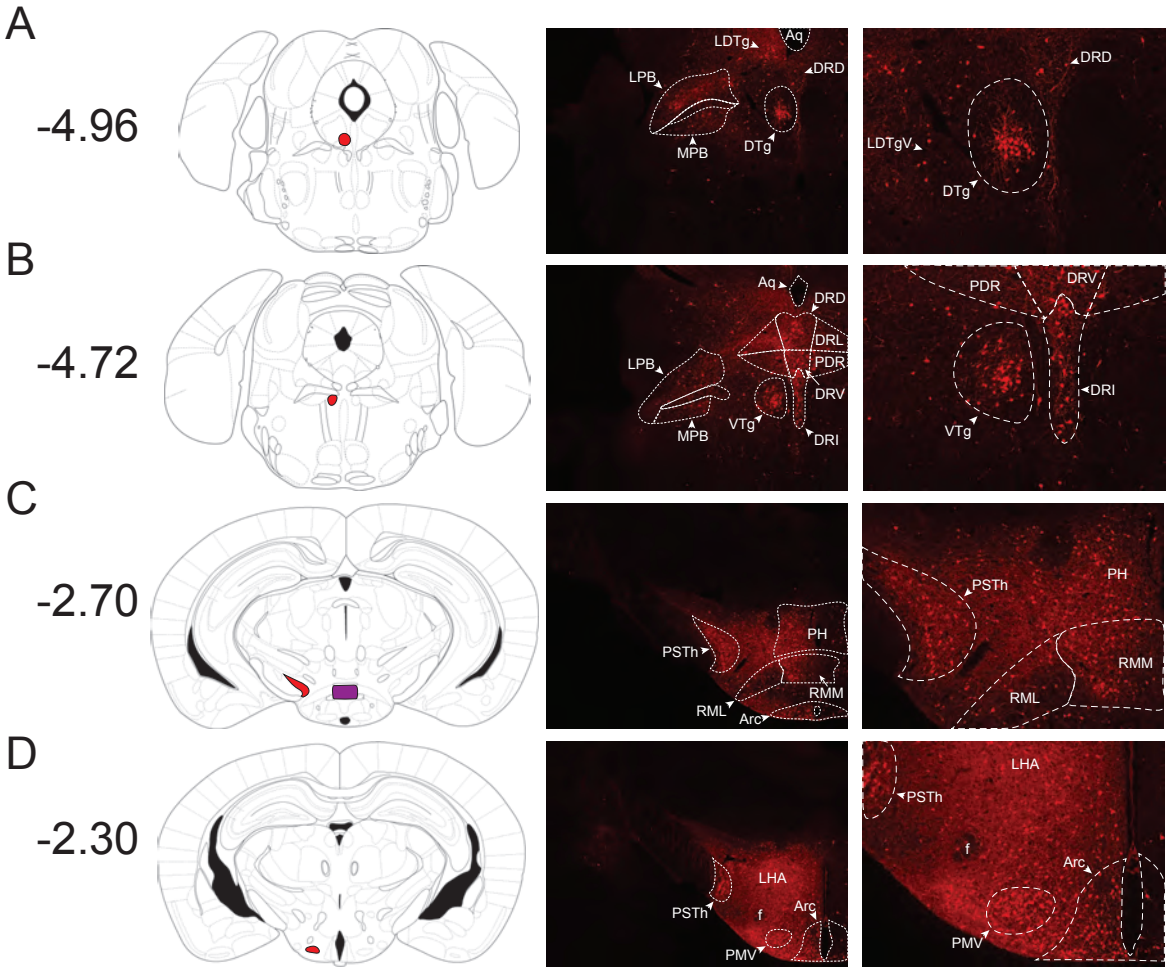


of the bed nucleus of the stria terminalis (BSTMPM/BSTMPI) **(Figure 4.2D)** and the core of the nucleus accumbens (AcbC) **(Figure 4.2F)** were identified to provide moderately dense inputs (++/+++ → +++) to Nts neurons in the LHA. For the AcbC, these contributions seemed to lie primarily within clusters along the medial aspect of the core **(Figure 4.2F)**. Additionally, LHA Nts neurons received some input from the dorsomedial hypothalamic nucleus (+/++ →++) **(Figure 4.2A)**. A rather sparse number of inputs (+ →++) were found in both the anterior hypothalamus (AH) **(Figure 4.2B)** and caudate putamen (CPu) **(Figure 4.2E)**; furthermore, the distribution of projections within the CPu was nearly exclusively ventromedial, with mCherry+ neurons positioned just dorsal to the ventral striatum and lateral to the lateral ventricle.

Additionally, we noted areas providing particularly dense inputs to LHA Nts neurons that had not been directly associated with energy balance. Regions providing dense inputs were regarded as areas with average density ratings of at least ++/+++ or more in all analyzed brains. The most caudal of these structures included both the dorsal (DTg) and ventral tegmental nuclei (VTg) **(Figure 4.3A and 4.3B)**. Both of these structures harbored very dense clusters of LHA Nts projections and were found immediately ventrolateral to the dorsal raphe nucleus. In addition, the medial portion of the retromammillary nucleus (RMM) consistently contained many projections (++/+++ → +++) **(Figure 4.3C)**. At the same bregma level, the parasubthalamic nucleus (PSTh) was found to contain numerous inputs (++/+++ → +++/++++), and this was despite a scarcity of projections from the neighboring subthalamic nucleus (STh) **(Figure 4.3C)**. Lastly, the ventral premammillary nucleus (PMV) always demonstrated a high level of

Figure 4.3. Additional structures, not implicated in AN, with high density inputs to LHA Nts neurons, regardless of risk factor.

Several regions not specifically associated with AN were found to contain a high density of neuronal projections to LHA Nts neurons. Structures assessed to have average input densities of at least ++/+++ across all three groups of mice were designated as high-input regions. From left to right, each row contains a bregma-numbered mouse atlas image [22], a 4x representative image from a wildtype mouse of a structure containing a high density of monosynaptic inputs to LHA Nts neurons, and a corresponding 10x image of the same structure. **A)** Bregma -4.96, **B)** Bregma -4.72, **C)** Bregma -2.70, **D)** Bregma -2.30.



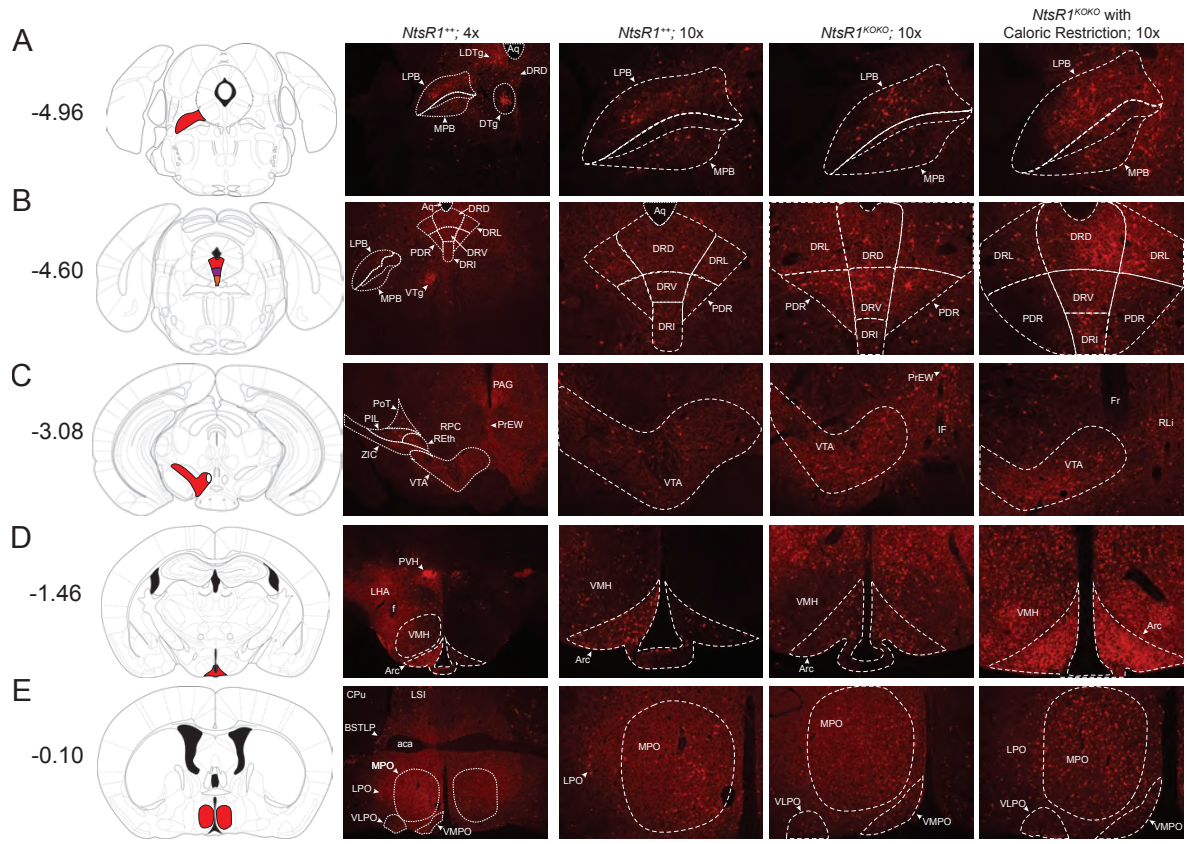
inputs to LHA Nts neurons **(Figure 4.3D)**.

NtsR1 Deficiency and Stress Alters Some Afferent Input to LHA Nts Neurons

Intriguingly, many brain regions implicated in AN provide afferents to LHA Nts neurons, the densities of which were increased in accordance with deficiency of NtsR1, either with or without adolescent stress exposure. The most caudal of these structures is the lateral parabrachial nucleus (LPB), which contained a modest number of projecting neurons in the wildtype females (+/++); however, both stress-exposed and non-exposed *NtsR1*^{KOKO} females exhibited moderate to many (++/+++) inputs to LHA Nts neurons **(Figure 4.4A)**. The dorsal raphe nucleus and ventral tegmental area (VTA), two structures within the midbrain, provided afferents to LHA Nts neurons **(Figure 4.4B and 4.4C)**. Within the dorsal raphe, modest inputs (++) were found to reside in the dorsal subnucleus (DRD) of both *NtsR1*$^{++}$ and *NtsR1*^{KOKO} mice; furthermore, the density of inputs was greater specifically in the DRD of *NtsR1*^{KOKO} mice exposed to adolescent stress **(Figure 4.4B)**. The ventral (DRV) and intermediate (DRI) aspects of the dorsal raphe in female wildtype mice provided sparse to moderate neuronal inputs (+/++) to LHA Nts neurons, and this projection density increased to many neuronal inputs in both stressed and non-stressed *NtsR1*^{KOKO} females (++/+++ → +++). Similar density ratings were assigned to neuronal projections confined to the VTA of NtsR1-null females, with few-to-moderate inputs in the VTA of wildtype animals and moderate-to-many inputs in the VTA of adolescent-stressed and non-stressed *NtsR1*^{KOKO} mice **(Figure 4.4C)**. Like the midbrain, the hypothalamus contained two structures associated with AN that provided LHA Nts neuron inputs, the densities of which increased in mice with both lack

Figure 4.4. Brainstem and hypothalamic structures implicated in AN with different density inputs to LHA Nts neurons, dependent upon risk factor.

Images of brainstem and hypothalamic structures involved in AN with inputs to LHA Nts neurons that increase in density with risk factors of lack of NtsR1 and/or adolescent stress exposure. From left to right, each row contains a bregma-numbered mouse atlas image [22], a 4x representative image from a wildtype mouse of a structure containing monosynaptic inputs to LHA Nts neurons, a 10x image of inputs from the same mouse, a representative 10x image of inputs from a *NtsR1*^{KOKO} mouse, and a representative 10x image of inputs from a *NtsR1*^{KOKO} mouse exposed to caloric restriction stress during adolescence. **A)** Bregma -4.96, **B)** Bregma -4.60, **C)** Bregma -3.08, **D)** Bregma -1.46, **E)** Bregma -0.10.

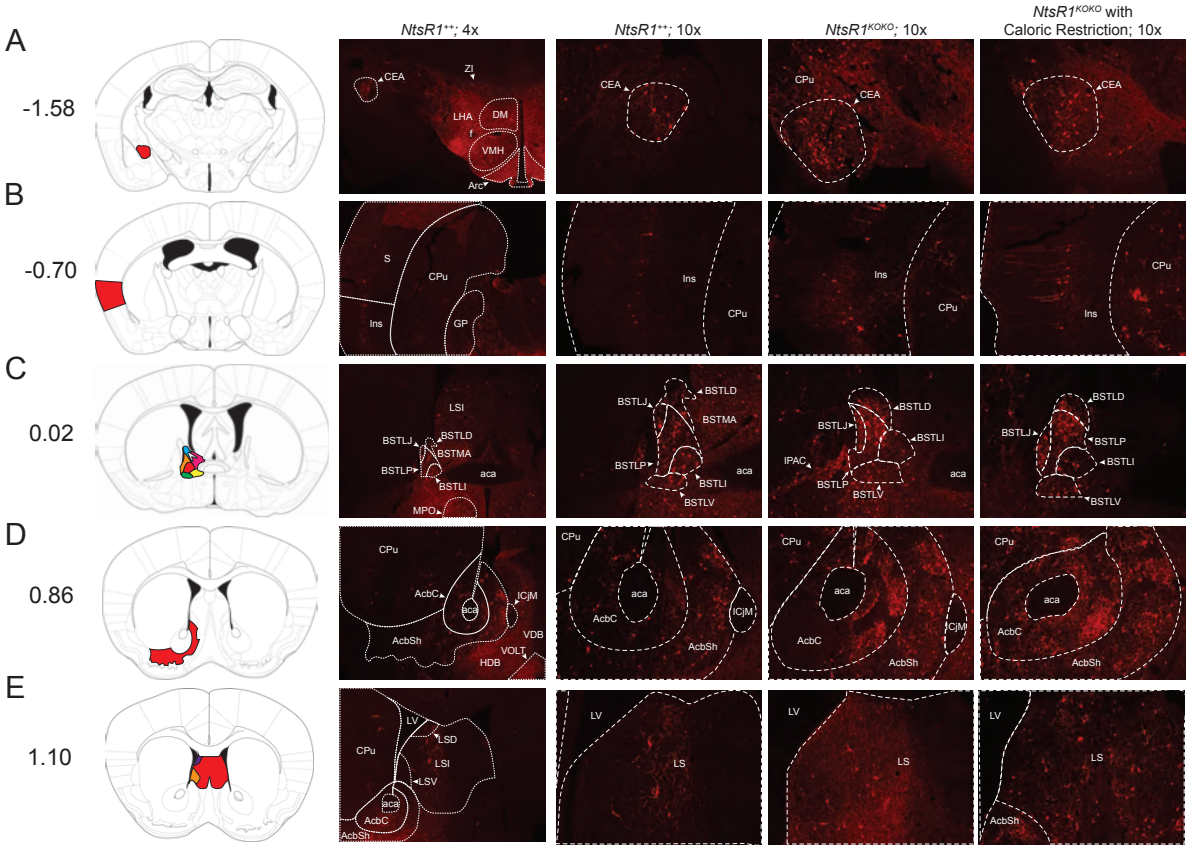


of NtsR1 and caloric restriction stress during adolescence. These regions included the Arcuate nucleus (Arc) **(Figure 4.4D)** and medial preoptic nucleus (MPO) **(Figure 4.4E)**. We noted many inputs from the Arc in *NtsR1*$^{++}$ and *NtsR1*^{KOKO} mice (+++), and this density was assessed to be even greater (++++) in the Arc of *NtsR1*^{KOKO} mice exposed to the adolescent stress paradigm **(Figure 4.4D)**. Relative to the Arc, the MPO displayed somewhat lower density inputs, with the MPO of adolescent-stressed *NtsR1*^{KOKO} females harboring numerous LHA Nts-projecting neurons (+++), the MPO of non-stressed *NtsR1*^{KOKO} females containing more modest densities (++/+++), and the MPO of NtsR1^{++} females encompassing fewer inputs (++) **(Figure 4.4E)**. Interestingly, the ventromedial hypothalamic nucleus (VMH) adjacent to the Arc contained very few afferents to LHA Nts neurons in *NtsR1*$^{++}$ and *NtsR1*^{KOKO} mice (++), but th VMH of adolescent stressed *NtsR1*^{KOKO} mice provided increased afferents (+++).

As with brainstem and hypothalamic structures implicated in the modulation and dysregulation of feeding that occurs in AN, a number of such regions within the forebrain and cortex were found to project to LHA Nts neurons with densities varying amongst the different groups of females. The central nucleus of the amygdala (CEA) provided few neuronal afferents to the LHA Nts neurons (+) within wildtypes, whereas *NtsR1*^{KOKO} mice displayed comparably more CEA inputs (++) to LHA Nts **(Figure 4.5A)**. Similar to the CEA, the insular cortex contained sparse inputs (0/+) in wildtype animals, whereas more inputs were identified in the insular cortex of stress-exposed *NtsR1*^{KOKO} females **(Figure 4.5B)**. Overall, the BNST provided ample afferents to LHA Nts neurons irrespective of whether or not mice contained intact NtsR1 and whether they

Figure 4.5. Forebrain and cortex structures associated with AN provide projections to LHA Nts neurons that differ in density depending upon risk factor.

Images of cortical and forebrain regions with inputs to LHA Nts neurons that increase in density with risk factors of deficiency of NtsR1, either with or without exposure to caloric restriction stress during adolescence. From left to right, each row contains a bregma-numbered mouse atlas image [22], a 4x representative from a wildtype mouse of a region containing monosynaptic inputs to LHA Nts neurons, a 10x image of inputs from the same mouse, a representative 10x image of inputs from the same structure in a *NtsR1*^{KOKO} mouse, and a 10x image of inputs in a *NtsR1*^{KOKO} mouse exposed to caloric restriction stress during adolescence. **A)** Bregma -1.58, **B)** Bregma -0.70, **C)** Bregma 0.02, **D)** Bregma 0.86, **E)** Bregma 1.10.



were or were not stressed during adolescence. While the more caudal BST subnuclei had fairly consistent input densities to LHA Nts neurons amongst groups of assessed females (BSTMPL, BSTMPM, BSTMPI, BSTLI, BSTMV), several rostral subnuclei, starting at Bregma level 0.02, contained projections with densities that increased either as a result of lacking NtsR1 alone and in combination with adolescent stress. These BST subregions with varying densities included the posterior and dorsal aspects of the lateral division (BSTLP and BSTLD), both of which were found to have moderate input densities in *NtsR1*$^{++}$ mice (++) and numerous input densities in both stressed and non-stressed *NtsR1*^{KOKO} mice (+++ → +++/++++) **(Figure 4.5C)**. A similar trend of greater neuronal projections specifically in *NtsR1*^{KOKO} mice was apparent in the ventral aspect of the lateral division (BSTLV); however, the densities of such projections was lower overall relative to the BSTLP and BSTLD, with very few inputs in the BSTLV of wildtype females (0/+) and modest inputs from the BSTLV of stressed and non-stressed *NtsR1*^{KOKO} mice (+/++). Lastly, the juxtacapsular portion of the lateral division (BSTLJ) was determined to have very dense inputs to LHA Nts neurons in solely adolescent-stressed *NtsR1*^{KOKO} mice (+++/++++), and this input density was lower in non-stressed mice (++), regardless of whether or not NtsR1 was intact **(Figure 4.5C)**. In contrast to the AcbC, the shell of the nucleus accubmens (AcbSh) was determined to contain densities of neuronal projections that varied in accordance with whether or not mice contained intact NtsR1. The AcbSh of wildtype mice demonstrated a moderate number of afferents to the LHA Nts neurons (++), whereas this is more pronounced (+++) in *NtsR1*^{KOKO} mice both exposed and not exposed to adolescent stress **(Figure 4.5D)**. As with the AcbC, inputs were distributed somewhat medially in dense clusters. The lateral

septal nucleus is the most rostral structure implicated in AN that holds a varying degree of inputs to LHA Nts neurons as a result of stress status during adolescence and absence or presence of NtsR1 **(Figure 4.5E)**. In the intermediate region of the lateral septum (LSI), female *NtsR1*$^{++}$ mice provided sparse projections to LHA Nts neurons (0/+) whereas female *NtsR1*^{KOKO} mice exposed to caloric restriction stress during adolescence contributed a moderate number of inputs (+/++) **(Figure 4.5E)**. The ventral portion (LSV) also demonstrated a similar projection differential, with few inputs in the wildtype mouse (+) and moderate-to-many (++ → ++/+++) within both the stress-exposed and non-stressed NtsR1-null mice.

Discussion

A number of structures identified to project to LHA Nts neurons have been implicated in AN. For instance, application of the Activity-based anorexia (ABA) model, which promotes a paradoxical increase in activity as a result of restricted access to food in rodents [23], has been shown to increase neuronal activity in the SO, Arc, DM, PVH, and DR [24]. We found that all of these structures provided direct inputs to LHA Nts neurons, and there were increased inputs from the Arc and DR of mice lacking NtsR1 that were exposed to adolescent stress-risk factors for AN. The roles of the Arc and DR in AN remain unclear, but estradiol administration in these regions promotes a dose-dependent decrease in food intake [25], which in the DR is mediated partly via serotonin [26]. Indeed, individuals with AN have shown enhanced 5-HT_{1A} receptor binding [27], an alternative decrease in 5-HT_{2A} binding [28,29], and increased levels of major serotonin metabolite 5-hydroxyindoleacetic acid (5-HIAA) in the cerebrospinal fluid (CSF) [30]. While previous work demonstrated that DR neurons project to the LHA [31,32], we are the first to show that they synapse onto Nts neurons specifically. In addition, while there is evidence to suggest that neuropeptide Y (NPY), α-melanin-stimulating hormone (α-MSH), and agouti-related peptide (AgRP) neurons within the Arc project to and modulate the activity of melanin-concentrating hormone (MCH) and orexin (OX) neurons within the LHA [33–35], we are the first to show that the Arc has direct inputs to LHA Nts neurons. Given that increased activity of Arc NPY neurons and NPY expression has been linked with worsening ABA and weight loss [36], it is possible that an augmented Arc NPY → LHA Nts circuit might contribute to some of these effects, and this deserves examination in the future.

Similar to the DR and Arc, the MPO of wildtype female mice provided modest projections to LHA Nts neurons that were augmented in adolescent-stressed *NtsR1*^{KOKO} females. These data suggest that interacting genetic and environmental risk factors may abnormally enhance the MPO → LHA Nts circuit. The MPO has been previously implicated in anorectic behavior, as estradiol administration in this region reduces feeding [25], possibly via inducing expression of Nts within the MPO itself [37,38]. While it was known that some MPO Nts neurons project to the LHA [32], we are the first to show that LHA Nts neurons receive direct afferents from the MPO, and going forward, it will be important to determine if MPO → LHA Nts circuitry contributes to estradiol-induced anorexia.

Functionally important LHA Nts afferents to the VTA are well characterized [10,39], but there are few reports of a reciprocal VTA → LHA connection [40], which we found here. Interestingly, substantial projections from the rostral linear nucleus (RLi) of the VTA to the LHA were shown to terminate primarily within the anterolateral LHA, which does not contain significant OX or MCH [41] but does contain Nts neurons. Hence, our current study confirms a VTA→LHA circuit, shows that it specifically targets LHA Nts neurons, and indicates that this circuit may be augmented by risk factors that increase vulnerability to develop AN. Indeed, enhanced VTA signaling has been implicated in AN. For example, some behaviors in the ABA paradigm modeling AN are thought to result from increased ghrelin-mediated activation of ghrelin receptor-expressing VTA dopamine neurons [42], as intra-VTA ghrelin administration increases both dopamine release to the nucleus accumbens as well as locomotor activity [43,44].

Conversely, wheel running activity in ABA rats is diminished with intra-VTA leptin [44,45]. Similar to ABA rats, AN patients display increases in plasma ghrelin and decreases in leptin levels [46,47], and these hormonal alterations may promote increased activity via the VTA. While our data suggest that loss of NtsR1, which is expressed on VTA dopamine neurons, promotes increased input to LHA Nts neurons from the VTA, it is ultimately unknown whether alterations in VTA →LHA Nts circuitry promotes development of AN.

Dysfunction of the striatum and dopamine-based reward has been observed in AN patients, and we also observed that genetic and environmental risk factors for AN increased striatal input density to LHA Nts neurons. Likewise, enhanced activation of the ventral striatum (including the AcbC and AcbSh) has been documented in women with AN [48,49], as have molecular alterations that could impact activity or synaptic function [50]. While we observed that all female mice studied had numerous AcbC inputs to the LHA Nts neurons, the AcbSh of both adolescent-stressed and non-stressed NtsR1-null mice had relatively more inputs to LHA Nts neurons than that of female wildtype controls. Interestingly, AcbSh D1R-expressing medium spiny neurons (MSNs) project to LHA GABA neurons, and their activation rapidly halts feeding [51]. Given that LHA Nts neurons also express GABA [52] (and in preparation), it is possible that AcbSh inputs to LHA Nts neurons contribute to this anorectic action. However, there are subpopulations of LHA GABA neurons [53], not all of which contain Nts and which differentially promote or suppress feeding [11,53]. Hence, it will be necessary to disentangle the exact function of these LHA subpopulations and to discern how the

specific AcbSh → LHA Nts projections documented here modify feeding. Intriguingly, the dorsal striatum, specifically the CPu, also provided projections to LHA Nts neurons, but they did not vary with genetic or environmental risk. The CPu has been implicated in increased harm avoidance in recovered AN individuals [50] and the hyper-responsiveness of AN patients to aversive stimuli [49,54]; however, since we did not observe differences in input level between groups, perhaps these actions are not the result of altered CPu regulation of the LHA.

We demonstrated that the BNST directly projects to LHA Nts neurons, and that some BNST inputs are augmented in mice lacking NtsR1 (BSTLP, BSTLD, and BSTLV), including when this is genetic deficiency is combined with adolescent stress exposure (BSTLJ). Notably, activation of GABAergic BNST → LHA neurons elicits voracious feeding behavior [55] by innervating glutamatergic LHA neurons [55]. However, due to the fact that LHA Nts neurons are GABAergic (in preparation) suggests that they are not targets of this previously-established, hyperphagia-inducing BNST→LHA circuit. Alternately, these dense BNST → LHA Nts connections might contribute to the anorectic response to stress. For example, some BNST neurons express Corticotropin-releasing factor (CRF), a neuropeptide invoked by stress, and intra-BNST administration of CRF evokes robust reductions in feeding in fasted rats [56]. In particular, the BNST is thought to mediate pathological behavioral responses to chronic stress exposure, as often occurs in individuals with anxiety disorders [57]. Thus, it is not altogether surprising that alterations in BNST afferents to the LHA were identified in mice with risk for development of AN-like behaviors, since AN is highly comorbid with

anxiety disorders. In particular, we observed a site-specific increase in LHA Nts inputs from the dorsolateral BNST in NtsR1^{KOKO} and adolescent-stressed mice. This is potentially relevant to stress-invoked anorexia since the dorsolateral BNST demonstrates heightened activation and increased expression of proteins implicated in reducing feeding and body weight [57–60]. Thus, it is possible that the increase in BNST input to LHA Nts neurons we observed in stress-prone mice is partly responsible for the BNST-mediated anorectic response to stress.

The CEA and insula have been well-studied in mechanisms of anorexia and AN, respectively, and we found increased afferents from these regions to LHA Nts neurons in *NtsR1*^{KOKO} female mice when exposed (Insula) or not exposed (CEA) to an adolescent stress. The CEA has known projections to the LHA, some of which express Nts [32,61]; however, our identification of direct CEA projections to LHA Nts neurons is novel. A specific subpopulation of lateral CEA neurons that express protein kinase C-δ (PKC-δ) receive inputs from the LPB and insula [62], and in turn mediate the suppression of feeding in response to anorexigenic signals. Going forward, it will be important to determine if these CEA PKC-δ neurons directly project to LHA Nts neurons to mediate anorexia and how. Given that some LPB neurons induce conditioned taste aversion [63], an LPB → CEA → LHA Nts circuit might suppress feeding by making food less desirable. Indeed, rats subjected to the ABA model of AN exhibit enhanced taste aversion [64]. We therefore speculate that *NtsR1*^{KOKO} females may be particularly prone to anorectic behaviors in part due to their increased LPB and CEA afferents to LHA Nts neurons.

The insula is suspected to provide input to the CEA PKC-δ anorectic circuit discussed above [62] and has been widely studied in the context of AN. A prior study in hamsters described insular cortex fibers that coursed through the lateral hypothalamus [65], but ours is the first report of direct connections from the insula to LHA Nts neurons. The insula is part of the primary gustatory cortex, which is largely responsible for relaying sensory taste information to higher-order structures and, thus, for taste processing [2,66]. This taste processing neurocircuitry is altered in individuals recovered from AN, who exhibit reduced activation of the anterior insula in response to taste of sucrose, which may ultimately reflect reduced hedonic valuation of sucrose taste [66]. Increased gray matter has also been discovered in the insular cortex of both ill and recovered AN patients [7]. Alterations in structure and circuitry in the insula may be responsible for a distorted perception of self, which is ultimately a core feature of AN [67,68]. While we observed few insula inputs to LHA Nts neurons compared to those from other structures, the fact that they were increased with addition of risk factors for AN (e.g. lacking NtsR1, adolescent stress) suggests that enhanced action via this circuit may contribute to the disorder.

Finally, we revealed a progressive increase in LS inputs to LHA Nts neurons from wildtype females to *NtsR1*^{KOKO} females and from *NtsR1*^{KOKO} females to adolescent-stressed *NtsR1*^{KOKO} females. Characterization of LS GABAergic inputs to the LHA has revealed that activation of these projections suppresses feeding [69]. Additionally, at least some of the targets of this inhibitory LS GABA → LHA circuit are LHA GABA neurons [69]. As discussed previously, it is highly likely that some of these LHA GABA

neurons are Nts neurons, and excess inhibitory LS GABA → LHA inputs in *NtsR1*^{KOKO} females exposed to adolescent stress may suggest that these mice in particular are predisposed to anorectic behavior.

Technical Limitations and Considerations

One caveat to the rabies virus tracing method includes the possibility for virus to spread to structures outside of the LHA. A neighboring anterior structure with numerous Nts neurons to which virus could potentially spread is the MPO. While moderate-to-many neurons were found to express mCherry within this structure, there was very little colocalization of mCherry with GFP+ Nts neurons (on average about 2 neurons per section). This provides some indication that these MPO neurons are indeed inputs to LHA Nts neurons and not the result of viral spread to the MPO. Another potential limitation to this model is the possibility that *NtsR1*^{KOKO} mice upregulate Nts expression to compensate for loss of Nts signaling. If *NtsR1*^{KOKO} mice did have global elevations in Nts expression or an increased number of Nts neurons, this could contribute to an overall increase in initial transduction and, thus, input labeling. To answer this question, the expression levels of Nts within the LHA of both NtsR1-null and wildtype mice could be determined via Quantitative Reverse Transcription PCR (qRT-PCR) to identify different expression levels between the two groups. In addition, the number of LHA Nts neurons could be quantified in both wildtype and NtsR1-null mice to resolve if differences exist, and this could be achieved by comparing cell counts between *Nts*^{Cre}*;GFP* brains, performing Nts immunohistochemistry in mice pretreated with colchicine, or by determining neuronal expression via Nts *ISH*.

Conclusion

In sum, we used genetic monosynaptic rabies virus tracing to map the afferents to LHA Nts neurons in an unbiased manner. Moreover, our work demonstrates that a potential genetic risk factor for AN (lacking NtsR1) and adolescent stress augment the density of specific neural circuits. Further work to systematically test these circuits is necessary to determine if they contribute to the development of AN, but our findings, at a minimum, provide a starting point for such functional studies.

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CHAPTER 5. Summary and Conclusions

Authors who contributed to this section were: Laura E. Schroeder and Gina M. Leinninger.

Overview and General Considerations

Within this thesis, I addressed the central hypothesis that Nts neurons in feeding centers are specifically regulated by top-down control centers implicated in AN, and altered Nts signaling disrupts feeding and body weight to promote AN-like behaviors. I showed that significant populations of Nts neurons do indeed exist within brain structures associated with feeding, and these data are the first to provide a comprehensive "map" of the Nts neurons within the mouse brain. Any of the major Nts populations I documented might contribute to energy balance and should be studied for this possibility in the future. Here, I opted to further examine lateral hypothalamic area (LHA) Nts neurons because they, and their synaptic targets, coordinate peripheral cues of energy status with appropriate feeding and locomotor behaviors necessary to maintain proper body weight [1,2]. Given that the connectome is altered in AN [3–5], I reasoned that synaptic inputs to LHA Nts neurons might be altered in this disorder, thus compromising their control of behaviors relevant to energy balance. Indeed, I demonstrated, via use of the monosynaptic rabies tracing technique, that LHA Nts neurons receive projections from numerous regions implicated in AN and that afferent density is enhanced in a female mouse model of AN. It makes sense that these neurons receive inputs from two different types of structures: 1) those, such as the LPB and CEA, that promote anorectic mechanisms in response to satiety cues, and 2) regions, including the ventral striatum, insula, and BSNT, that are implicated in the dysfunctional behaviors associated with AN, such as altered taste-processing, stress-response, and self-evaluation. In addition, I showed that projections to LHA Nts neurons from all of these anorexia-associated structures, as well as from others

involved in AN, are increased in mice that are susceptible to developing AN-like behaviors. Thus, my data support the hypothesis that changes to Nts circuitry are linked with AN. It remains to be determined whether these structural changes promote the disorder or are a result of it. Additionally, it will be important to define the nature of the enhanced input to LHA Nts neurons (activating or inhibitory), which will provide insight on how their function is altered and how this contributes to behavior. Going forward, these questions can be tested in mice using genetic and viral tools to gain a more nuanced mechanistic understanding of neural changes in AN.

My thesis has direct translational relevance to understanding AN as I also explored whether genetic disruption of the Nts-NtsR1 system might contribute to development of the disorder. To model the loss-of-function variants in the Nts-NtsR1 pathway discovered in AN patients, I studied *NtsR1*^{KOKO} male and female mice, both with and without exposure to an adolescent stress known to interact with genetic risk factors to promote AN-like behaviors [6]. These data revealed that *NtsR1*^{KOKO} female mice are particularly vulnerable to environmental stress and develop altered body composition, feeding behaviors, and locomotor activity as a result. Akin to the skewed prevalence of AN between men and women, genetically predisposed *NtsR1*^{KOKO} male mice were essentially resilient to environmental stress and were equivalent to wildtype controls with regards to all behavioral measures assessed. This skewed sex distribution of disease itself imparts predictive validity to the hypothesis that disrupted Nts-NtsR1 signaling promotes AN. In addition, major disruptions in body weight, feeding, and locomotor behaviors were observed specifically after female *NtsR1*^{KOKO}

mice were exposed to adolescent stress, and this supports the hypothesis that loss of Nts signaling is indeed a genetic risk factor, the full scale phenotypic effects of which are evoked by other types of risks (sex and environment). Interestingly, *NtsR1*^{KOKO} female mice in particular displayed both restrictive- and binge-feeding behaviors. This again provides legitimacy to the idea that disrupted Nts signaling promotes AN-like feeding behaviors, since AN patients may engage in both extremes of feeding [7,8]. Adolescent-stressed female *NtsR1*^{KOKO} mice also demonstrate increased activity when provided a wheel, suggesting that these mice may be prone to "addiction" to exercise-like behaviors, another feature of AN. Lastly, female NtsR1^{KOKO} mice in particular displayed enhanced inputs to LHA Nts neurons from regions implicated in AN, providing further evidence that genetic deficiency of NtsR1 is itself a risk for altered neurocircuitry, which we suspect promote these maladaptive behaviors.

All in all, our mouse studies support the hypothesis that the Nts system is anatomically and structurally positioned to modify energy balance and that at least some nodes of this system may be altered in AN. Furthermore, disruption of Nts-NtsR1 signaling confers risk for developing AN-like behaviors, similar to what has been observed in individuals with damaging gene variants in the Nts-NtsR1 pathway. These data suggest that Nts-NtsR1-based therapeutics may be viable candidates for pharmacotherapeutic intervention in AN. Before this can be considered, it will be essential to elucidate the precise mechanisms via which disrupted Nts-NtsR1 promotes the disordered feeding and locomotor behaviors of AN. These mechanisms can be defined in future work by using newly developed genetic mouse models that enable

conditional modulation of Nts and NtsR1 expression and/or activity. Thus, the data provided here are an important step supporting Nts as a potential contributor to AN as well as the need to study Nts signaling as it relates to the human disease.

Questions Raised by this Work: Do Specific Nts Neurons Contribute to AN?

While this dissertation supports a role for altered Nts-NtsR1 in AN, much work remains to be done to elucidate how it normally regulates feeding, activity, and how it goes awry in AN. One yet to be resolved question is if there is a specific source of endogenous Nts that is altered to contribute to AN. Chapter 1 includes a summary of the literature regarding the roles of Nts signaling in physiological energy balance and the pathophysiology of body weight disorders. The majority of past work regarding Nts occurred before the advent of Cre-based technologies and primarily involved use of pharmacologic agents and NtsR1 knockout mice; thus, direct manipulation of various Nts neuron populations, which pharmacological studies demonstrate promote inconsistent physiologic responses, was not feasible. An example of conflicting physiologic functions of spatially distinct Nts populations includes the fact that direct injection of Nts into the ventral tegmental area (VTA) promotes increased locomotor activity [9,10], whereas injection of Nts into the nucleus accumbens (NAc) results in reduced activity [11]. This chapter additionally highlighted how Nts involvement in AN is highly likely, but the mechanism by which altered Nts signaling contributes to eating disorders remains to be determined.

The second chapter provides a glimpse of the potential of Cre technologies in providing a better understanding of the function of specific Nts populations and subpopulations throughout the brain. Current technologies of *in situ hybridization* and immunofluorescence with colchicine pretreatments permit the detection of Nts neurons but prohibit their manipulation; however, use of *Nts^{Cre}; GFP* reporter mice will allow for both. Thus, the mouse *Nts^{Cre}; GFP* neuron atlas produced and found in this thesis provides the precise locations and coordinates of major populations of Nts neurons, which can be manipulated in future studies to define their distributed contributions to energy balance. One limitation of these reporter mice is the fact that Cre-dependent GFP expression is not temporally specific. Thus, neurons that expressed Nts solely during development will persistently express GFP in adulthood. While this may be considered a limitation, comparisons between the *Nts^{Cre}; GFP* reporter and the Allen Brain Atlas data revealed that most Nts-GFP populations in the *Nts^{Cre}; GFP* reporter match the density and distribution of Nts neurons in the adult mouse [12]. In addition, the regions where there was a mismatch between Allen Brain *In situ Hybridization* and the GFP reporter suggest primarily a developmental role of Nts in these structures. These areas included the principal sensory trigeminal nucleus (Pr5), dorso-lateral and –medial periaqueductal gray (DLPAG/DMPAG), intermediodorsal (IMD) and central medial (CM) thalamic nuclei, lateral mammillary nucleus (LM), ventral subiculum (VS), hippocampal CA1 pyramidal cell layer, restrosplenial (RSD/RSGc) and cingulate (Cg) cortices, ventral tenia tecta (VTT), and medial portion of the anterior olfactory area (AOM). Given that from birth, loss-of-function gene variants in Nts-NtsR1 are implicated in AN, it is possible that developmental loss of Nts expression in these sites could

contribute to the disorder and deserve further exploration. It is also likely that disruption of established Nts circuits by biological or environmental risks might also contribute to the altered behaviors observed in AN. Both possibilities warrant further testing. Despite these caveats regarding identifying actively-expressing Nts neurons in the *Nts^{Cre}; GFP* reporter mouse, chapter 2 provides an extensive atlas of Nts neurons throughout the brain, which will allow researchers to study the functions of relatively unexplored Nts populations as well as to manipulate known Nts populations to better understand their roles.

Does NtsR1-Deficiency Recapitulate Other Models of AN and Neuroendocrine Changes?

In Chapter 3, we showed that *NtsR1^{KOKO}* female mice in particular are susceptible to development of behaviors similar to AN. This was achieved by first assessing baseline differences between male and female *NtsR1^{++}* and *NtsR1^{KOKO}* mice, which revealed that *NtsR1^{KOKO}* females are predisposed to lower body weight and reduced feeding. These mice were additionally subjected to a multifactorial adolescent stress model of AN exhibited to elicit anorexia in genetically-susceptible mice [6]. We demonstrated that specifically adolescent-stressed *NtsR1^{KOKO}* females are prone to develop altered body composition, aphagic episodes, increased exercise-like ambulatory activity, reduced hedonic sucrose intake, and heightened responding for palatable sucrose reward, all of which translate to the human disease. While these data provide strong support of NtsR1 deficiency in promoting AN, it would be useful to validate this in an alternative rodent model of AN. For example, female *NtsR1^{KOKO}* and

NtsR1^{++} mice could be analyzed via the Activity-Based Anorexia (ABA) paradigm, which is the gold standard of animal models for anorexia and is based on the fact that restricted food access promotes paradoxical increases in wheel running activity and significantly diminished food intake and body weight [13]. Wildtype mice exposed to ABA exhibit profound anorexia and weight loss that leads to death, and these may be exacerbated in mice with additional risk factors for AN, leading to earlier mortality compared to wildtypes [13]. Based upon my findings suggesting that NtsR1 deficiency confers vulnerability in the multifactorial model of AN, I therefore predict that *NtsR1*^{KOKO} female mice will also be more vulnerable to the ABA model of AN and will reach mortality more rapidly than controls. There are limitations to using the ABA model: it does not factor in relevant environmental risks or the fact that multiple contributors are thought to induce disease, and this model does not replicate the psychological aspects of the disease [14]. Yet, the ABA model does have face validity when considering that it increases activity and weight loss, decreases feeding, and elicits amenorrhea and hypothermia [13]. Assessing response of *NtsR1*^{KOKO} females to this model will ultimately help to confirm that disrupted Nts signaling is a risk factor for AN.

In addition, it would be useful to assess the endocrine profile of *NtsR1*$^{++}$, *NtsR1*^{KOKO}, and adolescent-stressed *NtsR1*^{KOKO} females to determine if they exhibit changes in hormone concentration and/or signaling previously linked with disordered eating. For example, estradiol levels correlate with the degree of altered feeding behavior. Lower plasma estradiol is associated with binge eating in humans, genetic influences of disordered eating are moderated by estradiol, estradiol modulates

serotonin (particularly within the dorsal raphe), dopamine (DA), and HPA-axis activity, and while food intake is lowest when estradiol is highest in the estrous and menstrual cycles of mammals, patients with AN display extremely low levels of estradiol [15–18]. It would be interesting to determine if there are differences in estradiol levels between *NtsR1*^{KOKO} and wildtype female mice as well as between *NtsR1*^{KOKO} mice exposed and not exposed to the adolescent-stress paradigm. In some ways, *NtsR1*^{KOKO} female mice, which are prone to slight reductions in feeding, represent a state prior to disease development, whereas adolescent-stressed NtsR1-null females display significant alterations in feeding and behavior reflective of a diseased state. I hypothesize that *NtsR1*^{KOKO} female mice have heightened estradiol levels, whereas adolescent-stressed NtsR1-null females, which display some hallmarks of binge-eating behaviors, may instead have diminished estradiol relative to wildtype controls. Assessment of estradiol and other such indicators of disease, including cortisol, would be useful in validating that loss of NtsR1 is a genetic risk factor of AN.

Could Restoration of NtsR1 Improve Outcomes in AN?

With an eye toward the potential translational relevance of Nts-NtsR1 in AN, it would be of interest to determine if restoration of *NtsR1* in *NtsR1*^{KOKO} female mice rescues metabolic and behavioral dysfunction. One means of doing this would involve restoration of all central NtsR1 in adolescent mice. In the future this could be accomplished by obtaining “Knockout First” NtsR1^{tm1a} mice from the KOMP repository, in which a frt-flanked lacZ-neo blocking cassette upstream of the coding sequence for NtsR1 renders mice as functionally NtsR1-null. Injecting mice with a brain-wide AAV-

FlpO vector could then remove the frt-flanked blocking cassette to restore NtsR1 expression. Such brain-wide vector delivery is technically possible [19], and doing this brain-wide would help determine if systemic-based strategies to enhance all NtsR1 signaling might be useful in counteracting changes that contribute to AN.

Given the notable density of NtsR1 within midbrain DA neurons and the many demonstrations of disrupted DA signaling in AN, restoring NtsR1 selectively to the VTA might be sufficient to produce improvements. This could be achieved via generating Th^{Cre}; $NtsR1^{KOKO}$ female mice, where Cre is expressed specifically in the *Th*-rich DA neurons of the midbrain. Injecting AAV-DIO-NtsR1 into the midbrain of these mice will induce NtsR1 only in the Cre-expressing Th-DA neurons, and hence would restore NtsR1 selectively to this midbrain population. One might envision restoring NtsR1 expression in Th^{Cre}; $NtsR1^{KOKO}$ female mice before exposure to adolescent stress to determine if it could prevent development of AN-like behaviors. Conversely, restoring NtsR1 expression to the midbrain after the adolescent stress paradigm would suggest if this manipulation could improve body weight and behavior in established AN, and, hence, the treatment potential for individuals already exhibiting the disorder., Going forward, the use of various genetic mouse models and viral tools will allow the field to further understand the role of NtsR1 in risk for AN and the translational potential of modulating this system.

Future Considerations of the Circuit Changes in the Nts-NtsR1 System in AN

In Chapter 4, the rabies-based monosynaptic tracing technique was utilized to identify direct synaptic inputs to LHA Nts neurons, including in "AN-prone" models. The rabies tracing system has been previously utilized to explore how exposure to certain experiences or risks induces alterations in inputs onto neurons of interest [20]. Since loss-of-function variants in Nts-NtsR1 signaling genes have been identified in humans [7] and since we have demonstrated that *NtsR1*^{KOKO} female mice are particularly vulnerable to development of altered feeding and locomotor behaviors reminiscent of AN (chapter 3), we reasoned that deficiency of NtsR1 is a genetic risk factor with the potential to induce neurocircuitry alterations in vulnerable mice. In addition, stress during adolescence is a risk factor for development of eating disorders [6,21], and a multifactorial adolescent stress risk model of AN was applied to *NtsR1*^{KOKO} female mice to assess if environmental risks promote maladaptive changes. Our interest in LHA Nts neurons stems from their known role in the maintenance of energy balance [22], and we hypothesized that enhanced inputs onto this specific population of LHA neurons might drive the excessive locomotor and restrictive feeding behaviors characteristic of individuals with AN. Overall, we showed that deficiency of NtsR1, either with or without adolescent stress exposure, promotes enhanced inputs onto LHA Nts neurons. These inputs are derived from a number of structures directly implicated in AN or involved in mechanisms of anorexia and include the lateral parabrachial nucleus (LPB), dorsal raphe nucleus (DR), ventral tegmental area (VTA), arcuate nucleus (Arc), medial preoptic nucleus (MPO), central amygdala (CEA), insula (Ins), shell of the nucleus accumbens (AcbSh), lateral septal nucleus (LS), and dorsal, juxtacapsular, posterior,

and intermediate aspects of the lateral division of the bed nucleus of the stria terminalis (BSTLD/BSTLJ/BSTLP/BSTLI). These data suggest that genetic and environmental risk factors of AN enhance modulatory control of LHA Nts neurons by regions known to promote anorectic behaviors, which may ultimately result in disruption of the usual mechanisms by which LHA Nts neurons maintain energy balance.

This work provided a screen of structures that should be investigated in future studies concerning the potential role of LHA Nts neurons in AN. A relatively simple and accessible primary experiment to perform would be to determine if specific subpopulations of neurons with known involvement in anorexia target LHA Nts neurons. While we found that few Nts neurons within the CEA itself colocalize with PKC-δ neurons (Chapter 2), immunofluorescence for PKC-δ could be performed within these LHA-targeted Nts^{Cre} brains to determine if this particular group of neurons in the CEA projects to LHA Nts neurons. If CEA PKC-δ neurons colocalize with CEA inputs to Nts neurons, this would allude to a potential downstream mechanism via which CEA PKC-δ neurons mediate anorexia in response to satiety cues [23]. Another potential subpopulation of neurons to investigate would include the calcitonin gene-related peptide (CGRP) neurons within the lateral parabrachial nucleus, which not only mediate conditioned taste aversion [24] but have also been shown to project to CEA PKC-δ neurons [23]. It would not be altogether surprising if these CGRP neurons additionally project to LHA Nts neurons, and this question could easily be answered via immunohistochemical staining of rabies-injected, LHA-targeted Nts^{Cre} brains for CGRP. One last subpopulation of interest to examine would be dorsal raphe (DR) serotonergic

neurons. Estradiol has been exhibited to depolarize DR serotonin neurons, which is a mechanism via which estradiol inhibits binge-eating behavior [8]. In addition, it would be useful to define the classical neurotransmitter content of inputs from these regions, which would provide better understanding of whether enhanced drive from these regions might be inhibiting or activating LHA Nts neurons in AN. This could be possible via use of RNAScope analysis for GABAergic and glutamatergic markers in the rabies-injected, LHA-targeted *Nts*^{Cre} brains.

Once subpopulations of neurons with known inputs to LHA Nts neurons are neurochemically characterized, this would inform the design of experiments to test how these circuits functionally modulate LHA Nts neurons in both wildtype and *NtsR1*^{KOKO} female mice. It would be interesting to use dual recombinase technology to permit expression of either excitatory (hMD3q) or inhibitory (hMD4i) DREADDs within a subpopulation of interest (such as in CEA PKC-δ) with known LHA Nts inputs. After chronic activation or inhibition of these neurons via daily Clozapine-N-oxide (CNO) administration, monosynaptic rabies tracing could be performed for LHA Nts neurons to determine if the activity of known inputs causes altered input density [20]. Such a strategy could be used to validate whether increased top-down action leads to altered brain circuitry and might indicate upstream sites that could be targeted to attenuate these changes.

As previously highlighted, some limitations to the monosynaptic rabies-tracing technique include the possibility for virus to spread to Nts-containing structures outside

of the LHA as well as the possibility for differences in Nts expression between wildtype and *NtsR1*^{KOKO} mice. To verify that spread of virus did not occur outside of the confines of the LHA, brains were scanned for the presence of large populations of yellow neurons, indicating initial transduction in *Nts*^{Cre} neurons, in neighboring structures, such as the MPO. Since few yellow neurons were identified outside of the LHA, including in the MPO, it is likely that the rabies tracing vectors did not infect primary Nts-containing cell bodies beyond the LHA. Another potential caveat is the possibility that *NtsR1*^{KOKO} mice demonstrate an overall upregulation of Nts expression or number of Nts-expressing neurons to compensate for loss of Nts signaling, which would ultimately permit enhanced input labeling. Resolving this issue would require determination of both the expression levels of Nts as well as numbers of Nts neurons within the LHA of both NtsR1-null and wildtype mice with methods previously stated.

Collectively, these data indicate that altered Nts-NtsR1 signaling is implicated in the metabolic and behavioral dysfunction observed in AN. In addition to supporting a potential signaling and structural role for Nts-NtsR1 in AN, these data lay the foundation for subsequent studies to define the specific Nts-NtsR1 pathways contributing to this disorder. Ultimately, these studies highlight the possibility that targeting this signaling system has therapeutic potential in the treatment of AN.

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