Aus der Medizinischen Klinik mit Schwerpunkt Psychosomatik
der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

Phänotypische Charakterisierung von neuronal aktivierten Neuronen im Hirnstamm und Hypothalamus nach peripherer Injektion von CCK-8S oder Bombesin

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von

Kim-Marie Engster

aus Salzgitter

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Abkürzungsverzeichnis

ARC	Nucleus arcuatus
AP	Area postrema
ANOVA	Analysis of variance
BHS	Blut-Hirn-Schranke
BSA	Bovines Serumalbumin
CCK	Cholecystokinin
CCK1-Rezeptor	Cholecystokinin-1 Rezeptor
CCK2-Rezeptor	Cholecystokinin-2 Rezeptor
CCK-S	Sulfatiertes Cholecystokinin
°C	Grad Celsius
DABCO	1,4-Diazabicyclo[2.2.2]octan
DAPI	4′,6′-Diamidino-2-Phenylindol
DMV	Nucleus dorsalis nervi vagi
ER	Endoplasmatisches Retikulum
ERK	Extracellular regulated kinase
FITC	Fluorescein isothiocyanate
Fischer LSD Test	Fisher's least significant difference Test
GRP	Gastrin-releasing peptide
I.C.V.	Intracerebroventrikulär
IE	Internationale Einheit
Ig	Immunglobulin
I.p.	Intraperitoneal
KG	Körpergewicht
kg	Kilogramm
LSD-Test	Least significant difference test
M	Molare Masse
µg	Mikrogramm
µl	Mikroliter
mg	Milligramm
mmol	Millimol
mRNA	Messenger ribonucleic acid
NaCl	Natriumchlorid

nmol	Nanomol
NTS	Nucleus tractus solitarii
NUCB2	Nucleobindin2
P	Probabilitas
PBS	Phosphate-buffered saline
PVN	Nucleus paraventricularis
SEM	Standard error of the mean
S2CRK0	Serotonin 2C Rezeptor defizient
ZNS	Zentrales Nervensystem

Zusammenfassung

Hintergrund: Die Nahrungsaufnahme ist komplex reguliert und wird durch unterschiedliche Hormone gehemmt. In Hinblick auf die zunehmende Prävalenz von Übergewicht und Adipositas mit den damit verbundenen gesundheitlichen Folgen ist ein detailliertes Verständnis der Hunger-Sättigungsregulation bedeutend. Cholecystokinin (CCK) reduziert kurzfristig die Nahrungszufuhr des Organismus und interagiert hierbei mit verschiedenen Mediatoren, wie Bombesin, Nesfatin-1, Serotonin oder Neuronatin. In der vorliegenden Arbeit wurde in drei verschiedenen Studien untersucht, wie peripher verabreichtes CCK oder Bombesin die neuronale Aktivierung (c-Fos) von Sertonin-, NUCB2/ Nesfatin-1- oder Neuronatin-haltigen Neuronen in wichtigen Hunger-Sättigungszentren des Hirnstammes bzw. Hypothalamus beeinflusst.

Methodik: Für unsere Experimente nutzten wir *ad libitum* gefütterte männliche Sprague-Dawley Ratten. In der ersten Studie injizierten wir den Versuchstieren CCK-8S intraperitoneal (i.p.) und werteten immunhistochemisch Aktivierungsmuster in den Kerngebieten Nucleus tractus solitarii (NTS) und Nucleus dorsalis nervi vagi (DMV) des Hirnstammes sowie des Nucleus paraventricularis (PVN) und Nucleus arcuatus (ARC) im Hypothalamus mittels des neuronalen Aktivierungsmarkers c-Fos aus. Zusätzlich bestimmten wir die Fluoreszenzintensität von Serotonin in den genannten Kernbereichen. In der zweiten Studie wurde männlichen Sprague-Dawley-Ratten Bombesin i.p. verabreicht. Daraufhin wurde die Anzahl von c-Fos-positiven, sowie von NUCB2/Nesfatin-1-immunreaktiven Neuronen im NTS, PVN und ARC bestimmt. Die dritte Forschungsarbeit untersuchte die c-Fos-Aktivierung in Kombination mit der Neuronatin-Immunreaktivität von Neuronen im NTS und DMV des Hirnstammes nach peripherer Verabreichung von Bombesin oder CCK-8S.

Ergebnisse: I.p. verabreichtes CCK-8S führte dosisabhängig zu einer signifikanten Zunahme c-Fos-positiver Neurone im PVN und NTS im Vergleich zu den Kontrolltieren. Während im DMV und ARC keine Veränderungen der Anzahl c-Fos-positiver Neurone beobachtet wurden, kam es im NTS und DMV nach CCK-Injektion zu einer statistisch signifikanten Abnahme der Serotonin-Fluoreszenzintensität in den Nervenfasern.

Die i.p. Injektion von Bombesin bewirkte eine Zunahme c-Fos-positiver Neurone im PVN und im NTS. Gleichzeitig konnte ein signifikanter Anstieg von c-Fos-positiven Neuronen mit NUCB2/Nesfatin-1-Immunreaktivität beobachtet werden. Zusätzlich fand sich auch eine Zunahme von c-Fos-positiven Neuronen mit Kolokalisation von NUCB2/Nesfatin-1 und Tyrosinhydroxylase im NTS sowie NUCB2/Nesfatin-1 und Oxytocin im PVN.

Sowohl i.p. verabreichtes CCK, wie auch Bombesin bewirkte eine Zunahme von c-Fos-positiven Neuronen im NTS. Gleichzeitig führten CCK und Bombesin zu einer dosisabhängigen Steigerung der Anzahl aktivierter Neuronatin-Neurone.

Schlussfolgerung: Peripher verabreichtes CCK scheint bei der zentralen Signalweitergabe von Hunger-Sättigungsimpulsen über das Serotonin- sowie über das Neuronatinsystem zu wirken.

I.p. verabreichtes Bombesin führt zu einer Aktivierung von NUCB2/Nesfatin-1 Neuronen im Hirnstamm und Hypothalamus. Zusätzlich bewirkt Bombesin eine Aktivierung von Neuronatin-Neuronen im NTS, was für eine Beteiligung von Neuronatin an dem Nahrungsaufnahme-hemmenden Effekt von Bombesin sprechen könnte.

Abstract

Background: Food intake is regulated in a complex way and various hormones are involved in its short-term inhibition. Given the increasing prevalence of obesity and the associated comorbidities, a detailed understanding of hunger and satiety regulation is warranted. Cholecystokinin (CCK) is responsible for short-term downregulation of food-intake and interacts with various other mediators, among them serotonin, bombesin, NUCB2/nesfatin-1, and neuronatin. In the present study, we examined the involvement of these mediators on the respective neuronal regulatory circles after intraperitoneal administration of CCK or bombesin.

Methods: For all experiments *ad libitum* fed male Sprague-Dawley rats were used. In our first study, the animals were injected with CCK-8S and the immunohistochemical activation patterns in the neurons of the solitary tract (NTS) and the dorsal nucleus of the vagus nerve (DMV) in the brainstem, as well as in the paraventricular nucleus (PVN) and the arcuate nucleus (ARC) of the hypothalamus were examined using c-Fos immunoreactivity. Further, the fluorescence intensity of serotonin in nerve fibers of these nuclei was analyzed. In our second study, the number of c-Fos and NUCB2/nesfatin-1-immunoreactive neurons in the NTS, PVN, and ARC were examined after injection of bombesin. In our third setup, the rats were injected with CCK-8S or bombesin. Here, the activation of neuronatin immunoreactive neurons in the NTS and DMV of the brainstem were studied.

Results: Injection of CCK-8S dose-dependently significantly increased the number of c-Fos-positive neurons in the PVN and NTS, whereas there was no increase of c-Fos-positive neurons in the DMV and ARC. Serotonin-fluorescence in the brainstem significantly decreased in the neurons of the NTS and DMV. Injection of bombesin resulted in an increase of c-Fos-positive neurons in the NTS and PVN. At the same time, a significant increase of c-Fos-positive neurons with NUCB2/nesfatin-1 immunoreactivity could be observed. Further, there was an increase of c-Fos-positive neurons colocalizing with NUCB2/nesfatin-1 and tyrosine hydroxylase in the NTS and NUCB2/nesfatin-1 and oxytocin in the PVN. Administration of CCK and bombesin increased c-Fos-positive neurons in the NTS and also increased the number of activated neuronatin-neurons.

Conclusion: Peripherally administered CCK seems to act on central hunger satiety signals stimulating the serotonin- and neuronatin system. Bombesin activates NUCB2/nesfatin-1 neurons in the brainstem and hypothalamus, possibly mediated by a peripheral release of CCK. Further, bombesin activates neuronatin-neurons in the brainstem. Neuronatin in the brainstem could thus be involved in the mediation of the inhibitory effect of bombesin on food intake.

1. Einleitung

1.1 Regulation der Nahrungsaufnahme

Bei der Nahrungsaufnahme handelt es sich um einen komplex regulierten Vorgang, welcher durch das Ineinandergreifen verschiedener Mediatorsysteme gekennzeichnet ist. Der Magen-Darm-Trakt interagiert dabei direkt mit dem zentralen Nervensystem (ZNS), diese Verbindung wird als „Brain-Gut-Achse“ bezeichnet [1]. Aufgrund steigender Prävalenzen von Adipositas und Übergewicht [2] mit den damit verbundenen gesundheitlichen und ökonomischen Folgen gewinnt die detaillierte Kenntnis der Hunger-Sättigungsregulation zunehmend an Bedeutung. Die von unserer Arbeitsgruppe untersuchten Peptide spielen bei der Regulation der „Brain-Gut-Achse“ eine bedeutende Rolle und wirken kurzfristig hemmend auf die Nahrungsaufnahme.

Das anorexigen wirksame Peptidhormon Cholecystokinin (CCK) wird in den I-Zellen des Dünndarms produziert und bei Anwesenheit von Aminosäuren oder langen Fettsäuren ausgeschüttet [3], [4], [5]. CCK kommt im Körper in verschiedenen molekularen Formen vor. Das in der Studie verwendete CCK-8 ist die kürzeste Form mit voller biologischer Wirkung [6]. Über CCK1-Rezeptoren bindet CCK an vagale Afferenzen und bewirkt eine Steigerung der gastrointestinalen Motilität, der Gallenblasenkontraktion sowie der Pankreassekretion [7], [8], [9], [10]. Afferente Anteile des Nervus vagus führen zu einer neuronalen Aktivierung u.a. der katecholaminhaltigen A2-Zellgruppe des Nucleus tractus solitarii (NTS). Der NTS spielt in der Regulation der Nahrungsaufnahme eine Schlüsselrolle [11], [12]. Läsionsstudien haben gezeigt, dass die CCK-Wirkung im hohen Maße von der Intaktheit dieses Kerngebietes abhängig ist [13]. Ausgehend vom NTS ziehen nervale Projektionen zu hypothalamischen Kernarealen, die in die Regulation der Hunger- und Sättigungsphysiologie involviert sind [14], [15]. Zentral bewirkt peripher freigesetztes CCK auf diesem Wege eine kurzfristige Terminierung der Nahrungsaufnahme [16], [17]. Studien konnten belegen, dass eine Vagotomie den Sattheits-induzierenden Effekt von CCK aufhebt [10].
Verschiedene Arbeitsgruppen haben die Lokalisation von CCK1- und 2-Rezeptoren im ZNS nachgewiesen [18], [19], [20], [21]. Bekannt ist, dass CCK zentral nicht nur an der Hunger-Sättigungs-Regulation beteiligt ist, sondern auch an der Integration verschiedener kognitiver Leistungen und Emotionen, wie Schmerz, Angst, Sexualverhalten, Gedächtnis und Lernen [21], [22].

Ein weiterer Neurotransmitter, der neben der Nahrungsaufnahme diverse kognitive Leistungen beeinflusst, ist Serotonin. Serotonin wird peripher in enterochromaffinen Zellen des Dünndarms [3] und zentral in den Raphekernen des Hirnstammes produziert [23], [24]. Im Gastrointestinaltrakt wird Serotonin bei intraluminaler Anwesenheit von Nahrung sezerniert [25]. Ähnlich wie CCK bewirkt Serotonin eine veränderte Magen-Darm-Motilität und bindet an vagale Afferenzen [26]. Verschiedene Studien konnten belegen, dass Serotonin hemmend auf die Nahrungsaufnahme wirkt [27], [28].

Verschiedene Arbeitsgruppen konnten zeigen, dass Überlappungen zwischen dem CCK- und dem Serotoninsystem existieren [25], [29], [30], [28]. In diesem Zusammenhang wurde beobachtet, dass die Depletion des ausschließlich zentral vorkommenden Serotonin 2C Rezeptor (S2CRK0) bei Mäusen dazu führt, dass die periphere Gabe von CCK, anders als im Wildtyp, wirkungslos hinsichtlich der neuronalen Aktivierung (c-Fos) von NTS-Neuronen ist [31]. Auch der Sättigungseffekt von CCK 30 Minuten nach Injektion des Peptids war bei den S2CRK0 - Tieren im Gegensatz zu dem Wildtyp aufgehoben [31]. Hayes et al. fanden heraus, dass Mikroinjektion des Serotonin-Antagonisten Ondansetron in den medialen NTS bei Ratten die aufgenommene Menge von Zuckerlösung steigerten, während eine periphere Injektion von CCK die Menge aufgenommener Zuckerlösung senkte [30]. Der CCK-Effekt auf die Aufnahme von Zuckerlösung konnte durch die Zufuhr des Serotonin-Antagonisten teilweise rückgängig gemacht werden [30]. Helm und Kollegen zeigten, dass die Injektion von CCK und Serotonin unilateral in den Nucleus paraventricularis (PVN) des Hypothalamus die Nahrungsaufnahme stärker fördert als die Injektion der Einzelsubstanzen, ein Effekt, der über eine reine additive Wirkung hinauszugehen scheint, so konnte nach gemeinsamer Verabreichung eine gesteigerte Dopaminfreisetzung in Neuronen des PVN beobachtet werden [28].

Zwei weitere Peptide mit Hormoncharakter, welche ebenfalls im CCK-Signalweg eine Rolle zu spielen scheinen und anorexigen wirken, sind Bombesin und Nesfatin-1. Bombesin ist ein Peptidhormon, welches 1971 erstmals aus der Haut der Kröte *Bombina bombina* isoliert wurde [32]. Das Hormon ähnelt strukturell den in Säugetieren vorkommenden Gastrin-releasing peptide (GRP) und Neuromedin B, die nach Aufnahme von Nährstoffen im Gastrointestinaltrakt freigesetzt werden und die Nahrungsaufnahme reduzieren [33], [34]. Bombesin-Rezeptoren sind im Gastrointestinaltrakt und im ZNS weit verbreitet [33], [35], [36]. Intracerebroventrikuläre (i.c.v.) Injektionen von Bombesin in Bereiche des Hirnstammes und Hypothalamus unterdrücken die Nahrungsaufnahme [37], [38]. Während i.c.v.-injiziertes Bombesin zusätzlich

Fellpflegeverhalten bei Ratten stimuliert, scheint intraperitoneal (i.p.) injiziertes Bombesin bei partiell erhaltener Unterdrückung der Nahrungsaufnahme keine Auswirkung auf das Paarungs- und Pflegeverhalten der Tiere zu haben [39]. Bombesin könnte die Nahrungsaufnahme somit möglicherweise über periphere Mechanismen, ggf. CCK-vermittelt, hemmen [40]. Interessanterweise zeigten Erspamer et al. bereits 1974, dass die Injektion von Bombesin bei Hunden die CCK-Ausschüttung stimuliert [41], während die Applikation von einem Antagonisten gegen die humanen Bombesin-Analoga (BIM262226) beim Menschen die CCK-Ausschüttung unterdrückt [42].
Nesfatin-1 ist ein Peptid, welches durch Mori et al. entdeckt wurde und die Nahrungsaufnahme bei Ratten reduziert, ohne das Fellpflegeverhalten der Tiere zu beeinflussen [43]. Nesfatin-1 wird *in vivo* proteolytisch vom Vorläufermolekül NUCB2 abgespalten [43]. NUCB2 mRNA wurde in verschiedenen Hirnkernen detektiert [44], [45]. Noetzel et al. fanden heraus, dass sulfatiertes CCK-8 dosisabhängig zu einer neuronalen Aktivierung von NUCB2/Nesfatin-1 Neuronen im PVN und NTS führt [46]. Die neuronale Aktivierung wurde über den Aktivierungsmarker c-Fos bestimmt. Das Protoonkogen c-Fos ist Bestandteil des Transkriptionsfaktors AP-1 und wird als Antwort auf physikalische oder chemische Reize verstärkt synthetisiert. Durch die immunhistochemische Detektion von c-Fos können aktivierte Neurone in Reaktion auf externe Stimuli dargestellt werden [47].

CCK, Serotonin, Bombesin und Nesfatin-1 sind wahrscheinlich wichtige funktionelle Bestandteile in der Regulation der homöostatischen Nahrungsaufnahme. Durch eine Begrenzung der aufgenommenen Nahrungsmenge und eine kurzfristige Terminierung selbiger könnten sie dazu beitragen, den Energiebedarf des Organismus adäquat zu decken. Wie oben ausgeführt, scheinen die genannten Mediatoren zu diesem Zweck miteinander zu interagieren.

In der dritten Arbeit betrachteten wir die Wirkung von peripher verabreichtem CCK-8S und Bombesin auf Neuronatin-Neurone im NTS und im Nucleus dorsalis nervi vagi (DMV) des Hirnstamms. Neuronatin ist ein auf Chromosom 20 lokalisiertes Gen, welches für ein gleichnamiges Protein kodiert [48]. Das Neuronatin-Gen unterliegt einer epigenetischen Prägung und kann somit postnatal variabel exprimiert werden [48]. Die Wirkung des Genprodukts Neuronatin ist bisher noch nicht vollständig entschlüsselt. Auf zellulärer Ebene scheint das Protein Neuronatin vielfältige Prozesse zu beeinflussen, so ist Neuronatin an der neuronalen Differenzierung [49] und wahrscheinlich auch an der Stoffwechselregulation beteiligt [50], [51]. Die variable, paternal geprägte Methylierung des Neuronatin-Gens wird mit der genetischen

Determination von Fettleibigkeit in Verbindung gebracht [52]. Vor diesem Hintergrund ist es naheliegend, dass Neuronatin in die Nahrungsregulation involviert ist. Die Neuronatin mRNA wurde bereits in wichtigen Zentren der Hunger-Sättigungsregulation nachgewiesen [53].

1.2 Ziele der Arbeit

Ziel der Arbeit war die Phänotypisierung von neuronal aktivierten Neuronen im Hirnstamm und Hypothalamus nach peripherer Injektion von CCK-8S oder Bombesin.

In der ersten tierexperimentellen Studie überprüften wir, inwiefern peripher verabreichtes CCK-8S die Konzentration von Serotonin in Nervenfasern des Nucleus dorsalis nervi vagi und Nucleus tractus solitarii des Hirnstamms sowie Nucleus paraventricularis und Nucleus arcuatus (ARC) des Hypothalamus moduliert.

In der zweiten Studie untersuchten wir, ob die periphere Bombesin-Gabe die neuronale Aktivierbarkeit (c-Fos) von NUCB2/Nesfatin-1 immunreaktiven Neuronen im Nucleus tractus solitarii sowie Nucleus paraventricularis und Nucleus arcuatus beeinflusst.

In einer dritten tierexperimentellen Untersuchung haben wir evaluiert, ob die intraperitoneale CCK- bzw. Bombesin-Gabe die neuronale Aktivierung von Neuronatin-haltigen Neuronen auf Hirnstammebene im Nucleus tractus solitarii und Nucleus dorsalis nervi vagi beeinflusst.

2. Material und Methoden

2.1 Genehmigungen

Die Tierversuche, auf denen diese Arbeiten basieren, wurden durch das Landesamt für Gesundheitsschutz und technische Sicherheit Berlin bewilligt (Tierschutznummer G 0053/06 und ggf. Erweiterungsanträge und G 0067/15).

2.2 Versuchstiere

Die Haltung der Versuchstiere erfolgte experimentübergreifend stets nach dem gleichen Protokoll. Männliche Sprague-Dawley Ratten mit einem Initialgewicht zwischen 150 und 180 g wurden in Gruppen von vier Tieren pro Käfig (Makrolon-Käfig Typ IV) unter konstanten Bedingungen gehalten. Der Tag-Nacht-Rhythmus wurde mittels Zeitschaltuhr automatisiert, wobei eine Zeitphase immer 12 Stunden betrug. Die Lichtphase begann um 6:30 Uhr und endete um 18:30 Uhr. Die Temperatur wurde durchgehend auf 22 °C +/- 2 °C eingestellt, die Luftfeuchtigkeit betrug 60 %. Zwischen den einzelnen Experimenten hatten die Ratten freien Zugang zu Standardfutter (Altromin™, Lage, Deutschland) und Leitungswasser. Nach ihrer Ankunft in der Tierhaltung hatten die Versuchstiere sieben Tage Zeit zur Gewöhnung an die neuen Umgebungsbedingungen. Anschließend begannen wir mit dem Training der Tiere zur Vorbereitung auf das jeweilige Experiment. Die Ratten wurden im Rahmen des Trainings täglich in Kopftieflage gebracht und ihre Bauchdecke wurde einmal kurz beklopft, um eine i.p.-Injektion zu simulieren. Die Simulation des Versuchsablaufs und die Gewöhnung an den Untersucher sollten den Stress für die Tiere während der Durchführung der Experimente minimieren. Zu dem Zeitpunkt der Experimente hatten die Ratten ein Gewicht zwischen 250 und 300 g erreicht.

2.3 Publikation 1: Die Auswirkung von peripher injiziertem CCK-8S auf die Konzentration von Serotonin in Nervenfasern des Nucleus tractus solitarii, Nucleus dorsalis nervi vagi, Nucleus paraventricularis und Nucleus arcuatus

2.3.1 Peptide

Für die Studie wurde Cholecystokinin-Oktapeptid (CCK-8S, H-Asp-Tyr(SO3H)-Met-Gly-Trp-Met-Asp-Phe-NH2, Bachem AG, Heidelberg, Deutschland) in 1 ml *Aqua ad iniectabilia* mit 1 % (v/v) Ammoniumhydroxid (NH_4OH) gelöst, aliquotiert und bei -20 °C aufbewahrt. Unmittelbar vor Beginn des Experiments wurde das eingefrorene Peptid aufgetaut und mit steriler 0,15 M NaCl-Lösung (Braun, Melsungen, Deutschland) verdünnt, um eine Endverdünnung von 5,2 nmol/kg Körpergewicht (KG) und 8,7 nmol/kg KG (~6 bzw. ~10 µg/kg KG) zu erreichen. Das verdünnte Peptid wurde unmittelbar vor der i.p.-Injektion auf Eis gelagert. Die verwendeten Dosierungen gelten als etabliert [46], [54], [55].

2.3.2 Versuchsaufbau

Um die größtmögliche Konsistenz in der Versuchsdurchführung zu gewährleisten, wurden die Tierexperimente stets zur gleichen Zeit, zwischen 10 Uhr und 10:30 Uhr durchgeführt. Zwei Gruppen von Tieren erhielten 5,2 nmol/kg oder 8,7 nmol/kg CCK-8S als i.p.-Injektion. Einer Versuchsgruppe wurde sterile, isotone NaCl-Lösung als Kontrolllösung (Vehikellösung) verabreicht. Pro Tier wurde ein Volumen von 500 µl injiziert. 90 Minuten nach der ersten Injektion wurden die Tiere mittels 100 mg/kg Ketamin (Ketanest®, Curamed, Karlsruhe, Deutschland) und 10 mg/kg Xylazine (Rompun® 2%, Bayer, Leverkusen, Deutschland) tief narkotisiert. Zusätzlich erhielten die Tiere 2500 IE Heparin i.p. (Liquemin®, Hoffman-La Roche, Grenzach-Wyhlen, Deutschland), um eine spontane Thrombenbildung im Kreislaufsystem zu verhindern. Nach Überprüfung der Narkosetiefe wurden die Tiere über eine transkardiale Perfusion nach dem Protokoll von Kobelt et al. aus dem Jahr 2005 fixiert [56]. Die Gehirne wurden nach ihrer Entnahme in einer aufsteigenden Saccharose-Lösung dehydriert, kryokonserviert und bei -80 °C in Plastikgefäßen mit gefrorenem pulverisiertem Wasser als Schutz vor Austrocknung gelagert. Anschließend wurden am Kryostaten 25 µm dünne koronare Gefrierschnitte vom Hirnstamm und Hypothalamus angefertigt.

2.3.3 Immunhistochemie

2.3.3.1 Allgemeines

Die 25 µm dicken Gewebeschnitte wurden für die Antikörpermarkierung in Multiwell®-Zellkulturplatten (Becton Dickinson, Heidelberg, Deutschland) im "free-floating" Verfahren in Phosphatpuffer (phosphate-buffered saline, PBS, pH 7,4; Stammlösung bestehend aus 249,46 g Dinatriumhydrogenphosphat-Dihydrat ($2NaH_2PO_4x2H_2O$) und 35,28 g Natriumhydrogenphosphat-Monohydrat ($NaH_2PO_4xH_2O$) in 2 l aqua bidest, pH 7,4) bei 21 °C auf dem Horizontalschüttler aufbewahrt. Nach Abschluss der Immunmarkierungen wurden die Gewebeschnitte auf Objektträger aufgezogen und zum Schutz gegen Fluoreszenzverlust in Folge der Anregungswellenlängen durch die konfokale Lasermikroskopie mit 8 µl einer Antifading-Lösung (DABCO (100 mg/ml 1,4-Diazabicyclo [2.2.2] octane, Sigma, St. Louis, USA, in 90 % (v/v) Glycerin, 10 % (v/v) PBS, pH 7,4) eingebettet. Alle Gehirnschnitte wurden bis zur Auswertung unter dem konfokalen Lasermikroskop (cLSM 760, Carl Zeiss, Jena, Germany) bei -20 °C im Gefrierschrank aufbewahrt.

2.3.3.2 Immunhistochemischer Nachweis von c-Fos in Neuronen des Nucleus arcuatus, Nucleus paraventricularis, Nucleus tractus solitarii und dem Nucleus dorsalis nervi vagi

Zur Vorbereitung der Immunmarkierung wurden die Gehirnschnitte mit 0,1 M PBS gewaschen. Eine 15-minütige Behandlung mit 1 % (w/v) Natriumborhydrid (Carl Roth, Karlsruhe, Deutschland) sollte die durch Aldehyde induzierte Eigenfluoreszenz des Gewebes reduzieren. Nach einem ausführlichen Waschschritt folgte die 60-minütige Vorinkubation mit 1 % (w/v) Bovines Serumalbumin (BSA, Sigma, St. Louis, USA), 0,3 % (v/v) Triton-X-100 (Serva, Heidelberg, Deutschland), 0,1 % (w/v) NaN_3 und 0,05 % (v/v) Phenylhydrazin, um unspezifische Antikörperbindungen innerhalb des Gewebes zu verhindern. Die Zugabe von Phenylhydrazin blockiert die endogene Peroxidaseaktivität im Gewebe. Die anschließende 24-stündige Inkubation erfolgte mit dem 1:3000 verdünnten Primärantikörper anti-c-Fos (rabbit anti-c-Fos, Oncogene Research Products, Boston, USA), welcher in 1 % (w/v) BSA und 0,3 % (v/v) Triton X-100 gelöst wurde. Nach dreimaligem Waschen der Gewebeschnitte in PBS und einer erneuten 60-minütigen Vorinkubation mit PBS und 1 % (w/v) BSA wurde der 1:1000 verdünnte Zweitantikörper für 12 Stunden zugesetzt (Goat Biotin-SP-conjugated anti-Rabbit IgG, Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA; in 1 % (w/v) BSA in PBS). Erneut wurden die Gewebeschnitte gewaschen, um ungebundene Antikörper zu entfernen. Hiernach erfolgte die

Inkubation der Gehirnschnitte mit dem Avidin-Biotin-Peroxidasekomplex (ABC, Vector Laboratories, Peterborough, UK; 1:1000 in PBS, pH 7,4). Nach Ablauf von sechs Stunden, dreimaligem Waschen in PBS sowie einer Vorinkubation mit Boratpuffer (pH 8,5) erfolgte die Tetramethylrhodamintyramide-Amplifikation in einer Verdünnung von 1:150 (PerkinElmer, Waltham, MA, USA) für 10 Minuten. Nach erneutem Waschen in PBS wurden die Gewebeschnitte für 15 Minuten mit 4′,6-Diamidin-2-phenylindol (DAPI, Sigma, St. Louis, USA; 2 µg/ml in PBS) inkubiert, um das Zellchromatin sichtbar zu machen. Dieser Arbeitsschritt diente der Identifizierbarkeit der zu untersuchenden Kerngebiete. Alle Gehirnschnitte wurden im Anschluss, wie oben unter 2.3.3.1 beschrieben, erneut gewaschen, eingebettet und aufbewahrt.

2.3.3.3 Immunhistochemischer Nachweis von c-Fos in Neuronen des Nucleus arcuatus, des Nucleus paraventricularis, Nucleus tractus solitarii und dem Nucleus dorsalis nervi vagi sowie von Serotonin in umgebenden Nervenfasern

Der Nachweis des neuronalen Aktivitätsprotein c-Fos erfolgt nach dem oben beschriebenen Protokoll (2.3.3.2) mit Tetramethylrhodamintyramide-Amplifikation. Nach drei Waschschritten und einer Vorinkubation mit 1 % (w/v) BSA in PBS schloss sich die Inkubation mit dem zweiten, gegen Serotonin gerichteten Erstantikörper in einer Verdünnung von 1:1000 an (rabbit anti-serotonin, Novus Biologicals, Littleton, CO, USA; in 1 % (w/v) BSA in PBS, pH 7,4). Nach 24 Stunden der Inkubation wurde der ungebundene Antikörper durch dreimaliges Waschen mit PBS entfernt und der Sekundärantikörper Fluorescein isothiocyanate (FITC) conjugated goat anti-rabbit IgG (goat anti-rabbit IgG, Sigma Aldrich) in einer Verdünnung von 1:400 für 12 Stunden aufgetragen. Abschließend wurde erneut dreimal mit PBS gewaschen, sowie eine Gegenfärbung mit DAPI durchgeführt und die Gewebeschnitte auf Objektträger aufgezogen sowie mit Antifading-Lösung eingedeckt, wie unter 2.3.3.2 beschrieben. Nach Aufbringen eines Deckglases wurden die Gewebeschnitte bis zum Mikroskopieren bei -20 °C gelagert.

2.3.3.4 Dreifachmarkierung mit Antikörpern gegen c-Fos, Serotonin und Tyrosinhydroxylase im Nucleus tractus solitarii des Hirnstammes

Die Immunmarkierung folgte dem oben beschriebenen Protokoll (2.3.3.3). Ergänzend wurde mit einem Erstantikörper gegen Tyrosinhydroxylase aus der Maus (anti-tyrosine hydroxylase, Sigma Aldrich: 1:6000 in 1 % (w/v) BSA und 0,1 % (w/v) Natriumazid in PBS) für 24 Stunden inkubiert. Nach dreimaligem Waschen mit PBS wurde der gegen Tyrosinhydroxylase gerichtete Sekundärantikörper aus der Ziege in der Verdünnung 1:400 in 1 % (w/v) BSA und PBS (goat anti-

mouse IgG, Alexa Fluor® 633, MolecularProbes, Leiden, Niederlande) aufgetragen. Nach 12 Stunden wurde erneut dreimal mit PBS gewaschen. Das Zellchromatin färbten wir mittels DAPI (siehe 2.3.3.2). Im Anschluss an dreimaliges Waschen mit PBS zogen wir die Schnitte auf Objektträger auf und betteten sie in Antifading-Lösung ein.

2.3.4 Mikroskopische und statistische Auswertung der Daten

Neurone, die ein rotes Fluoreszenzsignal zeigen, wurden als c-Fos-positiv gewertet. Von allen aufeinanderfolgenden 25 µm dicken Hirnschnitten wurde jeder dritte Schnitt zur Auswertung der c-Fos-Markierung genutzt. Die Anzahl der c-Fos-positiven Neurone wurde bilateral im PVN (Bregma -2,21 bis -3,60 mm), im ARC (Bregma -2,12 bis -3,60 mm), im NTS (Bregma -13,24 bis -14,30 mm) und im DMV (Bregma -13,24 bis -14,30 mm) nach den Koordinaten von Paxinos und Watson [57] von einem verblindeten Untersucher quantifiziert. Die Anzahl c-Fos-positiver Neurone wurde für 3-5 Ratten pro Gruppe gemittelt. Die verbleibenden Hirnschnitte wurden für immunhistochemische Zweifach- und Dreifach-Markierungen verwendet. Mit stets der gleichen Einstellung wurde unter dem konfokalen Lasermikroskop die Fluoreszenzintensität von Serotonin und damit indirekt die Konzentration von Serotonin in den Nervenfasern bestimmt. Das Programm ImageJ 1,47v wurde für die semiquantitative Analyse der Fluoreszenzintensität genutzt. Die Fluoreszenzintensität wurde in unserem Experiment als Grauenstufenwert/Gewebeschnitt (Pixel/Gewebeschnitt) erfasst. Aus den gewonnenen Daten wurden Mittelwerte ± SEM errechnet und mittels Varianzanalyse (ANOVA) ausgewertet. Unterschiede zwischen den experimentellen Gruppen wurden durch den post-hoc Fisher's least significant difference (Fischer LSD) Test evaluiert und $p < 0,05$ wurde als signifikant gewertet. Die statistischen Analysen wurden mithilfe des Programms SigmaStat 3.1 (Systat, San Jose, USA) durchgeführt.

2.4 Publikation 2: Peripher verabreichtes Bombesin induziert c-Fos in NUCB2/Nesfatin-1 Neuronen im Nucleus tractus solitarii des Hirnstamms und im Nucleus paraventricularis des Hypothalamus

2.4.1 Peptide

Bombesin (Pyr-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH2, Bachem AG, Heidelberg, Deutschland, Bachem AG, Heidelberg, Deutschland) wurde in *Aqua ad iniectabilia* gelöst und bei -20 °C gelagert. Das Peptid wurde unmittelbar vor Beginn des Experiments

aufgetaut und mit steriler 0,15 M NaCl-Lösung verdünnt, um Konzentrationen von 26 und 32 nmol/kg KG (~42 µg/kg KG und ~52 µg/kg KG) zu erreichen. Die gewählten Dosierungen sind in Übereinstimmung mit bereits veröffentlichten Publikationen [58], [59].

2.4.2 Versuchsaufbau

Der Versuchsaufbau entspricht in der in 2.3.2 beschriebenen Abfolge. Wir untersuchten drei experimentelle Gruppen mit jeweils vier Versuchstieren, welchen Vehikellösung, 26 nmol/kg Bombesin oder 32 nmol/kg Bombesin i.p. injiziert wurde. Anschließend wurden die Tiere, wie zuvor beschrieben, transkardial mit Fixierlösung in tiefer Narkose perfundiert, die Gehirne wurden entnommen, dehydriert, kryokonserviert und bei -80 °C gelagert. Die anschließende Färbung erfolgte an 25 µm dicken Gewebeschnitten.

2.4.3 Immunhistochemie

2.4.3.1 Immunhistochemischer Nachweis von c-Fos im Nucleus tractus solitarii, Nucleus paraventricularis und Nucleus arcuatus

Der immunhistochemische Nachweis von c-Fos erfolgte nach dem in 2.3.3.2 beschriebenen Protokoll.

2.4.3.2 Doppelfärbung mit Antikörpern gegen c-Fos und NUCB2/Nesfatin-1 im Nucleus tractus solitarii, Nucleus paraventricularis und Nucleus arcuatus

C-Fos wurde nach dem in 2.3.3.2 beschriebenen Protokoll dargestellt. Nach der Tetramethylrhodamintyramide-Amplifikation in der Verdünnung 1:150 für 15 Minuten wurden die Gewebeschnitte nach dreimaligem Waschen mit dem zweiten Erstantikörper gegen NUCB2/Nesfatin-1 (rabbit anti-nesfatin-1, Phoenix Pharmaceuticals, Inc, Burlingame, USA; 1:1000 in PBS und 1 % (w/v) BSA) für 24 Stunden bei Raumtemperatur inkubiert. Anschließend wurde dreimal mit PBS gewaschen und ein Sekundärantikörper aus der Ziege (Fluorescein isothiocyanate (FITC) conjugated goat anti-rabbit IgG, Sigma Aldrich, 1:400 in PBS mit 1 % (w/v) BSA) auf die Gewebeschnitte aufgetragen. Nach 12 Stunden wurde der Zweitantikörper durch dreimaliges Waschen entfernt. Im Anschluss an die oben beschriebene Färbung des Zellchromatins mittels des Fluorochrom DAPI wurden die Gewebeschnitte ein letztes Mal mit

PBS gespült, auf Objektträger aufgezogen und mit DABCO eingebettet. Die Hirnschnitte wurden bei -20 °C bis zur mikroskopischen Auswertung im Kühlschrank gelagert (siehe auch 2.3.3.2).

2.4.3.3 Dreifachmarkierung mit Antikörpern gegen c-Fos, NUCB2/Nesfatin-1 und Oxytocin im Nucleus paraventricularis

Die Doppelmarkierung gegen c-Fos und NUCB2/Nesfatin-1 wurde, wie unter Punkt 2.4.3.2 beschrieben, durchgeführt. Anschließend wurde mit dem dritten Erstantikörper, der gegen Oxytocin gerichtet war (monoclonal mouse anti-oxytocin, Chemicon International, Hofheim am Taunus, Deutschland; 1:7000 in PBS mit 1 % (w/v) BSA) für 24 Stunden inkubiert. Nach der Antikörper-Inkubation wurden die Gewebeschnitte dreimal mit PBS gewaschen. Darauffolgend wurden die Hirnschnitte für 12 Stunden mit dem Sekundärantikörper inkubiert (goat anti-mouse IgG, Alexa Fluor® 633, MolecularProbes, Leiden, Niederlande). Erneut wurde dreimal mit PBS gewaschen, um den ungebundenen Antiköper zu entfernen. Das Zellchromatin wurde, wie zuvor unter Punkt 2.3.3.2 beschrieben, mit DAPI angefärbt. Die Hirnschnitte wurden erneut dreimal in PBS gespült, auf Objektträger aufgezogen und mit Antifading-Lösung bedeckt sowie bei -20 °C im Kühlschrank bis zur mikroskopischen Auswertung gelagert.

2.4.3.4 Dreifachmarkierung mit Antikörpern gegen c-Fos, NUCB2/Nesfatin-1 und Tyrosinhydroxylase im Nucleus tractus solitarii

Die Doppelmarkierung gegen c-Fos und NUCB2/Nesfatin-1 wurde ausgeführt, wie unter 2.4.3.2 beschrieben. Die Gewebeschnitte wurden dreimal aufeinanderfolgend mit PBS gewaschen. Alsdann wurde das Gewebe mit einem Erstantikörper gegen Tyrosinhydroxylase (mouse anti-tyrosine hydroxylase, Sigma Aldrich: 1:6000 in 1 % (w/v) BSA und 0,1 % Natriumazid in PBS) für 24 Stunden bei Raumtemperatur inkubiert. Nach dreimaligem Waschen mit PBS erfolgte die Inkubation der Gehirnschnitte mit dem gegen Tyrosinhydroxylase gerichteten Sekundärantikörper, der aus der Maus stammte und 1:400 in 1 % (w/v) BSA verdünnt wurde (goat anti-mouse IgG, Alexa Fluor® 633, MolecularProbes, Leiden, Niederlande). Um den nicht-gebundenen Sekundärantikörper zu entfernen, wurde erneut dreimal mit PBS gewaschen. Im Anschluss erfolgte, wie unter Punkt 2.3.3.2 beschrieben, die Gegenfärbung mit DAPI, das Aufziehen der Gewebeschnitte auf Objektträger und das Eindecken mit Antifading-Lösung sowie das Aufbringen von Deckgläsern.

2.4.4 Mikroskopische und statistische Auswertung der Daten

Neurone, die ein rotes Fluoreszenzsignal nach Fluoreszenzanregung zeigten, wurden als c-Fos-positiv gewertet. Von allen 25 µm dicken Hirnschnitten wurde jeder dritte Schnitt genutzt, um c-Fos-positive Neurone bilateral im PVN (Bregma -2,21 mm bis -3,60 mm), im ARC (Bregma -2,12 mm bis -3,60 mm) und im NTS (Bregma -13,24 mm bis -14,30 mm) gemäß der Koordinaten von Paxinos und Watson auszuzählen [57]. Alle Gewebeschnitte wurden unter dem konfokalen Lasermikroskop (cLSM 760, Carl Zeiss, Jena, Deutschland) visualisiert und ausgewertet. Der Mittelwert von c-Fos-positiven Neuronen wurde für vier (NTS), bzw. drei (PVN und ARC) Ratten pro Gruppe berechnet. Doppel- und dreifach-immunmarkierte Neurone wurden ebenfalls quantitativ erfasst. Der auswertende Untersucher war verblindet gegenüber der experimentellen Zuordnung der Versuchstiere. Die Daten wurden als Mittelwerte ± SEM dargestellt und mittels ANOVA, bzw. ANOVA *on ranks* analysiert. Unterschiede zwischen Gruppen wurden durch den Holm-Sidak und den Student-Newman-Keuls post-hoc Test ermittelt, dabei wurde ein von $p < 0,05$ als signifikant angenommen.

2.5 Publikation 3: Cholecystokinin und Bombesin aktivieren Neuronatin-Neurone im Nucleus tractus solitarii des Hirnstammes

2.5.1 Peptide

Für die dritte Studie verwendeten wir erneut CCK-8S (Bachem AG, Heidelberg, Deutschland) in der etablierten Endverdünnung von 5,2 und 8,7 nmol/kg KG (~6 bzw. ~10 µg/kg KG) sowie Bombesin (Bachem AG, Heidelberg, Deutschland) in den etablierten Konzentrationen 26 und 32 nmol/kg KG (~42 und ~52 µg/kg KG).

2.5.2 Versuchsaufbau

Der Versuchsaufbau glich dem unter 2.3.2 beschriebenen Vorgehen. Die Ratten wurden in fünf Gruppen unterteilt. Die erste Versuchsgruppe erhielt 500 µl Vehikellösung i.p. und umfasste sieben Versuchstiere. Jeweils vier Tieren wurde eine der Vehikellösung äquivalente Menge 26 nmol/kg Bombesin und 32 nmol/kg Bombesin i.p. injiziert. Wiederum jeweils vier Tieren wurden 5,2 und 8,7 nmol/kg CCK-8S i.p. verabreicht. Die Tiere wurden anschließend, wie unter Punkt 2.3.2, beschrieben narkotisiert, heparinisiert und transkardial mit Fixierlösung perfundiert. Nach

Entnahme der Gehirne wurde das Gewebe in einer aufsteigenden Saccharose-Lösung dehydriert, kryokonserviert und bei -80 °C gelagert.

2.5.3 Immunhistochemie

2.5.3.1 Dreifachmarkierung mit Antikörpern gegen c-Fos, Neuronatin und Tyrosinhydroxylase im Nucleus tractus solitarii und Nucleus dorsalis nervi vagi

Das Protoonkogen c-Fos wurde nach dem unter Punkt 2.3.3.2 ausgeführten Protokoll dargestellt. Im Unterschied zu den vorherigen Experimenten wurde der Primärantikörper gegen c-Fos jedoch in einer Verdünnung von 1:4000 eingesetzt (rabbit anti-c-Fos, Oncogene Research Products, Boston, USA, 1:4000 in 5 % (w/v) BSA, 0,1 % (v/v) Natriumazid und 0,3 % (v/v) Triton X-100). Nach Tetramethylrhodamintyramide-Amplifikation wurden die Gehirnschnitte mit dem zweiten Primärantikörper gegen Neuronatin (rabbit anti-neuronatin, Phoenix Pharmaceuticals, Inc, Burlingame, USA; 1:400 in 1 % (w/v) BSA in PBS mit 0,1 % (w/v) Natriumazid verdünnt) für 48 Stunden bei Raumtemperatur inkubiert. Nach dreimaligem Waschen mit PBS und Vorinkubation mit 1 % (w/v) BSA und PBS mit 0,1 % (w/v) Natriumazid wurde der Sekundärantikörper Fluorescein isothiocyanate (FITC) conjugated goat anti-rabbit IgG (Sigma Aldrich) 1:400 verdünnt mit PBS und 0,1 % (w/v) Natriumazid sowie BSA 1 % (w/v) für 12 Stunden aufgetragen. Nach erneutem dreimaligem Waschen erfolgte die 24-stündige Inkubation mit dem dritten Primärantikörper gegen Tyrosinhydroxylase (anti-tyrosine hydroxylase, Sigma Aldrich: 1:6000 in 1 % (w/v) BSA und 0,1 % (w/v) Natriumazid in PBS, pH 7,4) sowie nach dreimaligem Waschen mit PBS die 12-stündige Inkubation mit einem Sekundärantikörper gerichtet gegen Tyrosinhydroxylase-Antikörper (goat anti-mouse IgG, Alexa Fluor® 633, MolecularProbes, Leiden, Niederlande). Die Gewebeschnitte wurden wiederum gespült, das Zellchromatin wurde mit DAPI sichtbar gemacht und die Gewebeschnitte wurden eingedeckt mit Antifading-Lösung, wie unter Punkt 2.3.3.2 beschrieben.

2.5.4 Mikroskopische und statistische Auswertung der Daten

Zur quantitativen Analyse der 25 µm dicken Gehirnschnitte wurde die konfokale Lasermikroskopie verwendet (cLSM 760, Carl Zeiss, Jena, Deutschland). Die Auswertung von c-Fos-positiven Neuronen erfolgte bilateral im NTS und DMV (Bregma 13,24 mm bis 14,30 mm) [57]. Nervenzellen, die ein rotes Fluoreszenzsignal im Zellkern aufwiesen, wurden als c-Fos-positiv gewertet. Grüne Fluoreszenzsignale im Zytoplasma der Neurone wurden als Neuronatin-

positiv gewertet. Blaue Fluoreszenzsignale im Zytoplasma wurden als Tyrosinhydroxylase-positiv bewertet. Der Mittelwert der c-Fos-, Neuronatin- und Tyrosinhydroxylase-positiven Nervenzellen pro Gewebeschnitt wurde für jedes Tier berechnet. Weiterhin wurden einfach immunmarkierte Neurone (c-Fos, Neuronatin, Tyrosinhydroxylase), doppelt immunmarkierte Neurone (c-Fos/Neuronatin, c-Fos/Tyrosinhydroxylase, Neuronatin/Tyrosinhydroxylase) sowie dreifach immunmarkierte Neurone (c-Fos/Neuronatin/Tyrosinhydroxylase) in den Kerngebieten NTS und DMV quantitativ erfasst. Die Auswertung am Mikroskop erfolgte verblindet gegenüber der experimentellen Zuordnung der Versuchstiere. Die Daten wurden als Mittelwerte ± SEM dargestellt und mittels ANOVA bzw. ANOVA *on ranks* analysiert. Unterschiede zwischen den experimentellen Versuchsgruppen wurden mit Hilfe des Fischer LSD- oder Dunns-Test erfasst. Dabei wurde ein p-Wert $< 0{,}05$ als signifikant angenommen.

3. Ergebnisse

3.1 Publikation 1: Die Auswirkung von i.p. injiziertem CCK-8S auf die Konzentration von Serotonin in Nervenfasern des Nucleus tractus solitarii, Nucleus dorsalis nervi vagi, Nucleus arcuatus und Nucleus paraventricularis

Nach der intraperitonealen Injektion von CCK-8S in zwei verschiedenen Dosierungen (5,2 und 8,7 nmol/kg KG) kam es zu einem Dosis-abhängigen Anstieg der c-Fos Neurone im Bereich des NTS (5,2 nmol/kg CCK-8S: 72 ± 4 und 8,7 nmol/kg CCK-8S: 112 ± 5 Neurone/Hirnschnitt) verglichen mit der Vehikelgruppe (7 ± 2 Neurone/Hirnschnitt, $p < 0,001$). Im Bereich des DMV konnte dieser Effekt auf die neuronale Aktivierung nicht beobachtet werden (5,2 nmol/kg CCK-8S: 16 ± 4; 8,7 nmol/kg CCK-8S: 15 ± 3, Vehikelgruppe: 7 ± 1 Neurone/ Hirnschnitt, $p > 0,05$). Sowohl im NTS wie im DMV konnte eine Abnahme der Serotonin-Fluoreszenzintensität 90 Minuten nach i.p.-Injektion von CCK-8S beobachtet werden (5,2 nmol/kg CCK-8S: 46 ± 2 Pixel/Hirnschnitt; 8,7 nmol/kg CCK-8S: 49 ± 8 Pixel/Hirnschnitt; Vehikelgruppe: 81 ± 8 Pixel/Hirnschnitt, $p < 0,01$). Die Abnahme der Serotonin-Fluoreszenzintensität war nicht abhängig von der CCK-8S-Dosierung, entsprechend konnte kein statistisch signifikanter Unterschied zwischen den beiden experimentellen Gruppen beobachtet werden ($p = 0,81$). Die Doppelfärbung gegen c-Fos und Serotonin zeigte, dass c-Fos-positive Neurone in einem dichten Netzwerk von Serotonin-haltigen Nervenfasern eingebettet waren. Ergänzend ergab eine Dreifachimmunmarkierung gegen c-Fos, Serotonin und Tyrosinhydroxylase an Hirnschnitten des NTS, dass neuronal aktivierte Tyrosinhydroxylase-positive Neurone von Serotonin-positiven Nervenfasern umgeben waren.

Die Untersuchung von Hirnschnitten im PVN zeigte, dass es nach der i.p.- Injektion von CCK-8S zu einem dosisabhängigen Anstieg von c-Fos-positiven Neuronen in diesem hypothalamischen Gehirnkern kam (5,2 nmol/kg CCK-8S: 128 ± 14 Neurone/Hirnschnitt; 8,7 nmol/kg CCK-8S: 218 ± 15 Neurone/Hirnschnitt; Vehikelgruppe: 19 ± 5 Neurone/Hirnschnitt, $p < 0,01$). Die quantitative Analyse von Hirnschnitten aus dem Kerngebiet des ARC hingegen ergab keinen statistisch signifikanten Anstieg von c-Fos-Neuronen nach CCK-8S Injektion (5,2 nmol/kg CCK-8S: 5 ± 3 Neurone/Hirnschnitt; 8,7 nmol/kg CCK-8S: 8 ± 1 Neurone/Hirnschnitt; Vehikelgruppe: 7 ±1 Neurone/Hirnschnitt, $p > 0,05$). Hirnschnitte aus beiden Kerngebieten wiesen keine Veränderungen in der Serotonin-Fluoreszenzintensität nach i.p.-Injektion von CCK-8S auf (PVN: 5,2 nmol/kg CCK-8S: 16 ± 1 Pixel/Hirnschnitt; 8,7 nmol/kg CCK-8S: 15 ± 1 Pixel/Hirnschnitt;

ARC: 5,2 nmol/kg CCK-8S: 16 ± 2 Pixel/Hirnschnitt; 8,7 nmol/kg CCK-8S: 16 ± 1 Pixel/Hirnschnitt) im Vergleich zu der Vehikelgruppe (PVN: 14 ± 1 Pixel/Hirnschnitt, p = 0,389; ARC: 20 ± 1 Pixel/Hirnschnitt, p = 0,850).

3.2 Publikation 2: Peripher verabreichtes Bombesin induziert c-Fos in NUCB2/Nesfatin-1 Neuronen

Nach i.p.-Injektion von Bombesin in zwei verschiedenen Dosierungen (26 und 32 nmol/kg KG) konnte ein vier- bis siebenfacher Anstieg der Anzahl c-Fos-positiver Neurone im PVN (26 nmol/kg Bombesin: 70 ± 9; 32 nmol/kg Bombesin: 109 ± 8 Neurone/Hirnschnitt) verglichen mit der Vehikelgruppe (16 ± 4 Neurone/Hirnschnitt, $p < 0,05$) feststellt werden. Die Anzahl von NUCB2/Nesfatin-1 immunreaktiven Neuronen im PVN war unabhängig von der verwendeten Bombesin-Dosis. Allerdings zeigte die Doppelmarkierung gegen c-Fos und NUCB2/Nesfatin-1, dass sich zweifach positive Neurone im PVN nach der Bombesin-Injektion vervielfachten (26 nmol/kg Bombesin: 32 ± 3; 32 nmol/kg Bombesin: 57 ± 13 Neurone/Hirnschnitt) verglichen mit der Vehikel-Injektion (4 ± 0,5 Neurone/Hirnschnitt, $p < 0,05$). Die Anzahl von Oxytocin-positiven Neuronen änderte sich nach der Bombesin-Injektion unabhängig von der Dosierung nicht signifikant (26 nmol/kg Bombesin: 67 ± 19; 32 nmol/kg Bombesin: 63 ± 8 Neurone/Hirnschnitt und Vehikel: 51 ± 10 Neurone/Hirnschnitt, $p > 0,05$), jedoch zeigte sich auch hier, dass dreifach positive Neurone nach der Injektion von Bombesin zunahmen (26 nmol/kg Bombesin: 2,6 ± 1,3; 32 nmol/kg Bombesin: 9 ± 4 Neurone/Hirnschnitt), im Vergleich zur Vehikelgruppe (0,4 ± 0,2 Neurone/Hirnschnitt, $p < 0,05$).

Im NTS zeigte sich nach der Injektion von Bombesin ein dosisabhängiger, zehn- bis achtzehnfacher Anstieg von c-Fos-positiven Neuronen (26 nmol/kg Bombesin: 46 ± 1; 32 nmol/kg Bombesin: 91 ± 8 Neurone/Hirnschnitt und Vehikel: 5 ± 2 Neurone/Hirnschnitt, $p < 0,05$). Die Anzahl von NUCB2/Nesfatin-1 immunreaktiven Neuronen stieg ebenfalls statistisch signifikant nach der Injektion von Bombesin an (26 nmol/kg Bombesin: 105± 12 Neurone/Hirnschnitt; 32 nmol/kg Bombesin: 118 ± 1 Neurone/Hirnschnitt; Vehikel: 61 ± 4 Neurone/Hirnschnitt, $p < 0,05$). Weiterhin stieg die Anzahl von Neuronen, die sowohl für c-Fos, wie auch für NUCB2/Nesfatin-1 positiv waren, signifikant an (26 nmol/kg Bombesin: 17 ± 3 Neurone/Hirnschnitt und 32 nmol/kg Bombesin: 25 ± 3 Neurone/Hirnschnitt im Vergleich zu Vehikel: 1 ± 0,1 Neurone/Hirnschnitt, $p < 0,05$). Während sich die Anzahl von Tyrosinhydroxylase-positiven Neuronen nach Injektion von Bombesin in den Dosierungen 26 nmol/kg und 32 nmol/kg nicht signifikant änderte (26 nmol/kg Bombesin: 26 ± 5 Neurone/Hirnschnitt; 32 nmol/kg Bombesin: 25 ± 6 Neurone/Hirnschnitt und

Vehikel: 25 ± 4 Neurone/Hirnschnitt, $p > 0,05$), kam es zum Anstieg von dreifach positiven, c-Fos, NUCB2/Nesfatin-1 und Tyrosinhydroxylase Neuronen (26 nmol/kg Bombesin: 6,7 ± 0,9 Neurone/Hirnschnitt und 32 nmol/kg Bombesin: 8,9 ± 0,3 Neurone/Hirnschnitt verglichen mit Vehikel: 0,6 ± 0,2 Neurone/Hirnschnitt, $p < 0,05$).

Im Bereich des ARC ließ sich im Gegensatz zum PVN und NTS keine statistisch signifikante Zunahme in der Anzahl c-Fos-positiver Neurone feststellen (26 nmol/kg Bombesin: 17 ± 2 Neurone/Hirnschnitt; 32 nmol/kg Bombesin: 12 ± 1 Neurone/Hirnschnitt; Vehikel: 14 ± 2 Neurone/Hirnschnitt, $p > 0,05$).

3.3 Publikation 3: Cholecystokinin und Bombesin aktivieren Neuronatin-Neurone im Nucleus tractus solitarii des Hirnstammes

Die Injektion von CCK-8S in zwei verschiedenen Dosierungen (5,2 und 8,7 nmol/kg KG) führte zu einer statistisch signifikanten Zunahme in der Anzahl der c-Fos-positiven Neurone im NTS (30 ± 15 und 62 ± 14 Neurone/Hirnschnitt) im Vergleich zur Vehikelgruppe (1,2 ± 0,9 Neurone/Hirnschnitt, $p = 0,025$ und $p = 0,001$). Ebenso verhielt es sich mit i.p. verabreichtem Bombesin in den Dosierungen 26 nmol/kg und 32 nmol/kg KG. Beide Dosen führten zu einer starken Zunahme von c-Fos-positiven Neuronen (26 nmol/kg Bombesin: 38 ± 8 Neurone/Hirnschnitt; 32 nmol/kg Bombesin: 71 ± 8 Neurone/Hirnschnitt) verglichen mit der Vehikelgruppe ((1,2 ± 0,8 Neurone/Hirnschnitt, $p = 0,007$ und $p = 0,001$). Die Zunahme der neuronalen c-Fos-Aktivierung war sowohl im Falle von CCK-8S wie auch im Falle von Bombesin signifikant dosisabhängig (5,2 nmol/kg CCK-8S: 30 ± 15 Neurone/Hirnschnitt; 8,7 nmol/kg CCK-8S: 62 ± 14 Neurone/Hirnschnitt; 26 nmol/kg Bombesin: 38 ± 8 Neurone/Hirnschnitt; 32 nmol/kg Bombesin 71 ± 8 Neurone/Hirnschnitt, $p = 0,027$ und $p = 0,023$). Im DMV hingegen löste weder die Injektion von CCK-8S (5,2 nmol/kg CCK-8S: 0,13 ± 0,13 Neurone/Hirnschnitt; 8,7 nmol/kg CCK-8S 0,63 ± 0,63 Neurone/Hirnschnitt) noch die Gabe von Bombesin (26 nmol/kg Bombesin: 2,2 ± 1,3 Neurone/Hirnschnitt; 32 nmol/kg Bombesin: 2,01 ± 0,5 Neurone/Hirnschnitt) eine neuronale c-Fos-Aktivierung aus im Vergleich zu der Behandlung mit Vehikellösung (0 ± 0 Neurone/Hirnschnitt, $p > 0,05$).

Unabhängig von der experimentellen Gruppenzuordnung konnte sowohl im Bereich des NTS wie auch im Bereich des DMV eine Anzahl Neuronatin-positiver Neurone detektiert werden. Interessanterweise wurden nicht nur die Nervenzellkörper durch den Neuronatin Antikörper dargestellt, sondern auch Nervenfaser- und intrazelluläre Vesikelstrukturen. Im Vergleich zur Kontrollgruppe (62 ± 5 Neurone/Hirnschnitt) stieg die Anzahl Neuronatin-positiver Neurone nach

Injektion von CCK-8S (5,2 nmol/kg CCK-8S: 69 ± 5 und 8,7 nmol/kg CCK-8S: 69 ± 10 Neurone/Hirnschnitt, $p > 0,05$) oder Bombesin (26 nmol/kg Bombesin: 71 ± 4 und 32 nmol/kg Bombesin: 58 ± 3 Neurone/Hirnschnitt, $p > 0,05$) nicht signifikant an. Gleiches galt für den DMV (Vehikel: 17 ± 1 Neurone/Hirnschnitt, 5,2 nmol/kg CCK-8S 17 ± 3; 8,7 nmol/kg CCK-8S 19 ± 3 Neurone/Hirnschnitt, 26 nmol/kg Bombesin 27 ± 12; 32 nmol/kg Bombesin 18 ± 1 Neurone/Hirnschnitt, $p > 0.05$).

Die Doppelmarkierung gegen c-Fos und Neuronatin zeigte eine Zunahme von neuronal aktivierten Neuronatin-positiven Neuronen nach Injektion von CCK-8S (5,2 nmol/kg CCK-8S: 19 ± 9 und 8,7 nmol/kg CCK-8S: 38 ± 7 Neurone/Hirnschnitt) im Gegensatz zur Injektion von Vehikellösung (0,8 ± 0,6 Neurone/Hirnschnitt, $p = 0,011$ und $p = 0,001$). Auch Bombesin bewirkte eine deutliche Zunahme der zweifach positiven c-Fos / Neuronatin-Neurone (26 nmol/kg Bombesin: 25 ± 4 und 32 nmol/kg Bombesin: 45 ± 3 Neurone/Hirnschnitt) verglichen mit der Kontrollgruppe (0,8 ± 0,6 Neurone/Hirnschnitt, $p = 0,02$ und $p = 0,01$). Es fanden sich signifikante Unterschiede zwischen den Behandlungsgruppen mit hoher und niedriger CCK-8S Dosierung (8,7 nmol/kg CCK-8S: 38 ± 7 und 5,2 nmol/kg CCK-8S: 19 ± 9 Neurone/Hirnschnitt, $p < 0,017$) und hoher und niedriger Bombesin-Dosierung (32 nmol/kg Bombesin: 45 ± 3 und 26 nmol/kg Bombesin: 25 ± 4 Neurone/Hirnschnitt, $p < 0,013$).

In allen experimentellen Gruppen fanden sich Tyrosinhydroxylase-positive Neurone im NTS, während im DMV, unabhängig von der Behandlung, keine Tyrosinhydroxylase-haltigen Neurone nachgewiesen werden konnten.

Die Anzahl von Tyrosinhydroxylase-haltigen Neuronen wurde durch CCK-8S (5,2 nmol/kg CCK-8S: 20 ± 3 und 8,7 nmol/kg CCK-8S: 18 ± 3 Neurone/Hirnschnitt) oder Bombesin (26 nmol/kg Bombesin: 19 ± 3 und 32 nmol/kg Bombesin: 24 ± 8 Neurone/Hirnschnitt) im Vergleich zur Vehikelgruppe (10 ± 3 Neurone/Hirnschnitt, $p > 0,05$) nicht beeinflusst. Tyrosinhydroxylase-positive Neurone, die gleichzeitig auch für c-Fos-positiv waren, nahmen in ihrer Anzahl sowohl nach Verabreichung von CCK-8S (5,2 nmol/kg CCK-8S: 10 ± 6 und 8,7 nmol/kg CCK-8S: 11 ± 3 Neurone/Hirnschnitt) wie auch nach Injektion von Bombesin (26 nmol/kg Bombesin: 9 ±2 und 32 nmol/kg Bombesin: 12 ± 4 Neurone/Hirnschnitt) zu, verglichen mit der Kontrollgruppe (0,2 ± 0,1 Neurone/Hirnschnitt, $p < 0,05$). Teilweise konnten Neurone identifiziert werden, die sowohl Tyrosinhydroxylase- wie auch Neuronatin-positiv waren. Die Anzahl dieser Neurone wurde jedoch weder durch CCK-8S (5,2 nmol/kg CCK-8S: 19 ± 4 und 8,7 nmol/kg CCK-8S: 16 ± 2 Neurone/Hirnschnitt) noch durch Bombesin (26 nmol/kg Bombesin: 18 ± 4 und 32 nmol/kg Bombesin: 22 ± 8 Neurone/Hirnschnitt) im Vergleich zu den Vehikeltieren (9 ± 2 Neurone/Hirnschnitt, $p > 0,05$) signifikant verändert.

Durch die Dreifachmarkierung gegen c-Fos, Neuronatin und Tyrosinhydroxylase konnte eine Erhöhung dreifach positiver Neurone nach Injektion von Bombesin ohne dosisabhängigen Effekt (26 nmol/kg Bombesin: 9 ± 2 und 32 nmol/kg Bombesin: 11 ± 4 Neurone/Hirnschnitt) nachgewiesen werden, im Vergleich zur Vehikelgruppe (0,2 ± 0,1 Neurone/Hirnschnitt, $p = 0,09$ und $p = 0,001$). Nach Gabe von CCK-8S fand sich kein statistischer Effekt hinsichtlich der Zunahme dreifach positiver Neurone im Vergleich zu der Vehikelgruppe (5,2 nmol/kg CCK-8S: 5 ± 3 und 8,7 nmol/kg CCK-8S: 5 ± 1 versus 0,2 ± 0,1 Neurone/Hirnschnitt, $p > 0,05$).

4. Diskussion

In der ersten Studie konnten wir zeigen, dass i.p. injiziertes CCK-8S eine Zunahme von c-Fos-positiven Neuronen in den Kerngebieten NTS und PVN bewirkt. Zusätzlich fand sich eine Abnahme der Serotonin-Fluoreszenzintensität in den Kerngebieten DMV und NTS des Hirnstammes, während die Serotonin-Fluoreszenzaktivität im PVN und ARC des Hypothalamus nach CCK-Injektion unverändert blieb [60]. Die CCK-abhängige Zunahme von c-Fos Neuronen im NTS und PVN konnte in der Vergangenheit oft nachgewiesen werden und wird durch die im Rahmen der vorliegenden Arbeit erhobenen Daten erneut bestätigt [60], [61], [62].

Wie bereits ausgeführt, werden nach Freisetzung von CCK über CCK1-Rezeptoren vagale Afferenzen aktiviert, welche zu NTS-Neuronen des Hirnstammes projizieren, von wo eine weitere neuronale Verschaltung unter anderem zum PVN des Hypothalamus erfolgt [62], [63]. Wir gehen davon aus, dass die in unserer tierexperimentellen Studie beobachtete Abnahme der Serotonin-Fluoreszenzintensität im NTS und DMV auf eine lokale Freisetzung des Neurotransmitters Serotonin zurückzuführen ist [60]. Vermutlich wurde der Neurotransmitter durch die vorgenommene transkardiale Perfusion ausgewaschen [60]. Andere Arbeitsgruppen wiesen nach, dass der NTS serotonerg von der Area postrema (AP), den Raphe-Kernen und dem Nervus vagus versorgt wird [64], [65], [66], [67]. Calza et al. haben beobachtet, dass Serotonin auch in Neuronen des NTS vorkommt [68].

Ultrastrukturelle Analysen ergaben, dass Serotonin lokal aus NTS-Neuronen freigesetzt werden kann [68]. Die i.p.-Gabe von CCK-8S induziert eine neuronale Aktivierung von NTS-Neuronen und bewirkt gleichzeitig eine Abnahme der Serotonin-Fluoreszenzintensität. Vermutlich kann CCK über den Nervus vagus Neurone im Hirnstamm aktivieren, welche Serotonin freisetzen [60]. Im Unterschied zum NTS konnten wir im direkt benachbarten DMV nach i.p.-Injektion von CCK-8S keinen Anstieg c-Fos-positiver Neurone detektieren [60]. Die fehlende neuronale Aktivierbarkeit des DMV durch CCK in unserer Untersuchung wird durch bereits publizierte Daten unterstützt [69], [70]. Allerdings konnten wir im DMV eine Abnahme der Serotonin-Fluoreszenzintensität nach i.p. verabreichtem CCK-8S beobachten [60]. Aufgrund der direkten anatomischen Nachbarschaft beider Kerngebiete ist es wahrscheinlich, dass Serotonin-haltige Nervenfasern von NTS-Neuronen durch den DMV ziehen und bei neuronaler Aktivierung von NTS-Neuronen im DMV Serotonin-haltige Vesikel aus diesen Nervenfasern entleert werden [60]. Bekannt ist, dass Serotonin nicht nur am synaptischen Endknöpfchen freigesetzt werden kann,

sondern auch entlang der Axone aus sogenannten „*dense-core*“ Vesikeln ausgeschüttet wird [71]. Ob sich die Abnahme der Serotonin-Fluoreszenzintensität im DMV bei gleichzeitig fehlender c-Fos-Aktivierung tatsächlich dadurch erklären lässt, dass Fortsätze von NTS-Neuronen durch den DMV ziehen, muss in zukünftigen Studien untersucht werden. Ein möglicher Ansatzpunkt wäre hier die *in vivo* Applikation eines geeigneten neuronalen Tracers.
Interessanterweise fanden wir im NTS Tyrosinhydroxylase-haltige Neurone, welche von einem dichten Netzwerk Serotonin-haltiger Nervenfasern umgeben waren [60]. Lam et al. beobachteten, dass der Serotoninrezeptor-Agonist meta-Chlorphenylpiperazin Tyrosin-haltige Neurone im NTS zu aktivieren vermag [72]. Vor diesem Hintergrund ist es möglich, dass auch peripheres CCK-8S via vagale Afferenzen zu einer Freisetzung von Serotonin im NTS des Hirnstamms und dort zu einer Rekrutierung katecholaminhaltiger Zellgruppen führt, mit nachfolgender Verschaltung zu Kerngebieten des Hypothalamus [60].

In der Gesamtheit sprechen die Ergebnisse der ersten Studie dafür, dass die bekannte anorexigene Wirkung von peripherem CCK-8S im NTS u.a. über das Serotonin-System vermittelt wird. Bisher publizierte Daten anderer Forschungsgruppen unterstützen diese Hypothese [25], [73], [74], [75], [76], [29], [28], [27]. Allerdings muss erwähnt werden, dass vereinzelt Ergebnisse vorliegen, die keinen synergistischen Effekt von CCK und Serotonin nachweisen [77].

Die zweite Arbeit zeigt einen signifikanten Anstieg der Anzahl c-Fos-positiver Neurone im NTS und PVN nach i.p.-Gabe von Bombesin bei gleichzeitiger und signifikanter statistischer Zunahme von NUCB2/Nesfatin-1 immunreaktiven Neuronen im NTS. Wir wiesen außerdem eine erhöhte Anzahl von Neuronen mit gleichzeitigem Nachweis von c-Fos und NUCB2/Neuronatin-1 Immunreaktivität in den Kerngebieten NTS und PVN nach peripherer Verabreichung von Bombesin nach, sodass wir von einer signifikanten Aktivierung von NUCB2/Nesfatin-1 Neuronen im NTS und PVN ausgehen. Bemerkenswerterweise waren die aktivierten NUCB2/Neuronatin-1 Neurone im NTS teilweise mit Tyrosinhydroxylase und im PVN mit Oxytocin kolokalisiert [78].

Sowohl Bombesin wie auch NUCB2/Nesfatin-1 sind gastrointestinal [35] und zentral [33] vorkommend, entfalten ihre Wirkung zum Teil Vagus-vermittelt, hemmen die Nahrungsaufnahme und verändern das Mahlzeitenmuster [36], [34]. Beide Mediatoren aktivieren darüber hinaus die Stressachse im Gehirn [79], [80] und beeinflussen die Kreislaufregulation [81]. Wie einleitend erwähnt, interagieren Bombesin und NUCB2/Nesfatin-1 mit dem CCK-System [46], [82], [83]. In der zweiten Arbeit haben wir eine direkte Interaktion von Bombesin und Nesfatin-1 auf Basis der

neuronalen Aktivierbarkeit von NUCB2/Nesfatin-1 Neuronen in Abhängigkeit von der peripheren Bombesin-Gabe überprüft [78]. Wir haben zunächst den NTS als Kerngebiet untersucht, da bereits zuvor demonstriert wurde, dass der NTS eine wichtige Funktion bei der Integration zentraler Bombesin-vermittelter Effekte einnimmt [33], [37]. Gezielte Läsionen der AP oder des NTS führten zu einer Aufhebung des hemmenden Bombesin-Effektes auf die Nahrungsaufnahme [36]. In Einklang mit diesen Studienergebnissen wiesen wir eine neuronale Aktivierung von NTS-Neuronen durch peripher injiziertes Bombesin nach. Der Erkenntnisgewinn liegt hier zusätzlich in der Charakterisierung der aktivierten NTS-Neurone als NUCB2/Nesfatin-immunreaktiv. Interessanterweise fanden wir im NTS auch eine neuronale Aktivierung von NUCB2/Nesfatin-1 immunreaktiven Neuronen in Kolokalisation mit dem Enzym Tyrosinhydroxylase [78]. Bereits zuvor konnten Noetzel et al. zeigen, dass NUCB2/Nesfatin-1 Neurone von einem dichten Netzwerk katecholaminerger Nervenfasern umgeben sind [46]. In Tracing-Studien wurde nachgewiesen, dass Projektionen von katecholaminergen Neuronen des NTS zum PVN entsendet werden [84]. Im PVN kann eine besonders hohe neuronale Aktivierung durch NUCB2/Nesfatin-1 Gabe erzielt werden [85]. Nesfatinerge Signalwege im Hypothalamus könnten bedeutend an der Weitervermittlung peripherer, z.B. Bombesin-assoziierter Sättigungs-Impulse beteiligt sein [78]. Interessanterweise stellten wir eine neuronale Aktivierung von NUCB2/Nesfatin-1 immunreaktiven Neuronen in Kolokalisation mit Oxytocin im PVN fest [78]. Verschiedene Arbeitsgruppen fanden in der Vergangenheit bereits Hinweise auf eine Aktivierung des Oxytocin-Systems durch NUCB2/Nesfatin-1 [86], [87]. Kirchgessner et al. konnten zeigen, dass Oxytocin-haltige Neurone im PVN von katecholaminergen Projektionen der A2-Zellgruppe des Hirnstammes innerviert werden [88]. Zusammenfassend gehen wir davon aus, dass die zentrale Signalweiterleitung von peripher appliziertem Bombesin über NUCB2/Nesfatin-1 vermittelt werden könnte [78]. Im NTS scheint NUCB2/Nesfatin-1 katecholaminerge Zellgruppen zu aktivieren, während im PVN Oxytocin-haltige Zellgruppen angesprochen werden [89], [90]. Im Widerspruch hierzu konnten z.B. Plamondon et al. keinen Hinweis auf die Vermittlung von Bombesin-Effekten über Oxytocin-Signalwege finden [91], [92]. Es bleibt anzumerken, dass wir in unseren Experimenten zwar auf zwei verschiede Dosierungen von Bombesin zurückgriffen (26 und 32 nmol/kg), aber keine dosisabhängige Auswirkung auf die neuronale Aktivierung (c-Fos) im PVN demonstrieren konnten. Uns gelang lediglich der Nachweis gradueller Effekte [78]. Bei der Auswahl der Dosierungen von Bombesin haben wir uns auf veröffentlichte Daten anderer Arbeitsgruppen gestützt [58], [59]. Wären die Dosierungen mit deutlicherem Abstand voneinander gewählt worden, hätten wir statt gradueller Effekte eventuell eine spezifische neuronale

Aktivierung von NUCB2/Nesfatin-1 immunreaktiven Neuronen durch Bombesin im PVN nachweisen können [78].

In den beiden bereits diskutierten Untersuchungen unserer Arbeitsgruppe konnten wir weitreichende Überschneidungen der anorexigen wirksamen Mediatoren feststellen. Interessant wäre es, darüber hinaus eine gemeinsame Endstrecke des physiologischen Wirkens zu detektieren. Der zugrundeliegende zelluläre Mechanismus der Terminierung der Nahrungsaufnahme ist bisher unbekannt. Sofern der Mechanismus identifiziert werden kann, stellt dieser einen vielversprechenden Ansatz einer pharmakologischen Intervention z.B. bei Adipositas dar.

Einen Anhaltspunkt für die mögliche gemeinsame Endstrecke fanden wir in der dritten Arbeit. Hierzu haben wir die bereits zuvor untersuchten Peptide CCK und Bombesin erneut peripher verabreicht und ihre Auswirkungen auf das Protein Neuronatin untersucht. Wir fanden nach i.p.-Gabe von CCK / Bombesin eine neuronale Aktivierung von Neuronatin-Neuronen im NTS [93]. Zusätzlich wurden Tyrosinhydroxylase-positive Neurone durch CCK und Bombesin aktiviert. Darüber hinaus zeigte sich auch eine dosisabhängige neuronale Aktivierung von Neuronen mit Kolokalisation von Neuronatin und Tyrosinhydroxylase nach Verabreichung von Bombesin [93]. Im Vergleich zum NTS konnten die genannten Effekte im DMV nicht nachgewiesen werden [93].

Wie zuvor ausgeführt, stellt der NTS einen Knotenpunkt in der Vermittlung anorexigener Impulse aus dem gastrointestinalen Trakt dar. Über das zentrale Aktivitätsmuster von Neuronatin-Neuronen im Bereich des NTS als Reaktion auf periphere Sättigungssignale ist bisher in der Literatur wenig bekannt. Unter anderem konnte gezeigt werden, dass die periphere Gabe des Sättigungshormons Leptin Einfluss auf die mRNA-Expression von Neuronatin hat [53]. Auch eine Kolokalisation mit Neuropeptiden der Appetitregulation wurde für Neuronatin nachgewiesen [53]. Die von uns dargestellte neuronale Aktivierung von Neuronatin-Neuronen im NTS als Antwort auf CCK und Bombesin deutet auf eine weiterführende physiologische Rolle von Neuronatin in der Hunger-Sättigungsregulation hin [93]. Eingangs wurde bereits erwähnt, dass Neuronatin auf zellulärer Ebene in bedeutende regulierende Prozesse (neuronale Differenzierung, Stoffwechselregulation) eingreift [49, 50] [51]. Durch eine Antagonisierung der Calcium-ATPase im Endoplasmatischen Retikulum hebt Neuronatin die intrazelluläre Calciumkonzentration an, was zu einer Phosphorylierung und Aktivierung bestimmter Kinasen führt [49]. Über eine Aktivierung der *extracellular regulated kinase* (ERK) als Transkriptionsfaktor trägt Neuronatin via Freisetzung von spezifischen Botenstoffen zur zellulären Differenzierung, Embryogenese und

Apoptose bei [49]. Auch exogen zugeführtes CCK aktiviert den ERK-Signalweg im NTS [94]. Babic et al. zeigten, dass eine Blockade dieses Signalwegs den Nahrungsaufnahme-hemmenden Effekt von CCK vermindert [95]. Neuronatin könnte die Wirkung von CCK auf zelluläre Mechanismen vermitteln, eventuell gilt Gleiches auch für Bombesin. [93]. Zuvor wurde bereits erwähnt, dass peripher verabreichtes Bombesin zumindest teilweise über die Ausschüttung von CCK wirkt [38], auf diesem Wege könnte indirekt auch eine neuronale Aktivierung auf Hirnstammebene bewirkt werden. Allerdings scheint Bombesin auch CCK-unabhängig die Nahrungsaufnahme zu beeinflussen. Experimentell führt eine Vagotomie im Falle von Bombesin im Gegensatz zu CCK nicht zu einer Aufhebung des Nahrungsaufnahme-modulierenden Effekts, zudem wirkt Bombesin stärker auf die Aufnahme fester Nahrung als CCK [10], [96]. Es kann demnach postuliert werden, dass Bombesin im NTS neben der neuronalen Neuronatin-Aktivierung einen weiteren wichtigen Prozess anstößt. Gegebenenfalls ist der in der zweiten Arbeit detektierte Bombesin-aktivierte NUCB2/Nesfatin-1 Signalweg hier zwischengeschaltet. NUCB2/Nesfatin-1 wiederum scheint auf Ebene des Hypothalamus eine Aktvierung des ERK-Signalwegs auszulösen [97]. Durch Einsatz von Morpholino-Oligomeren (Nukleinsäure-Analoga) gegen NUCB2 wurde eine Herabregulation von ERK im Hypothalamus erreicht, welche mit einer gesteigerten Nahrungsaufnahme einherging [97]. Vor diesem Hintergrund wäre interessant zu untersuchen, ob Neuronatin an der Vermittlung dieses Prozesses mitbeteiligt ist.

Wie beschrieben, leiten katecholaminerge Neurone die im NTS ankommenden Impulse aus dem gastrointestinalen Trakt an den Hypothalamus weiter [98], [15]. Tyrosinhydroxylase ist ein Enzym, welches den geschwindigkeitsbestimmenden Schritt in der Synthese von Katecholaminen katalysiert. Babic und Kollegen konnten nachweisen, dass die CCK-induzierte ERK-Phosphorylierung vor allem Tyrosinhydroxylase-haltige Neurone im NTS betrifft und dass die hierdurch bewirkte Aktivierung von Tyrosinhydroxylase-Neuronen wesentlich zu dem Nahrungsaufnahme-hemmenden Effekt von CCK beiträgt [94].

Zusammenfassend konnten wir feststellen, dass peripher verabreichtes CCK-8S und Bombesin eine c-Fos-Aktivierung von Neuronatin-Nervenzellen des NTS auslösen. Bombesin könnte diese Aktivierung auch indirekt, über eine Ausschüttung von CCK, bewirken. Neuronatin scheint an der Aktivierung bestimmter Kinasen beteiligt zu sein, welche auf externe Faktoren reagieren und auf zellulärer Ebene unterschiedliche regulierende Prozesse anstoßen. Gegebenenfalls könnten Bombesin und CCK Neuronatin vermittelt, im NTS eine Aktivierung des

Katecholaminstoffwechsels induzieren, mit nachfolgender katecholaminerger Weiterleitung des peripheren Sättigungsimpulses an den Hypothalamus.

Ebenfalls denkbar wäre ein zweiter, NUCB2/Nesfatin-1 abhängiger Mechanismus der neuronalen Verschaltung zwischen NTS und Hypothalamus, welcher direkt durch Bombesin aktiviert wird. Wie oben ausgeführt, scheint Bombesin, zumindest teilweise, CCK-unabhängig eine kurzfristige Sättigung zu induzieren. Es wäre interessant zu untersuchen, ob NUCB2/Nesfatin-1 im Hirnstamm als Mediator dieses Bombesin-vermittelten, CCK-unabhängigen Sättigungssignals an den Hypothalamus fungiert.

Im NTS ist möglicherweise eine CCK-abhängige Serotoninaktivierung der Neuronatin-Aktivierung vorgeschaltet. Die im Kontext dieser Arbeit durchgeführten Experimente erbrachten sowohl Hinweise für eine Aktivierung des serotonergen Systems, wie auch des Neuronatin-Systems auf Hirnstammebene durch CCK. Vorstellbar wäre jedoch auch eine Verstärkung des CCK-induzierten Sättigungsimpulses durch eine redundante Organisation der Signalkaskade, Serotonin und Neuronatin könnten im NTS nach Aktivierung durch CCK unabhängig voneinander den Katecholaminstoffwechsel im Hirnstamm anregen.

In zukünftigen Studien könnte die Aktivierbarkeit wichtiger Mediatoren wie Serotonin, Neuronatin und NUCB2/Fesfatin-1 durch CCK bzw. Bombesin auf mRNA-Ebene näher untersucht werden. Zudem bedarf es weiterer Forschung auf zellulärer Ebene, sowie Folgestudien, die die gezielte Antagonisierung einzelner Mediatoren nutzen, um die komplexe Mikrostruktur der Nahrungsaufnahme weiter offenzulegen.

5. Literaturverzeichnis

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Eidesstattliche Versicherung

Ich, Kim-Marie Engster, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: „Phänotypische Charakterisierung von neuronal aktivierten Neuronen im Hirnstamm und Hypothalamus nach peripherer Injektion von CCK-8S oder Bombesin“ selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.
Alle Stellen, die wörtlich oder dem Sinne nach auf Publikationen oder Vorträgen anderer Autoren beruhen, sind als solche in korrekter Zitierung kenntlich gemacht. Die Abschnitte zu Methodik (insbesondere praktische Arbeiten, Laborbestimmungen, statistische Aufarbeitung) und Resultaten (insbesondere Abbildungen, Graphiken und Tabellen) werden von mir verantwortet.
Ich versichere ferner, dass ich die in Zusammenarbeit mit anderen Personen generierten Daten, Datenauswertungen und Schlussfolgerungen korrekt gekennzeichnet und meinen eigenen Beitrag sowie die Beiträge anderer Personen korrekt kenntlich gemacht habe (siehe Anteilserklärung). Texte oder Textteile, die gemeinsam mit anderen erstellt oder verwendet wurden, habe ich korrekt kenntlich gemacht.
Meine Anteile an etwaigen Publikationen zu dieser Dissertation entsprechen denen, die in der untenstehenden gemeinsamen Erklärung mit dem Erstbetreuer angegeben sind. Für sämtliche im Rahmen der Dissertation entstandenen Publikationen wurden die Richtlinien des ICMJE (International Committee of Medical Journal Editors; www.icmje.og) zur Autorenschaft eingehalten. Ich erkläre ferner, dass ich mich zur Einhaltung der Satzung der Charité – Universitätsmedizin Berlin zur Sicherung Guter Wissenschaftlicher Praxis verpflichte.

Weiterhin versichere ich, dass ich diese Dissertation weder in gleicher noch in ähnlicher Form bereits an einer anderen Fakultät eingereicht habe.

Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§156, 161 des Strafgesetzbuches) sind mir bekannt und bewusst.

Datum Unterschrift

Anteilserklärung an erfolgten Publikationen

Kim Marie Engster hatte folgenden Anteil an der folgenden Publikation:

Engster KM, Frommelt L, Hofmann T, Nolte S, Fischer F, Rose M, Stengel A, Kobelt P. Peripheral injected cholecystokinin-8S modulates the concentration of serotonin in nerve fibers of the rat brainstem. Peptides, 2014. 59: p. 25-33.

Beitrag im Einzelnen:
Durchführung aller Tierexperimente und Laborarbeiten, Mikroskopie und statistische Auswertung der Rohdaten, Erstellen aller Fotos und Abbildungen, Verfassen des Manuskripts und der Revisionen inklusive der „Point - by - Point response“ für die Gutachter.

Engster KM, Kroczek AL, Rose M, Stengel A, Kobelt P. Peripheral injection of bombesin induces c-Fos in NUCB2/nesfatin-1 neurons. Brain Res, 2016. 1648(Pt A): p. 46-53.

Beitrag im Einzelnen:
Durchführung aller Tierexperimente und Laborarbeiten, Mikroskopie, statistische Auswerten der Rohdaten und Erstellen aller Fotos und Abbildungen sowie Verfassen des Manuskripts und der Revisionen inklusive der „Point - by - Point response“ für die Gutachter.

Guggenberger M, **Engster KM**, Hofmann T, Rose M, Stengel A, Kobelt P. Cholecystokinin and bombesin activate neuronatin neurons in the nucleus of the solitary tract. Brain Res, 2020. 1746: p. 147006.

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Peripheral injected cholecystokinin-8S modulates the concentration of serotonin in nerve fibers of the rat brainstem

Kim-Marie Engster, Lisa Frommelt, Tobias Hofmann, Sandra Nolte, Felix Fischer, Matthias Rose, Andreas Stengel, Peter Kobelt*

Medical Clinic, Department of Psychosomatic Medicine, Charité – Universitätsmedizin Berlin, Germany

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ABSTRACT

Serotonin and cholecystokinin (CCK) play a role in the short-term inhibition of food intake. It is known that peripheral injection of CCK increases c-Fos-immunoreactivity (Fos-IR) in the nucleus of the solitary tract (NTS) in rats, and injection of the serotonin antagonist ondansetron decreases the number of c-Fos-IR cells in the NTS. This supports the idea of serotonin contributing to the effects of CCK. The aim of the present study was to elucidate whether peripherally injected CCK-8S modulates the concentration of serotonin in brain feeding-regulatory nuclei. Ad libitum fed male Sprague-Dawley rats received 5.2 and 8.7 nmol/kg CCK-8S ($n = 3$/group) or 0.15 M NaCl ($n = 3$–5/group) injected intraperitoneally (ip). The number of c-Fos-IR neurons, and the fluorescence intensity of serotonin in nerve fibers were assessed in the paraventricular nucleus (PVN), arcuate nucleus (ARC), NTS and dorsal motor nucleus of the vagus (DMV). CCK-8S increased the number of c-Fos-ir neurons in the NTS (mean ± SEM: 72 ± 4, and 112 ± 5 neurons/section, respectively) compared to vehicle-treated rats (7 ± 2 neurons/section, $P < 0.05$), but did not modulate c-Fos expression in the DMV or ARC. Additionally, CCK-8S dose-dependently increased the number of c-Fos-positive neurons in the PVN (218 ± 15 and 128 ± 14, respectively vs. 19 ± 5, $P < 0.05$). In the NTS and DMV we observed a decrease of serotonin-immunoreactivity 90 min after injection of CCK-8S (46 ± 2 and 49 ± 8 pixel/section, respectively) compared to vehicle (81 ± 8 pixel/section, $P < 0.05$). No changes of serotonin-immunoreactivity were observed in the PVN and ARC. Our results suggest that serotonin is involved in the mediation of CCK-8's effects in the brainstem.

Introduction

Hunger and satiety, food intake and body weight are regulated by various hormonal and neural signals [51]. Cholecystokinin (CCK) is synthesized by a population of endocrine cells in the small intestine [27], and the release of the peptide is stimulated by food intake [16,30]. Gibbs et al. were the first researchers who demonstrated that CCK terminates the intake of solid and fluid food [11,12]. The endogenous form of CCK, i.e. CCK-58, decreases food intake without a compensatory reduction of the subsequent inter-meal interval [14], while after intraperitoneal (ip) injection of the most studied form, the fragment CCK-8, shortens the inter-meal interval indicating an effect of CCK-8 on satiation only [34]. CCK is likely to reduce food intake via its CCK_1 receptors localized on afferent vagal nerve terminals localized in the gastrointestinal mucosa [44,50]. The vagus nerve relays signals to the hindbrain, especially to the nucleus of the solitary tract (NTS), where noradrenergic projections from the A2-cell group are activated [43]. Consecutively, both catecholaminergic and non-catecholaminergic neurons of the NTS project to several hypothalamic brain areas [42,46,47].

The mapping of brain neuronal circuits recruited by hormone signals has been widely obtained by assessing changes of the proto-oncogene c-Fos, which allows identification of activated neurons at the cellular level [45]. Several studies have revealed that peripherally injected CCK induces c-Fos-immunoreactivity (c-Fos-IR) in different autonomic brain nuclei, namely in the paraventricular nucleus of the hypothalamus (PVN), locus coeruleus (LC), nucleus of the solitary tract (NTS), and the area postrema (AP), indicating that a complex neural network in the brain is activated by CCK [4,7,29,33,43].

The neurotransmitter serotonin could be involved in the mediation of CCK's brainstem effects as it shows similarities with the activation pattern of CCK. Serotonin is produced centrally in the

* Corresponding author at: Department of Medicine, Division Psychosomatic Medicine and Psychotherapy, Charité – Universitätsmedizin Berlin, Campus Mitte, Sauerbruchweg 5, 10117 Berlin, Germany. Tel.: +49 30 450 559739; fax: +49 30 450 559939.
E-mail address: peter.kobelt@charite.de (P. Kobelt).

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0196-9781/

raphe nuclei of the brainstem and peripherally in enterochromaffin cells scattered in the small intestinal and colonic mucosa [10,22]. As described for CCK, the peripheral release of serotonin is stimulated by the presence of food [49]. Serotonin binds to receptor terminals of vagal afferent fibers localized in the mucosa of the gastrointestinal tract [35] and delays gastric emptying [41]. As established for CCK, serotonin is known to reduce food intake [38]. High densities of the serotonin receptor were found on neurons in the NTS, AP and the dorsal motor nucleus of the vagus (DMV) [35].

Taken together, CCK and serotonin not only have similar effects on food intake but also both act via the vagus nerve and partly influence the same brain nuclei. Previous investigations have therefore examined a possible interaction between CCK and serotonin. Hayes et al. observed an increased intake of sucrose solution in rats after microinjection of the serotonin antagonist ondansetron into the medial NTS [18]. Interestingly, they also showed a reduced intake of sucrose after injection of CCK, an effect that was blunted by the serotonin type-3 (5-HT3) receptor antagonist, ondansetron [18]. Moreover, Helm et al. injected CCK and serotonin unilaterally into the PVN and observed a significant inhibition of food intake in the early dark period which was associated with a greater increase of dopamine than injection of each transmitter alone [20]. According to the results given above, both the NTS and the PVN are likely to play a role in the combined mediation of cholecytokinergic and serotonergic effects on food intake inhibition. However, more detailed neurophysiological studies are needed to corroborate this hypothesis.

The aim of the present study was to investigate whether peripherally injected CCK modulates the concentration of serotonin in nerve fibers of the NTS, DMV, arcuate nucleus (ARC) and the PVN. CCK-8S injection is associated with a well-established distinct neuronal activation pattern in the PVN and NTS. Consequently, we also investigated the influence of CCK injected ip on the density of c-Fos neurons in the PVN and NTS, as well as DMV and ARC which served as control nuclei. In addition, serotonin-immunoreactivity in the NTS was determined to assess the distribution of serotonin nerve fibers in relation to activated neurons colocalized with tyrosine hydroxylase a marker for catecholaminergic cells.

Materials and methods

Animals

Male Sprague-Dawley rats (Harlan-Winkelmann Co., Borchen, Germany) weighing 250–300 g were housed under conditions of controlled illumination (12:12 h light/dark cycle, lights switched on at 6 a.m.), humidity, and temperature (22 ± 2 °C) for at least 21 days prior to the experiments. Animals were fed with a standard rat diet (Altromin®, Lage, Germany) and tap water ad libitum. All animals were accustomed to the experimental conditions for a period of 14 days by handling them daily and putting them in the position to mimic the procedure of ip injection. Animal care and experimental procedures followed institutional ethic guidelines and conformed to the requirements of the state authority for animal research conduct (protocol 0053/06).

Peptide

In this study we used sulphated CCK-8 (CCK-8S) which is the most commonly used form of CCK in behavioral and pharmacological studies on food intake. CCK-8S (Bachem AG, Heidelberg, Germany) was dissolved in water with 1% (v/v) 1 N NH_4OH, aliquoted and stored at −20 °C. Immediately before starting the experiments, the peptide was diluted in vehicle solution consisting of sterile 0.15 M NaCl (Braun, Melsungen, Germany) to reach the final concentration of 5.2 and 8.7 nmol/kg body weight (b wt; 6 and 10 μg/kg b wt). Peptide solutions were kept on ice for the duration of the experiments. The doses of CCK-8S were selected based on our previous studies [26,32,37].

Experimental protocol

Experiments were started at the same time of day (between 10:00 a.m. to 10:30 a.m.), i.e. 3.5–4 h after the start of the light phase to achieve maximum consistency. Ad libitum fed rats received an ip injection (final volume: 500 μl) of 5.2 and 8.7 nmol/kg CCK-8S ($n = 3$/group) or vehicle solution (0.15 M NaCl; $n = 3$–5). At 90 min after CCK-8S injection, rats were deeply anesthetized with ip 100 mg/kg ketamine (Ketanest®, Curamed, Karlsruhe, Germany) and 10 mg/kg xylazine (Rompun® 2%, Bayer, Leverkusen, Germany) and heparinized with 2500 IU heparin injected ip (Liquemin®, Hoffmann-La Roche, Grenzach-Whylen, Germany). Transcardial perfusion was performed as described in detail before [23].

Immunohistochemistry

Single staining for c-Fos detection in the arcuate nucleus, in the paraventricular nucleus, in the nucleus of the solitary tract, and in the dorsal motor nucleus of the vagus in the brainstem

Free-floating 25 μm brain sections were pre-treated with 1% (w/v) sodium borohydride in phosphate buffered saline (PBS) for 15 min. Subsequently, sections were incubated in a solution containing 1% (w/v) bovine serum albumin (BSA) and 0.3% (v/v) Triton X-100, and 0.05% (v/v) phenylhydrazine in PBS for 60 min to block unspecific antibody binding. Thereafter, the diluted primary antibody solution (rabbit anti-c-Fos, Oncogene Research Products, Boston, MA, USA; 1:3000 in a solution of 1% (w/v) BSA, and 0.3% (v/v) Triton X-100 in PBS) was applied for 24 h at room temperature. After washing in PBS, sections were incubated with the secondary antibody solution (goat biotin-SP-conjugated anti rabbit IgG, Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA; 1:1000 in 1% (w/v) BSA in PBS) for 12 h at room temperature. After rinsing in PBS three times, sections were incubated in avidin-biotin peroxidase complex (ABC; 1:200; Vector Laboratories, UK) in PBS for 6 h. Subsequently, sections were rinsed in PBS three times again, and then incubated in TSA™ tetramethyl rhodamine tyramide in amplification solution (PerkinElmer, Waltham, MA, USA) for 10 min at room temperature. After washing in PBS, sections were stained with 4′,6-diamidino-2-phenylindole (DAPI) for 15 min to counterstain cell chromatin. Brain sections were finally embedded in 8 μl anti-fading solution (100 mg/ml 1,4-diazabicyclo [2.2.2] octane, Sigma, St. Louis, USA; in 90% (v/v) glycerine, 10% (v/v) PBS, pH 7.4) and analyzed using confocal laser scanning microscopy (cLSM 760, Carl Zeiss, Germany).

Double staining for c-Fos and serotonin in the arcuate nucleus, in the paraventricular nucleus of the hypothalamus, and in the nucleus of the solitary tract, and in the dorsal motor nucleus of the vagus of the brainstem

Staining for c-Fos followed the protocol described above (see section 'Single staining for c-Fos detection in the arcuate nucleus, in the paraventricular nucleus, in the nucleus of the solitary tract, and in the dorsal motor nucleus of the vagus in the brainstem'). Sections were then incubated with the second primary antibody solution (rabbit anti-serotonin; Novus Biologicals; Littleton, CO, USA; 1:1000 in PBS containing 1% (w/v) BSA) for 24 h at room temperature. After rinsing in PBS three times, fluorescein isothiocyanate (FITC) conjugated goat anti-rabbit IgG (Sigma–Aldrich) in PBS was applied for 12 h at room temperature. After washing in PBS three times, sections were stained with DAPI for 15 min. Finally, sections were rinsed in PBS three times and embedded in 8 μl

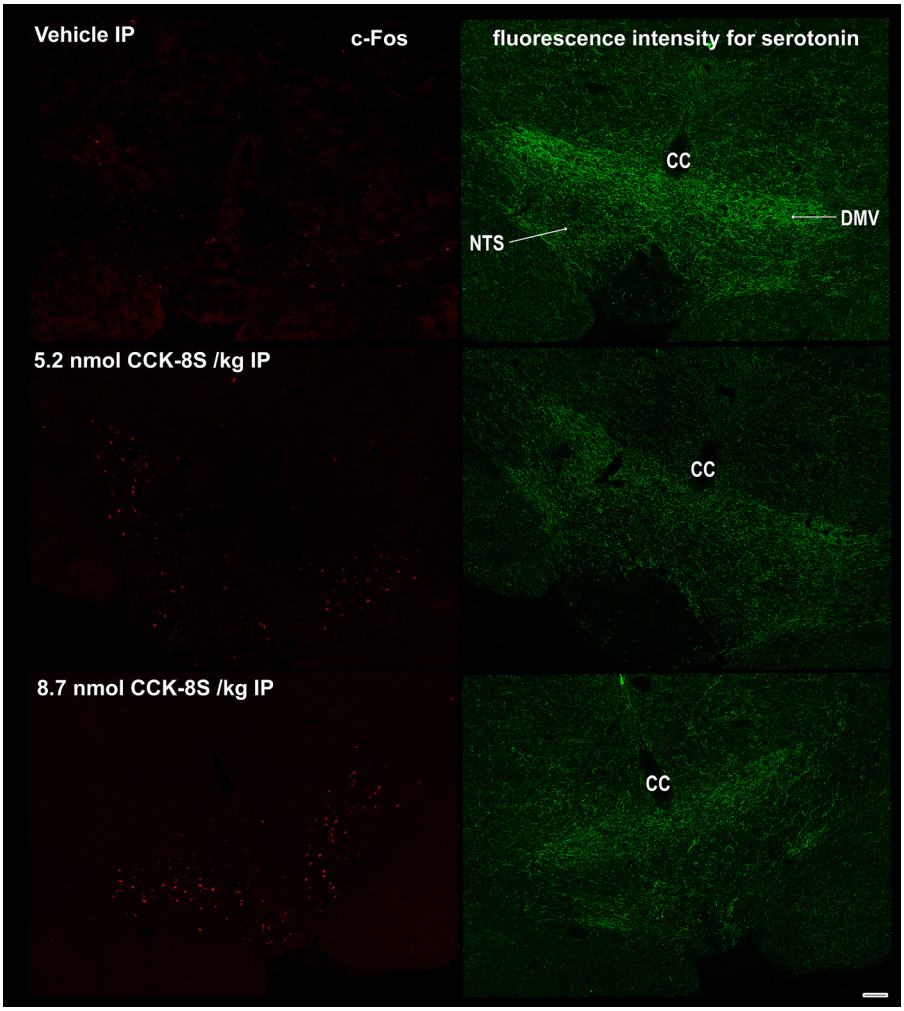

Fig. 1. CCK-8S injected ip increased the number of c-Fos-IR neurons (red) in the NTS in a dose depended manner compared to vehicle, while in the DMV no modulation of c-Fos was observed (left panel). In the NTS and DMV, serotonin-IR decreased in rats treated with CCK-8S compared to vehicle animals as assessed semi-quantitatively by fluorescence intensity (green) (right panel). The white scale bar represents 100 μm. cc, canalis centralis; NTS, nucleus tractus solitary; DMV, dorsal motor nucleus of the vagus. (For interpretation of the references to color in figure legend, the reader is referred to the web version of the article.)

anti-fading solution as described above and analyzed using confocal laser microscopy (cLSM 760).

Triple staining for c-Fos, serotonin and tyrosine hydroxylase in the nucleus of the solitary tract of the brainstem

For triple staining against c-Fos, serotonin and tyrosine hydroxylase we used the protocol as described above and further incubated the sections with the third primary antibody solution (anti-tyrosine hydroxylase; Sigma–Aldrich; 1:6000 in 1% (w/v) BSA, and 0.1% (v/v) sodium azide in PBS) for 24 h at room temperature. After washing in PBS three times, brain sections were incubated overnight with the secondary goat anti-mouse IgG antibody, Alexa Fluor® 633 (Molecular Probes, Leiden, Netherlands; 1:400 in 1% (w/v) BSA). Then, sections were washed, counterstained with DAPI, embedded with anti-fading solution and analyzed using confocal laser microscopy (cLSM 760).

Data assessment and statistical analysis

Neurons with red nuclear staining were considered as Fos-IR. Every third of all consecutive coronal 25 μm sections was counted for Fos-IR staining bilaterally in the PVN (Bregma −1.30 to −2.12 mm), bilaterally in the ARC (Bregma −2.12 to −3.60 mm), bilaterally in the NTS (Bregma −13.24 to −14.30 mm) and bilaterally in the DMV (Bregma −13.24 to −14.30 mm) according to the coordinates by Paxinos and Watson [36]. The remaining (second and third) sections were used for immunohistochemical double and triple staining. The average number of Fos-IR cells per section for the brain nuclei mentioned above was calculated for three to five rats per experimental group by an investigator blinded to the treatment of the animals. Using the same setup of the confocal laser microscope, the concentration of serotonin was determined by analyzing the fluorescent intensity. For semi-quantitative analysis, the NIH image processing program ImageJ 1.47v was used. Data on fluorescent intensity are presented in number of pixel per section (px/section). All data are presented as mean ± SEM and were analyzed by Analysis of Variance (ANOVA). Differences between treatment groups were evaluated with the Fisher LSD post hoc test and $P < 0.05$ was considered significant.

Results

Intraperitoneal injection of CCK-8S (5.2 and 8.7 nmol/kg) dose-dependently increased the number of c-Fos neurons in the NTS (mean ± SEM: 72 ± 4 and 112 ± 5 neurons/section, respectively) compared to vehicle-treated rats (7 ± 2 neurons/section, $P < 0.001$; Figs. 1 and 2). In contrast, in the DMV we did not detect a significant difference in neuronal activation between rats treated

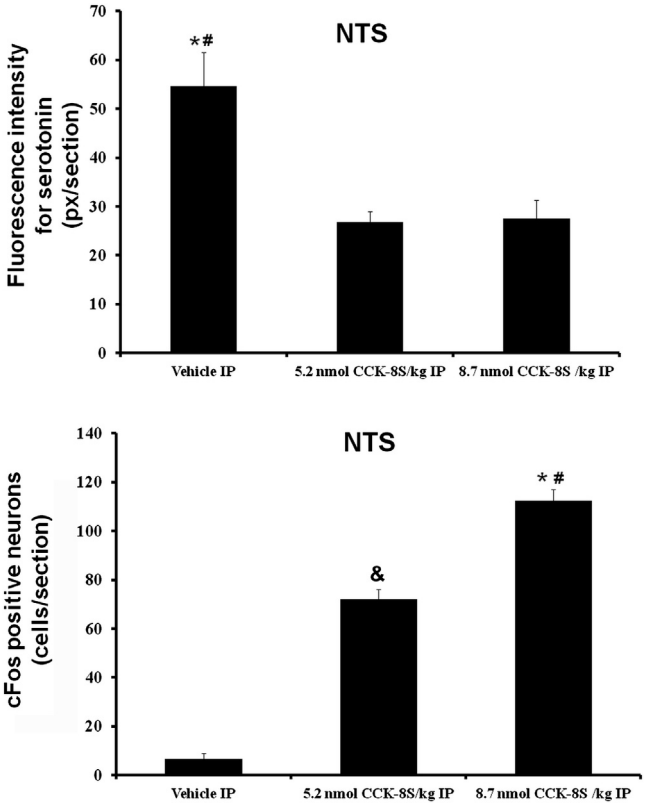

Fig. 2. In the NTS, serotonin-IR was decreased at 90 min after ip injection of CCK-8S compared to vehicle (upper panel). CCK-8S dose-dependently increased the number of c-Fos neurons in the NTS compared to vehicle treated rats (lower panel). Data are mean ± SEM of 3–5 rats/group. Upper panel: $^{\#}P = 0.01$ vs. 5.2 nmol CCK-8S/kg; $^{*}P = 0.015$ vs. 8.7 nmol CCK-8S/kg. Lower panel: $^{\#}P < 0.001$ vs. 5.2 nmol CCK-8S/kg; $^{*}P < 0.001$ vs. vehicle; $^{\&}P < 0.001$ vs. vehicle.

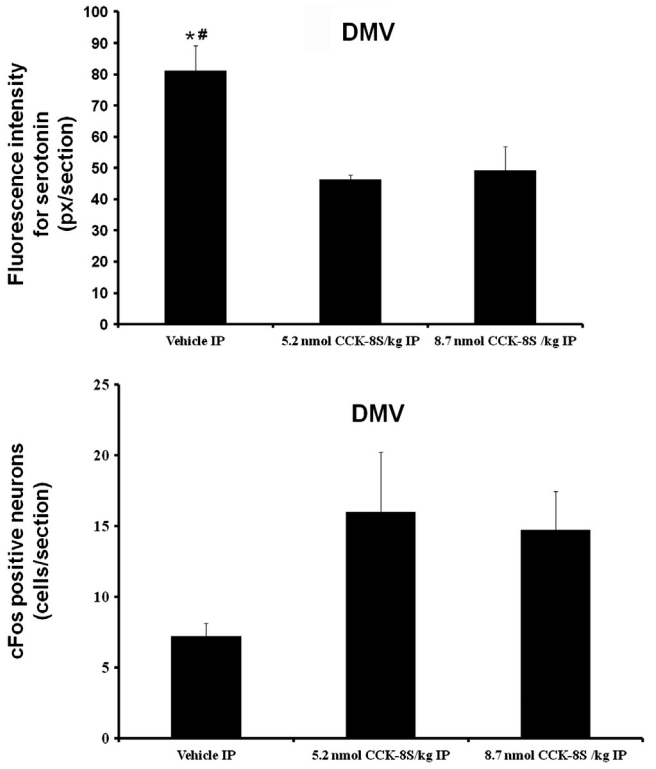

Fig. 3. Serotonin-IR in the DMV was decreased 90 min after ip injection of CCK-8S compared to vehicle (upper panel). CCK-8S had no effect on c-Fos activation in the DMV. Upper panel: $^{\#}P = 0.01$ vs. 5.2 nmol CCK-8S/kg; $^{*}P < 0.015$ vs. 8.7 nmol CCK-8S/kg.

with both doses of CCK (16 ± 4 and 15 ± 3 neurons/section, respectively) and vehicle-treated animals (7 ± 1 neurons/section, $P = 0.054$; Figs. 1 and 2). In the NTS and DMV we observed a decrease of serotonin-immunoreactive fluorescence intensity at 90 min after ip injection of CCK-8S (46 ± 2 and 49 ± 8 px/section, respectively) compared to vehicle (81 ± 8 px/section, $P < 0.01$; Figs. 1–3). No dose-dependency in the serotonin-immunoreactive fluorescence intensity was observed between the two doses of CCK-8S ($P = 0.81$; Figs. 2 and 3). Double-staining indicated that c-Fos-immunoreactive neurons are embedded in a dense network of serotonin- immunoreactive nerve fibers (Fig. 4). In addition, triple immunostaining in the NTS against c-Fos, serotonin and tyrosine hydroxylase showed that activated (c-Fos-positive) tyrosine hydroxylase-positive neurons were encircled by a very dense network of serotonin-immunoreactive nerve fibers (Fig. 5).

In the PVN we observed that the number of c-Fos-positive neurons also increased dose-dependently after ip injection of CCK-8S (128 ± 14 and 218 ± 15 neurons/section, respectively, $P = 0.002$; Figs. 6 and 7) compared to vehicle-treated rats (19 ± 5 neurons/section, $P < 0.001$; Figs. 6 and 7). In contrast, the number of c-Fos positive neurons in the ARC did not change following ip injection of CCK-8S (5 ± 3 and 8 ± 1 neurons/section, respectively; Figs. 8 and 9) compared to vehicle treatment (7 ± 1 neurons/section, $P = 0.635$; Figs. 7 and 9). In the PVN and the ARC no differences were detected in the fluorescence intensity of serotonin-IR after injection of the two doses of CCK-8S (16 ± 1 and 15 ± 1 px/section, respectively in the PVN, 16 ± 2 and 16 ± 1 px/section, respectively in the ARC; Figs. 6 and 7) compared to vehicle (14 ± 1 px/section in the PVN, $P = 0.389$; 20 ± 1 px/section in the ARC, $P = 0.850$; Figs. 7 and 9).

Discussion

In the present study we showed that ip injected CCK-8S at both doses significantly reduced the fluorescence intensity of serotonin-nerve fibers in the NTS and DMV compared to vehicle-treated rats giving rise to an ip CCK-8S-induced release of serotonin from nerve fibers located in these nuclei. No differences between serotonin fluorescence intensities were observed in the ARC and PVN of the hypothalamus after peripheral injection of CCK-8S.

The gastrointestinal peptide CCK acts via the vagus nerve and several studies showed that CCK-8S activates distinct brain nuclei in the hypothalamus and brainstem [13,24,40]. It has already been demonstrated that peripheral injection of CCK-8S increases the number of c-Fos neurons in the NTS and PVN [9,24]. These results are in agreement with the present study showing that CCK-8S dose-dependently increased the number of c-Fos neurons in the NTS and PVN. As mentioned above, CCK-8S induces the activation of a complex neuro-hormonal system in the hypothalamus and brainstem, which results in a short-term inhibition of food intake in rats. In addition to CCK, serotonin is another neurotransmitter which may play a physiological role in this system. Several studies already indicated a possible interaction between CCK and serotonin [2,5,6,15,17,19,20,38]. The ip injection of the serotonin antagonist, ondansetron attenuated the CCK-induced suppression of food intake in rats [19]. When ondansetron was microinjected directly into the medial NTS (mNTS), the suppression of food intake by CCK was completely reversed [18]. Therefore, Hayes et al. suggested that serotonin receptors within the mNTS control meal size and mediate CCK-induced satiation [18]. Although these data suggest a consecutive action of both peptides with a signaling of serotonin downstream to CCK, a synergistic interaction was not detected in a rodent study using CCK and serotonin agonists [53].

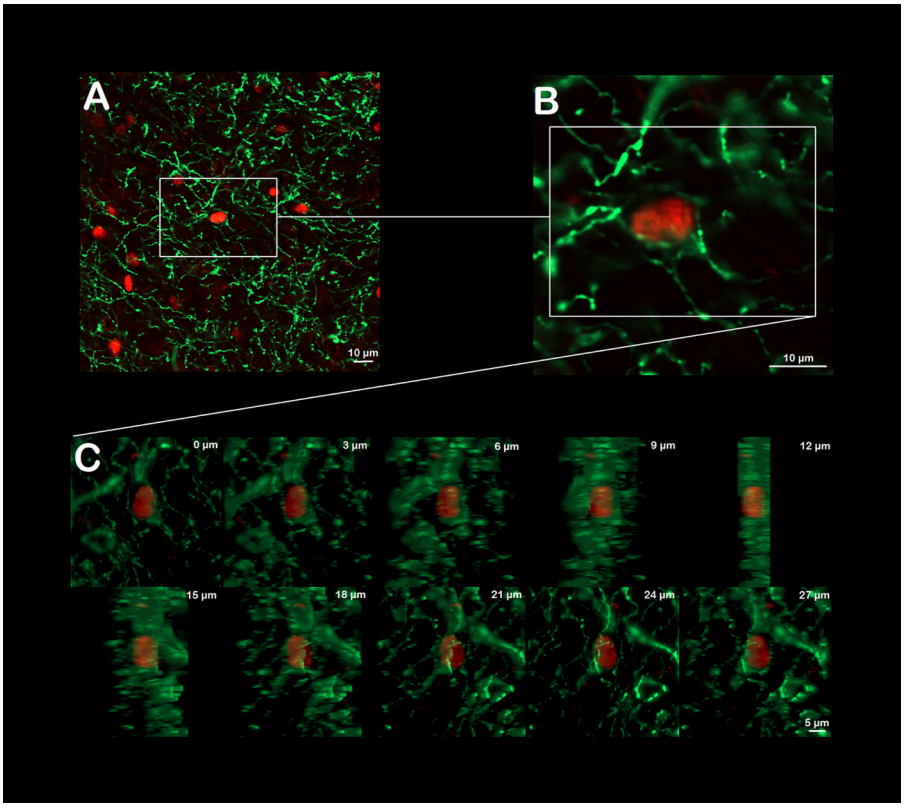

Fig. 4. Representative picture of double immunostaining with c-Fos and anti-serotonin in the NTS of the brainstem (A). Double-staining showed that c-Fos-IR neurons (red) are embedded in a dense network of serotonin-IR nerve fibers (green) (B). (C) A y-stack of the same neuron demonstrating the close proximity of serotonin-IR fibers that surround c-Fos-IR neuron in the NTS. The white scale bar represents 10 μm in (A) and (B), and 5 μm in (C). (For interpretation of the references to color in figure legend, the reader is referred to the web version of the article.)

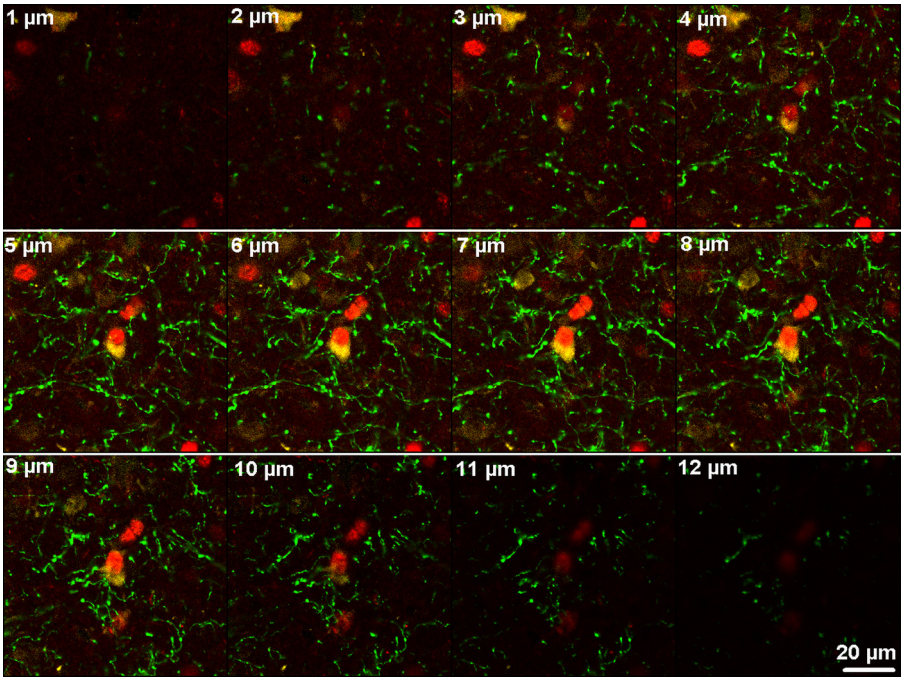

Fig. 5. Representative picture showing triple immunostaining with anti-tyrosine hydroxylase, anti-c-Fos, and anti-serotonin in the NTS. Triple immunostaining in this cLSM z-stack indicated that c-Fos-IR neurons (red) partly contain tyrosine hydroxylase (yellow). These activated neurons are embedded in a dense network of serotonin-immunoreactive nerve fibers (green). The white scale bar represents 20 μm. (For interpretation of the references to color in figure legend, the reader is referred to the web version of the article.)

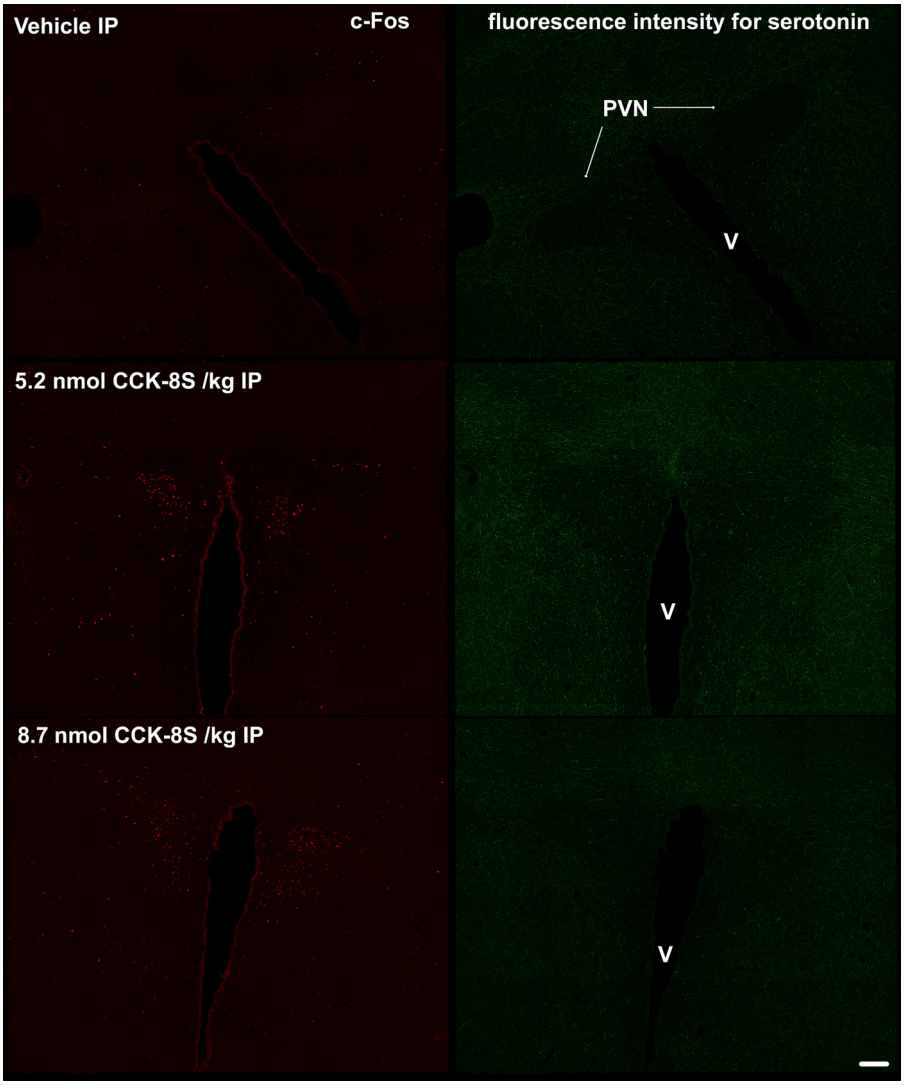

Fig. 6. Representative image showing double immunostaining with anti-c-Fos and anti-serotonin in the PVN. CCK-8S dose-dependently increased the number of c-Fos neurons (red) in the PVN compared to vehicle (left panel). No effect on serotonin-immunoreactive fluorescence intensity (green) was observed between treatment groups after ip injection of vehicle and CCK-8S at both doses (right panel). The white scale bar represents 100 μm. V = third ventricle. (For interpretation of the references to color in figure legend, the reader is referred to the web version of the article.)

Our results support the assumption of the NTS playing a role in the serotonergic mediation of cholecystokinergic effects. The decrease of serotonin-IR in the NTS and DMV after peripheral administration of CCK-8S likely reflects a local release of serotonin. We expect that the released serotonin is not detectable extracellularly because it was washed out in the process of the transcardial perfusion and immunohistochemical processing. A direct measurement of serotonin release on the cellular level will be subject to future in vitro studies. The NTS receives serotonergic innervation from brainstem raphe nuclei [48], the AP [8] and vagal afferents [39,52]. Moreover, serotonin is present in neurons residing within the NTS [3]. Accordingly, ultrastructural analysis showed that serotonin can be locally released by NTS neurons [21]. In our studies we found a significant activation of NTS neurons with a simultaneous significant decrease of serotonin-IR after peripheral injection of CCK-8S. Therefore, we suggest that peripheral CCK acts via the vagus nerve to locally release serotonin. Injection of CCK-8S was also accompanied by a significant decrease of serotonin-IR in the DMV, whereas a significant neuronal activation of DMV cells after administration of CCK could not be observed. Our results confirm other studies that did not detect an activation of DMV neurons after ip injection of CCK-8S [1,28]. However, axons of NTS neurons can be found throughout the DMV, as both brain nuclei have a close proximity. Activation of NTS neurons by CCK could therefore lead to a release of serotonin from axon segments that originate in the NTS and pass through the DMV. This hypothesis is in line with the finding that serotonin can be released from dense core vesicles distant to the synapse [31]. We therefore hypothesize that the effects of CCK cannot be assigned to DMV neurons themselves, but to NTS neurons leading through the DMV instead. Further retrograde tracing experiments are warranted to corroborate this hypothesis.

We detected that NTS tyrosine hydroxylase (TH)-immunoreactive neurons are embedded in a very dense network of serotonin-IR nerve fibers. It has been shown before that administration of CCK activates c-Fos expression in catecholaminergic neurons within the NTS that project to the PVN [43]. This CCK-induced activation of TH-containing neurons could be mediated by serotonin, as serotonin receptor agonists are known to activate TH-containing neurons within the NTS [25]. Future studies using electron microscopy are needed to support this hypothesis on a

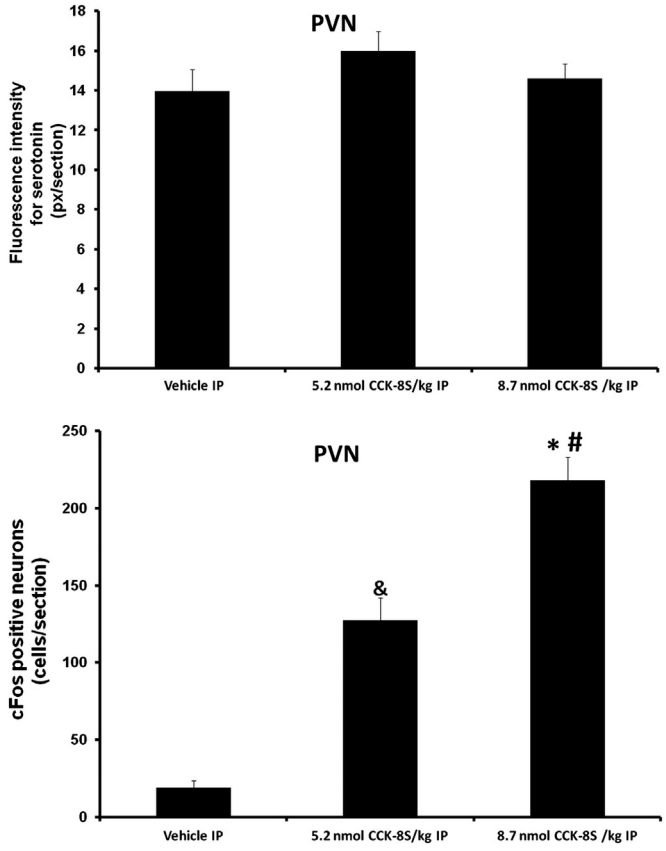

Fig. 7. In the PVN no effect on serotonin-immunoreactive fluorescence intensity was observed between treatment groups at 90 min after ip injection (upper panel). CCK-8S dose-dependently increased the number of c-Fos neurons in the PVN compared to vehicle (lower panel). Data are mean ± SEM of 3 rats/group. Lower panel: $^{\#}P = 0.002$ vs. 5.2 nmol CCK-8S/kg; $^{*}P < 0.001$ vs. vehicle; $^{\&}P < 0.001$ vs. vehicle.

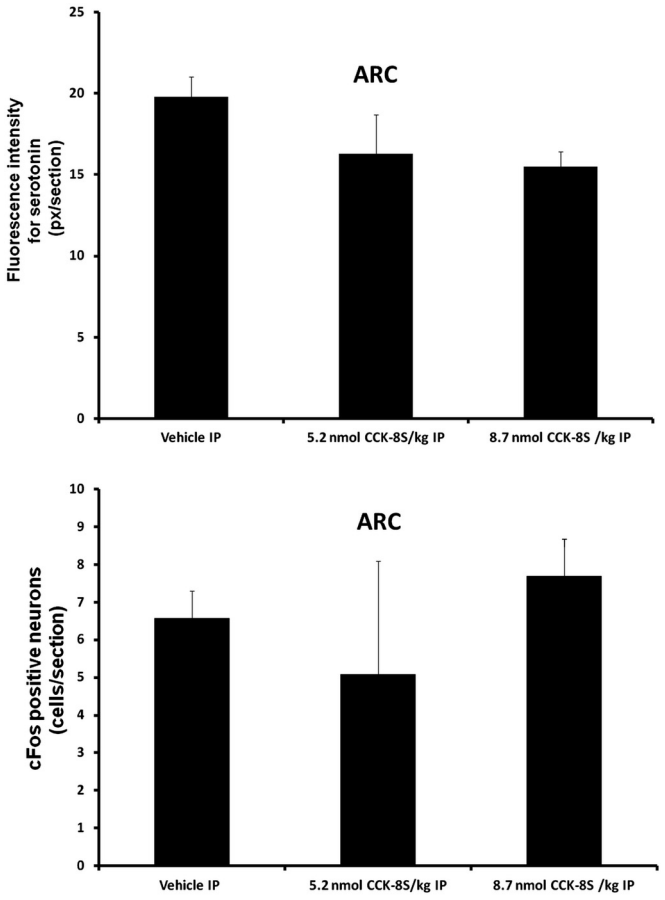

Fig. 9. In the ARC injection of CCK-8S did not affect serotonin-IR (upper panel). There were no differences in the number of c-Fos neurons between treatment groups (lower panel). Data are mean ± SEM of 3 rats/group. $P = 0.850$.

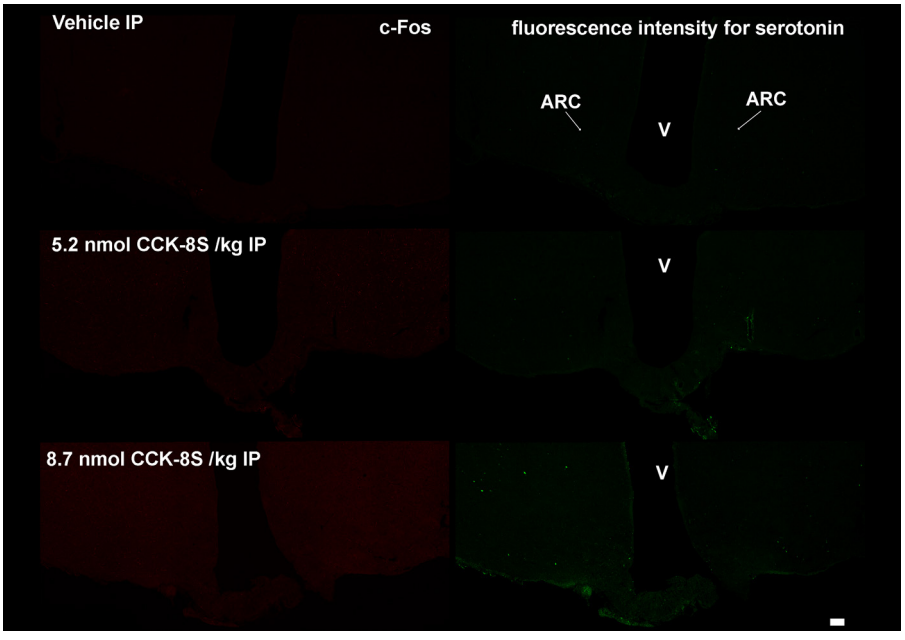

Fig. 8. Representative image showing double immunostaining with anti-c-Fos and anti-serotonin in the ARC. In the ARC ip administration of CCK-8S did not affect serotonin-IR (green) (right panel). There were no differences in the number of c-Fos neurons between treatment groups (red) (left panel). The white scale bar represents 100 μm. V = third ventricle. (For interpretation of the references to color in figure legend, the reader is referred to the web version of the article.)

subcellular level. While neurons in the PVN might be activated via ascending catecholaminergic pathways activated by serotonin, we did not detect any alterations of serotonin-IR intensity in the ARC or PVN of the hypothalamus after peripheral administration of CCK-8S pointing toward a downstream signaling of serotonin in the brainstem following peripheral injection of CCK.

In summary, ip injection of CCK-8S activates neurons of the NTS that likely leads to a downstream activation of serotonin in this nucleus resulting in a stimulation of catecholaminergic signaling projecting to the PVN which ultimately results in the short-term anorexigenic response that has already been well-established for CCK-8S.

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Research report

Peripheral injection of bombesin induces c-Fos in NUCB2/nesfatin-1 neurons

Kim-Marie Engster, Arthur L. Kroczek, Matthias Rose, Andreas Stengel, Peter Kobelt *

Charité Center for Internal Medicine and Dermatology, Department of Psychosomatic Medicine, Charité – Universitätsmedizin Berlin, Germany

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ABSTRACT

As anorexigenic hormones bombesin and nucleobindin2 (NUCB2)/nesfatin-1 decrease food intake in rodents. Both hormones have been described in brain nuclei that play a role in the modulation of hunger and satiety, like the paraventricular nucleus of the hypothalamus (PVN) and the nucleus of the solitary tract (NTS). However, the direct interaction of the two hormones is unknown so far. The aim of study was to elucidate whether bombesin directly interacts with NUCB2/nesfatin-1 neurons in the PVN and NTS. Therefore, we injected bombesin intraperitoneally (ip) at two doses (26 and 32 nmol/kg body weight) and assessed c-Fos activation in the PVN, arcuate nucleus (ARC) and NTS compared to vehicle treated rats (0.15 M NaCl). We also performed co-localization studies with oxytocin or tyrosine hydroxylase. Bombesin at both doses increased the number of c-Fos positive neurons in the PVN ($p < 0.05$) and NTS ($p < 0.05$) compared to vehicle, while in the ARC no modulation was observed ($p > 0.05$). In the PVN and NTS the number of c-Fos positive neurons colocalized with NUCB2/nesfatin-1 increased after bombesin injection compared to vehicle treatment ($p < 0.05$). Moreover, an increase of activated NUCB2/nesfatin-1 immunoreactive neurons that co-expressed oxytocin in the PVN ($p < 0.05$) or tyrosine hydroxylase in the NTS ($p < 0.05$) was observed compared to vehicle. Our results show that peripherally injected bombesin activates NUCB2/nesfatin-1 neurons in the PVN and NTS giving rise to a possible interaction between bombesin and NUCB2/nesfatin-1 in the modulation of food intake.

1. Introduction

Bombesin is an anorexigenic tetradecapeptide that was isolated in 1971 from the skin of the European toad *Bombina bombina* (Anastasi et al., 1971). There are several homologs of bombesin in mammals, including gastrin-releasing peptide (GRP) and neuromedin B (Moody and Merali, 2004). Bombesin-like peptides are released in the gastrointestinal tract after the uptake of nutrients (Schusdziarra et al., 1986b). Bombesin binds with high affinity to the GRP receptor (BB_2), and to the neuromedin receptor (BB_1) distributed in the gastrointestinal tract (GI) and in the central nervous system (CNS) (Moody and Merali, 2004; Moran et al., 1988; Sayegh, 2013; Schusdziarra et al., 1986b). High densities of bombesin binding sites were observed in the hippocampus, hindbrain, midbrain, and amygdala and in the hypothalamus (Moody and Merali, 2004; Wada et al., 1991; Zarbin et al., 1985). In line with these findings, microinjections of bombesin into specific brain areas, like hypothalamic nuclei or the nucleus of the solitary tract (NTS) of the brainstem were effective in suppressing food intake (Johnston and Merali, 1988; Kyrkouli et al., 1987). Additionally, it has been observed that the satiating effects of bombesin are attenuated in animals with lesions of hindbrain areas (*e.g.* NTS or the dorsal motor nucleus of the vagus), whereas forebrain lesions did not affect bombesin's effects on food intake (Bellinger and Bernardis, 1984; Geary et al., 1986; Sayegh, 2013, West et al., 1982). This underlines the key role of central – predominantly hindbrain – areas in the bombesin-induced suppression of food intake.

The mapping of brain neuronal circuits recruited by hormone signals has been obtained by assessing changes of the proto-oncogene c-Fos, which allows the identification of activated neurons at the cellular level (Sagar et al., 1988). Studies have shown that intraperitoneally (ip) injected bombesin increased the number of c-Fos positive neurons in the paraventricular nucleus of the hypothalamus (PVN), and in the NTS (Bonaz et al., 1993; Li and Rowland, 1996), an activation likely underlying the peripheral anorexigenic effect of the peptide observed in rodents and humans (Denbow, 1989; Taylor and Garcia, 1985; Woods et al., 1983; Muurahainen et al., 1993; Kulkosky et al., 1982). However, relevant differences between peripheral and central administration of bombesin have been observed with regards to food intake. In

* Correspondence to: Charité Center for Internal Medicine and Dermatology, Department of Psychosomatic Medicine, Charité – Universitätsmedizin Berlin, Campus Mitte, Sauerbruchweg 5, 10117 Berlin, Germany.
E-mail address: peter.kobelt@charite.de (P. Kobelt).

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contrast to animals icv injected with bombesin, rats injected ip with bombesin display a normal frequency of grooming and do not show a decrease in drinking behavior (Kulkosky et al., 1982). Kulkosky et al. concluded that the specific anorexigenic effect of ip administered bombesin cannot be explained solely by the increase of bombesin in the cerebrospinal fluid (Kulkosky et al., 1982). It can be assumed that ip injected bombesin suppresses feeding additionally through spinal afferent fibers and vagal afferent mechanisms (Michaud et al., 1999; Sayegh, 2013; Stuckey et al., 1985).

Besides bombesin, in rodents the neuropeptide nesfatin-1 suppresses food intake when injected centrally or peripherally (Shimizu et al., 2009; Oh-I et al., 2006; Stengel et al., 2009; Atsuchi et al., 2010). Nesfatin-1 reduces food intake *via* a reduction of meal size and meal frequency in mice (Goebel et al., 2011). Nesfatin-1 is proteolytically cleaved from the precursor protein nucleobindin (NUCB2) and was first described in Oh-I et al. (2006) as "*N*ucleobindin 2-*e*ncoded *s*atiety-and *fat i*nfluencing prote*in*" (Oh-I et al., 2006). Also NUCB2 is biologically active and has the same anorexigenic effect as nesfatin-1 (Oh-I et al., 2006). NUCB2 mRNA and protein expression were detected in different brain nuclei, like hypothalamic nuclei and the NTS (Goebel et al., 2009; Foo et al., 2008).

Administration of sulfated cholecystokinin-8 (CCK-8S) was shown to dose-dependently induce c-Fos in NUCB2/nesfatin-1 immunoreactive neurons in the PVN and NTS (Noetzel et al., 2009) which may contribute to CCK's anorexigenic effects. Interestingly, bombesin induces a BB_2 receptor-stimulated activation of gastric vagal afferent discharge mediated partly through a CCK pathway (Yoshida-Yoneda et al., 1996; Schwartz et al., 1997). In line with this finding, also bombesin might interact with NUCB2/nesfatin-1 to induce its anorexigenic effects. As both anorexigenic hormones induce an activation of hypothalamic and brainstem nuclei, we considered the PVN and the NTS as possible sites of interaction.

Thus, the aim of the present study was to determine whether bombesin injected ip modulates the neuronal activity of NUCB2/nesfatin-1 immunoreactive neurons in the PVN or NTS. Neuronal activity was assessed by measuring c-Fos expression in relevant brain nuclei. It has been reported that oxytocin might be involved in the downstream anorexigenic signaling of nesfatin-1 (Foo et al., 2008; Oh-I et al., 2006; Kohno et al., 2008). Therefore, we also determined whether activated NUCB2/nesfatin-1 immunoreactive neurons are co-localized with ocytocin in the PVN after ip injection of bombesin. Since there is also evidence that oxytocin containing neurons in the PVN are innervated by noradrenergic projections of the A2-cell group in the NTS (Sawchenko and Swanson, 1981, 1982), we lastly quantified activated NUCB2/nesfatin-1 immunoreactive neurons co-localized with tyrosine hydroxylase.

2. Results

2.1. Effects of ip injected bombesin on c-Fos expression and the number of NUCB2/nesfatin-1 immunoreactive neurons in hypothalamic and medullary nuclei

2.1.1. Paraventricular nucleus

After ip injection of bombesin (26 and 32 nmol/kg body weight) we observed a gradual increase in the number of c-Fos positive neurons in the PVN (mean ± SEM: 70 ± 9 and 109 ± 8 neurons/section, respectively) compared to vehicle treated rats (16 ± 4 neurons/section, $p < 0.05$; Figs. 1A and 2A). The number of NUCB2/nesfatin-1 immunoreactive neurons in the PVN did not change after bombesin treatment (237 ± 21 and 220 ± 7 neurons/section, respectively) compared to vehicle treatment (214 ± 3 neurons/section, $p > 0.05$; Fig. 2B). Double labeling showed that the number of c-Fos positive and NUCB2/nesfatin-1 immunoreactive double-labeled neurons in the PVN increased more than tenfold after ip injection of bombesin (32 ± 3 and 57 ± 13 neurons/section, respectively) compared to the vehicle group (4 ± 0.5 neurons/section, $p < 0.05$; Fig. 2A). However, the gradual effect of the two doses of bombesin on c-Fos and NUCB2/nesfatin-1 immunoreactive double-labeling did not reach statistical significance ($p > 0.05$; Fig. 2A).

Triple-staining indicated that c-Fos-positive and NUB2/nesfatin-1 immunoreactive neurons partly co-localized with oxytocin (Figs. 1A,B and 2B). While the number of oxytocin immunoreactive neurons did not change after injection of bombesin (67 ± 19 and 63 ± 8, respectively compared to 51 ± 10 neurons/section; $p > 0.05$ Fig. 2B), this treatment increased the number of c-Fos positive and NUCB2/nesfatin-1 immunoreactive neurons co-localized with oxytocin (2.6 ± 1.3 and 9 ± 4 neurons/section, respectively) compared to vehicle treated rats (0.4 ± 0.2 neurons/section, $p < 0.05$; Fig. 2C).

2.1.2. Nucleus of the solitary tract

In the NTS bombesin administration also led to a gradual increase of c-Fos immunoreactive neurons (46 ± 1 and 91 ± 8 neurons/section, respectively) compared to vehicle treatment (5 ± 2 neurons/section, $p < 0.05$; Figs. 3A and 4A). In contrast to the PVN, the number of NUCB2/nesfatin-1 immunoreactive neurons significantly increased after ip injection of bombesin in the NTS (105 ± 12 and 118 ± 1 neurons/section, respectively) compared to vehicle treatment (61 ± 4 neurons/section, $p < 0.05$; Figs. 3A and 4B). Interestingly, we observed some NTS neurons whose projections were stained by nesfatin-1 antibody (Fig. 3C).

As observed for oxytocin in the PVN, in the NTS the number of tyrosine hydroxylase positive neurons did not change after ip injection of bombesin (26 ± 5 and 25 ± 5 neurons/section, respectively) compared to vehicle treated rats (25 ± 4 neurons/section, $p > 0.05$; Fig. 4B). The number of c-Fos-positive and NUCB2/nesfatin-1 immunoreactive neurons in the NTS significantly increased after ip injection of bombesin (17 ± 3 and 25 ± 3 neurons/section, respectively) compared to the injection of vehicle solution (1 ± 0.1 neuron/section, $p < 0.05$; Fig. 4A). No difference was observed between the two doses of bombesin ($p > 0.05$).

Triple labeling against c-Fos, NUCB2/nesfatin-1 and tyrosine hydroxylase showed that c-Fos-positive and NUCB2/nesfatin-1 immunoreactive neurons were scarcely co-localized with tyrosine hydroxylase in the NTS (Figs. 3A, B and 4C). Quantification of these neurons showed an increase of triple-labeled neurons positive for NUCB2/nesfatin-1, c-Fos and tyrosine hydroxylase after ip injection of bombesin (6.7 ± 0.9 and 8.9 ± 0.3 neurons/section, respectively) compared to vehicle treatment (0.6 ± 0.2 neurons/section, $p < 0.05$; Figs. 3A and 4C).

2.1.3. Arcuate nucleus

In contrast to the PVN and NTS, no changes of c-Fos immunoreactivity were detected in the ARC after bombesin treatment (17 ± 2 and 12 ± 1 neurons/section, respectively) compared to vehicle treatment (14 ± 2 neurons/section, $p > 0.05$; data not shown).

3. Discussion

The present study shows that peripherally injected bombesin affects the activity of NUCB2/nesfatin-1 immunoreactive neurons in conscious non fasted rats. As a response to bombesin injected ip, we observed a four- to seven-fold increase in the number of c-Fos neurons in the PVN and a ten- to eighteen-fold increase in the number of c-Fos neurons in the NTS. In contrast to the PVN, we did not detect any changes in number of c-Fos neurons in the ARC

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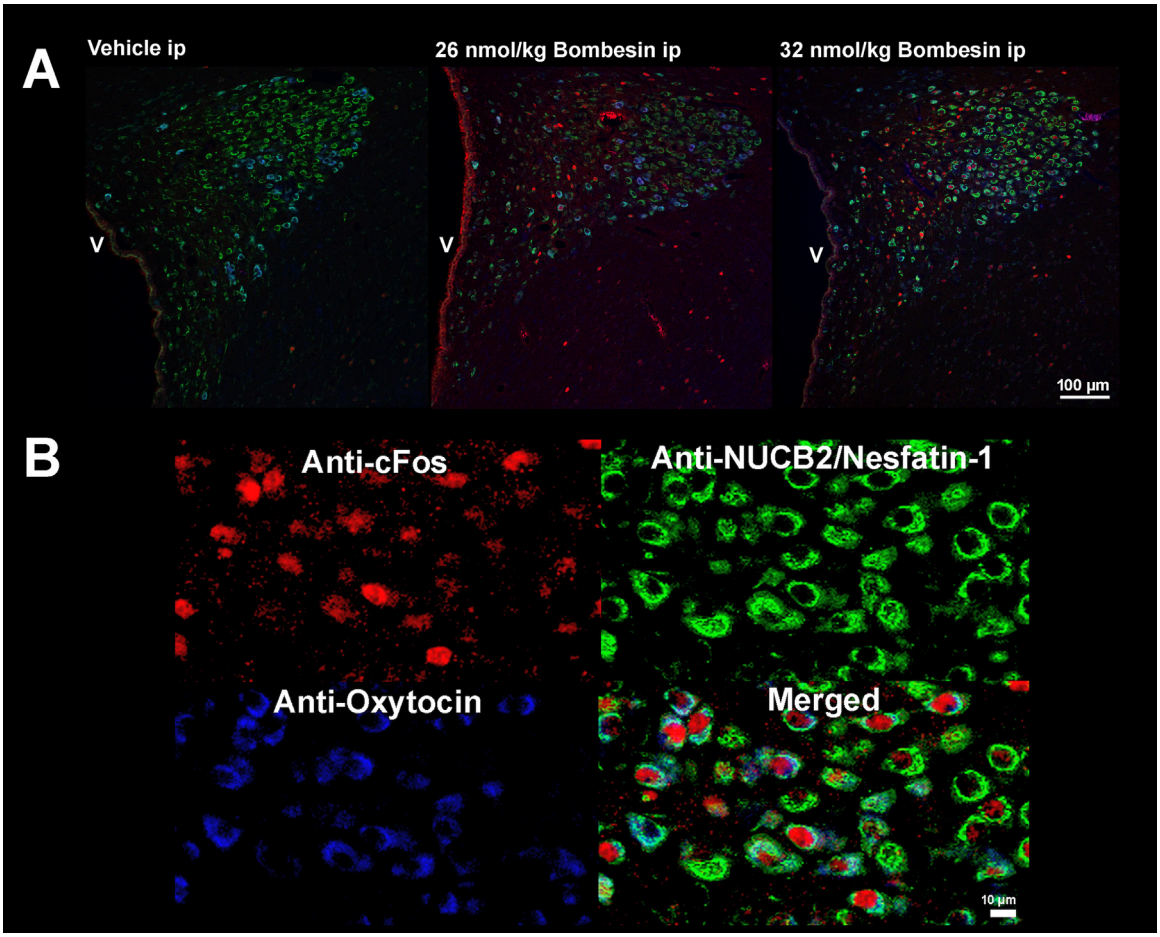

Fig. 1. Bombesin injected intraperitoneally gradually increased the number of c-Fos immunoreactive neurons in the paraventricular nucleus. While the number of NUCB2/nesfatin-1 immunoreactive neurons (green fluorescence) expressing c-Fos (red fluorescence) gradually increased after bombesin injection, the total number of NUCB2/nesfatin-1 immunoreactive and oxytocin immunoreactive neurons (blue fluorescence staining) did not change compared to vehicle treatment (**A**). Panel (**B**) shows a higher magnification (from 32 nmol/kg bombesin image) with triple-labeling against c-Fos (red fluorescence), NUCB2/nesfatin-1 (green fluorescence) and oxytocin (blue fluorescence). A merge of the single images resulted in turquoise fluorescent coloring of the cytoplasm of labeled neurons. The white scale bar represents 100 µm in (**A**) and 10 µm in (**B**). Abbreviation: ip, intraperitoneal; V, third ventricle. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

after bombesin injection, although high densities of bombesin binding sites in this nucleus have been reported (Moody and Merali, 2004). In both, the PVN and NTS the number of activated NUCB2/nesfatin-1 immunoreactive neurons significantly increased. We further observed a trend towards an increase of activated NUCB2/nesfatin-1 immunoreactive neurons colocalized with oxytocin or tyrosine hydroxylase.

Bombesin and nesfatin-1 are two anorexigenic peptides, which share some relevant similarities: peripheral as well as central administration of both peptides reduce food intake and alter meal patterns (Goebel et al., 2011; Stuckey and Gibbs, 1982; Flynn, 1991; Lynch and Babcock, 1993; Thaw et al., 1998; Yamada et al., 2002). Bombesin and NUCB2/nesfatin-1 immunoreactivity can be detected in the gastrointestinal tract as well as in the CNS (Moody and Merali, 2004; Schusdziarra et al., 1986a; Moran et al., 1988; Mohan and Unniappan, 2012; Stengel et al., 2013; Goebel-Stengel et al., 2011) giving further rise to the function as gut-brain peptides. Both peptides have a wide range of function: *e.g.* nesfatin-1 not only influences food intake and blood pressure, but also stress-related and anxiogenic behaviors (Yosten and Samson, 2010; Okere et al., 2010; Könczöl et al., 2010; Yoshida et al., 2010). Like nesfatin-1, centrally injected bombesin elicits hypertensive effects and activates the HPA axis (Erspamer et al., 1972; Kent et al., 2001a, 2001b). Researchers demonstrated that icv injection of bombesin stimulates corticotropin-releasing factor (CRF) release in the hypothalamus (Kent et al., 2001a), while nesfatin-1 increases the excitability of CRF positive neurons when administered directly onto PVN neurons *in vitro* (Price et al., 2008). Interestingly, pretreatment with CRF antagonists blocked the effect of centrally injected bombesin as well as centrally administered nesfatin-1 on food intake (Stengel et al., 2009; Gotoh et al., 2013; Plamondon and Merali, 1997).

In order to reduce food intake, bombesin and nesfatin-1 interact with different gut peptides, like CCK (Jansen and Lamers, 1984; Erspamer et al., 1974; Degen et al., 2001; Noetzel et al., 2009). In rats, injection of bombesin stimulated the release of CCK (Erspamer et al., 1974). Accordingly, in humans, a bombesin-like peptide antagonist (BIM26226) inhibited CCK release (Degen et al., 2001). Furthermore, it has been shown that CCK-8 S dose-dependently activated c-Fos positive NUCB2/nesfatin-1 immunoreactive neurons in the PVN and NTS (Noetzel et al., 2009). Considering these existing data, an indirect interaction between bombesin and nesfatin-1 through CCK can be hypothesized. Because of the broad similarities between bombesin and nesfatin-1 mentioned above, we further anticipated that both peptides might also directly interact *via* central neuronal pathways. We especially focused on the PVN as a possible site of interaction, since a high density of bombesin binding sites was found in the hypothalamus (Moody and Merali, 2004) and the greatest number of c-Fos-labeled neurons after icv injection of nesfatin-1 was detected in the PVN (Moreau and Ciriello, 2013).

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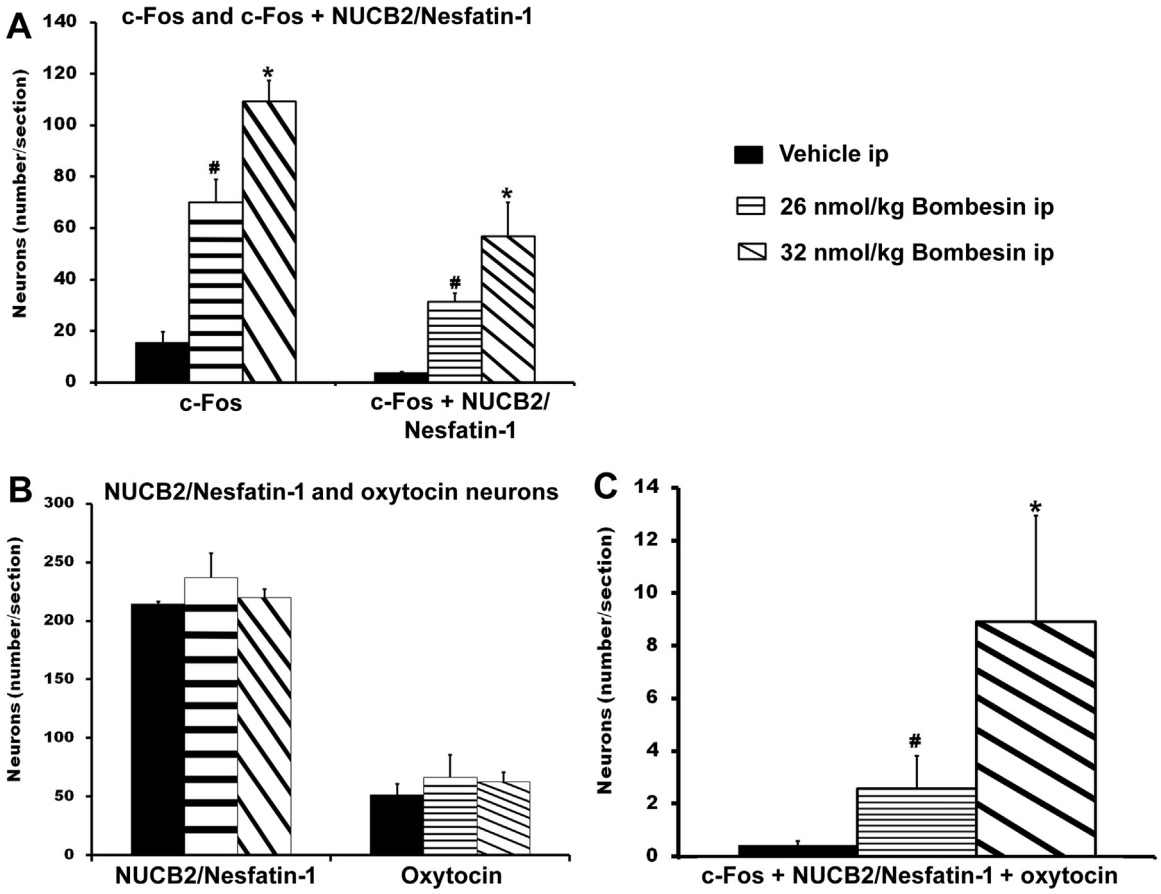

Fig. 2. Quantification of activated neurons in the paraventricular nucleus. Bombesin gradually increased the number of c-Fos immunoreactive neurons and the number of c-Fos positive NUCB2/nesfatin-1 immunoreactive neurons in the paraventricular nucleus (**A**), whereas the number of NUCB2/nesfatin-1 immunoreactive neurons and oxytocin immunoreactive neurons was not affected by bombesin injected ip (**B**). C-Fos positive NUCB2/nesfatin-1 immunoreactive neurons were partly co-localized with oxytocin. Quantification of these neurons showed a gradual increase of the number of triple-labeled neurons after ip injection of bombesin (**C**). Data are expressed as mean ± SEM of 4 rats/group. $^{*,\#} < 0.05$ *vs.* vehicle. Abbreviations: ip: intraperitoneal.

Interestingly, we found an increase in the number of c-Fos-labeled NUCB2/nesfatin-1 immunoreactive neurons in the PVN after bombesin administration, which points to an activation of nesfatinergic pathways through bombesin. Since it has been demonstrated that NUCB2/nesfatin-1 immunoreactive neurons in the PVN are colocalized with oxytocin (Noetzel et al., 2009; Kohno et al., 2008) we examined whether bombesin activates those neurons that express both, oxytocin and nesfatin-1. Although the activation of NUCB2/nesfatin-1 and oxytocin double-labeled neurons did not significantly increase, we found a trend toward significance. It may be speculated that the central oxytocin system serves as a downstream mediator for the mediation of the anorexigenic and/or hypertensive effects of bombesin *via* a cascade bombesin → nesfatin-1 → oxytocin. This assumption is supported by results of Maejima et al. who found an activation of oxytocin positive neurons in the PVN as assessed by c-Fos expression and Ca^{2+} influx into these neurons after injection of nesfatin-1 (Maejima et al., 2009). It has also been shown that nesfatin-1 induces oxytocin release from PVN neurons (Maejima et al., 2009). In addition, the oxytocin receptor antagonist H4928 blocked the food intake suppressive and hypertensive effects of nesfatin-1 after central injection (Yosten and Samson, 2010). However, bombesin administration failed to alter the pituitary secretion of oxytocin in rats (Verbalis et al., 1988) and central pretreatment with the oxytocin receptor antagonist vasotocin failed to alter bombesin-induced behaviors (Plamondon and Merali, 1997), suggesting the absence of direct interaction of these two peptides.

According to the fact that the NTS is sensitive and specific to the food intake suppressant effects of bombesin (Johnston and Merali, 1988), we also examined the increase in the number of c-Fos-labeled nesfatin-1 immunoreactive neurons in this brain nucleus after peripheral injection of bombesin. Comparable to the PVN, bombesin increased the number of activated NUCB2/nesfatin-1 immunoreactive neurons in the NTS. Some of these activated NUCB2/nesfatin-1 immunoreactive neurons also were tyrosine hydroxylase positive. Tracing studies showed that catecholaminergic neurons in the NTS send projections to the PVN (Sawchenko and Swanson, 1981). A blockade of noradrenergic transmission in the PVN inhibited oxytocin neurons (Ueta et al., 1993) and there is evidence that oxytocin containing neurons in the PVN are innervated by noradrenergic projections of the A2-cell group in the NTS (Kirchgessner et al., 1988). We showed before that NUCB2/nesfatin-1 immunoreactive neurons in the PVN are embedded in a dense network of catecholaminergic fibers (Noetzel et al., 2009). NUCB2/nesfatin-1 and oxytocin immunoreactive neurons in the PVN might therefore be activated by catecholaminergic projections originating from the A2-cell group.

It is important to note that we also found an increase in the number of NUCB2/nesfatin-1 immunoreactive neurons in the NTS (unlike the PVN), suggesting that bombesin might upregulate NUCB2 mRNA expression and protein translation in this nucleus. Therefore, the NTS might be the main target of peripheral bombesin.

Additionally, we observed some NTS neurons whose projections were stained by the nesfatin-1 antibody. So far NUCB2/

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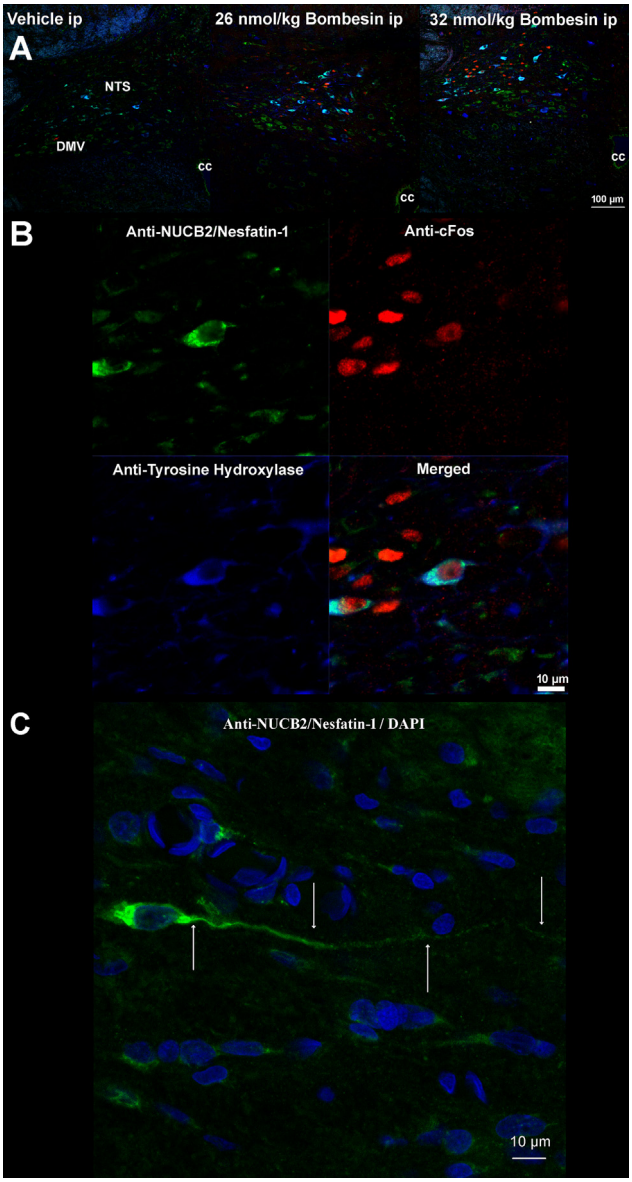

Fig. 3. In the nucleus of the solitary tract (NTS) intraperitoneal injection of bombesin resulted in an increase of c-Fos positive neurons (red fluorescence). The number of activated nesfatin-1 immunoreactive neurons (green fluorescence) also positive for tyrosine-hydroxylase (blue fluorescence) increased as well (turquoise fluorescence) (**A**). Panel (**B**) a higher magnification (from 32 nmol/kg bombesin image) with triple-labeling against c-Fos (red fluorescence), NUCB2/nesfatin-1 (green fluorescence) and tyrosine-hydroxylase (blue fluorescence). A merge of the single images resulted in turquoise fluorescent coloring of the cytoplasm of labeled neurons. In the NTS some NUCB2/nesfatin-1 immunoreactive neurons were not only stained by the anti-nesfatin-1 antibody in the regions of the soma but also the projections (**C**). DAPI was used for nuclear staining (blue fluorescence). The white scale bar represents 100 μm in (**A**) and 10 μm in (**B, C**). Abbreviations: cc, canalis centralis; DMV, dorsal motor nucleus of the vagus nerve; ip, intraperitoneal; NTS, nucleus of the solitary tract. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

nesfatin-1 staining in the brain was restricted to the soma and proximal processes of the neurons (Goebel et al., 2009; Goebel-Stengel et al., 2011; Foo et al., 2008). However, since also the nerve bodies can release cellular contents (Bustos et al., 2004; Landgraf and Neumann, 2004) NUCB2/nesfatin-1 was still thought to act as an extracellular transmitter. The present immunohistochemical finding supports this assumption.

In our present study we used two different doses of ip injected bombesin (26 and 32 nmol/kg). It could be argued that it would have been reasonable to administer a second dose of bombesin lower than 26 nmol/kg. This could have resulted in an even more distinct experimental outcome. However, the observed gradual effect of the two different bombesin doses point to a specific modulation of the number of c-Fos positive neurons in the PVN and NTS. Finally, the decision to use doses of 26 and 32 nmol/kg is supported by other experimental groups (Li and Rowland, 1996; Scarpignato and Bertaccini, 1981).

In conclusion, our current study shows that bombesin injected ip at two different doses gradually increases neuronal activation in the NTS and PVN, as assessed by changes in the number of c-Fos positive neurons. A proportion of activated neurons in the PVN and NTS was NUCB2/nesfatin-1 immunoreactive. Therefore, we postulate that NUCB2/nesfatin-1 immunoreactive neurons, particularly in the NTS, centrally mediate bombesin effects.

4. Experimental procedures

4.1. Animals

Male Sprague-Dawley rats (Harlan-Winkelmann Co., Borchen, Germany) with a body weight of 250–300 g were kept in groups of 4 rats/cage under controlled conditions of illumination (12:12 h light/dark cycle, lights on/off: 6:30 a.m./6:30 p.m.), temperature (22 ± 2 °C) and humidity. Animals were fed with a standard rat diet (Altromin™, Lage, Germany) and tap water *ad libitum*. For 14 days before the beginning of experiments, all animals were accustomed to experimental conditions and procedures. During the handling phase, the back position was repeatedly performed in order to make the rats familiar with receiving an ip injection.

Animal care and experimental procedures followed institutional ethic guidelines and conformed to the requirements of the state authority for animal research conduct.

4.2. Peptide and treatment

Bombesin (Pyr-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2 trifluoro-acetate salt, Bachem AG, Heidelberg, Germany) was dissolved in distilled water and stored at −20 °C. Immediately before performing the experiments, the peptide was diluted in vehicle solution consisting of sterile 0.15 M NaCl (Braun, Melsungen, Germany) to achieve a final concentration of 42 or 51 μg/kg (26 and 32 nmol/kg). Doses of bombesin were selected in accordance with previous studies (Li and Rowland, 1996; Scarpignato and Bertaccini, 1981). As we demonstrated before bombesin injected ip at doses of 4 and 8 μg/kg has no effect on the number of c-Fos positive neurons in the PVN or NTS (Kobelt et al., 2006). Therefore, we chose a higher dose of bombesin. It has to be mentioned that other experimental groups used even higher doses of bombesin (100 μg/kg injected ip) for c-Fos induction in the rat brain (Bonaz et al., 1993; Li and Rowland, 1996). Peptide solutions were kept on ice during the experiments.

Freely fed rats received an ip injection (final volume 0.5 ml) of vehicle solution (0.15 M NaCl, n = 4) or bombesin at two doses (26 and 32 nmol/kg body wt, n = 4) at the end of the dark phase. Animals continued to have *ad libitum* access to food and water. 90 min after the ip injection, animals were deeply anesthetized with ip injections of 100 mg/kg ketamine (Ketanest™, Curamed, Karlsruhe, Germany) and 10 mg/kg xylazine (Rompun™ 2%, Bayer, Leverkusen, Germany) and heparinized with 2.500 U heparin ip (Liquemin™, Hoffmann-La Roche, Grenzach-Whylen, Germany). Transcardial perfusion was performed as described before (Kobelt et al., 2004). After perfusion, brains were kept in a 5% w/v sucrose solution overnight and subsequently cut into 1.0–4.5 mm coronal blocks enclosing the respective hypothalamic regions using a brain

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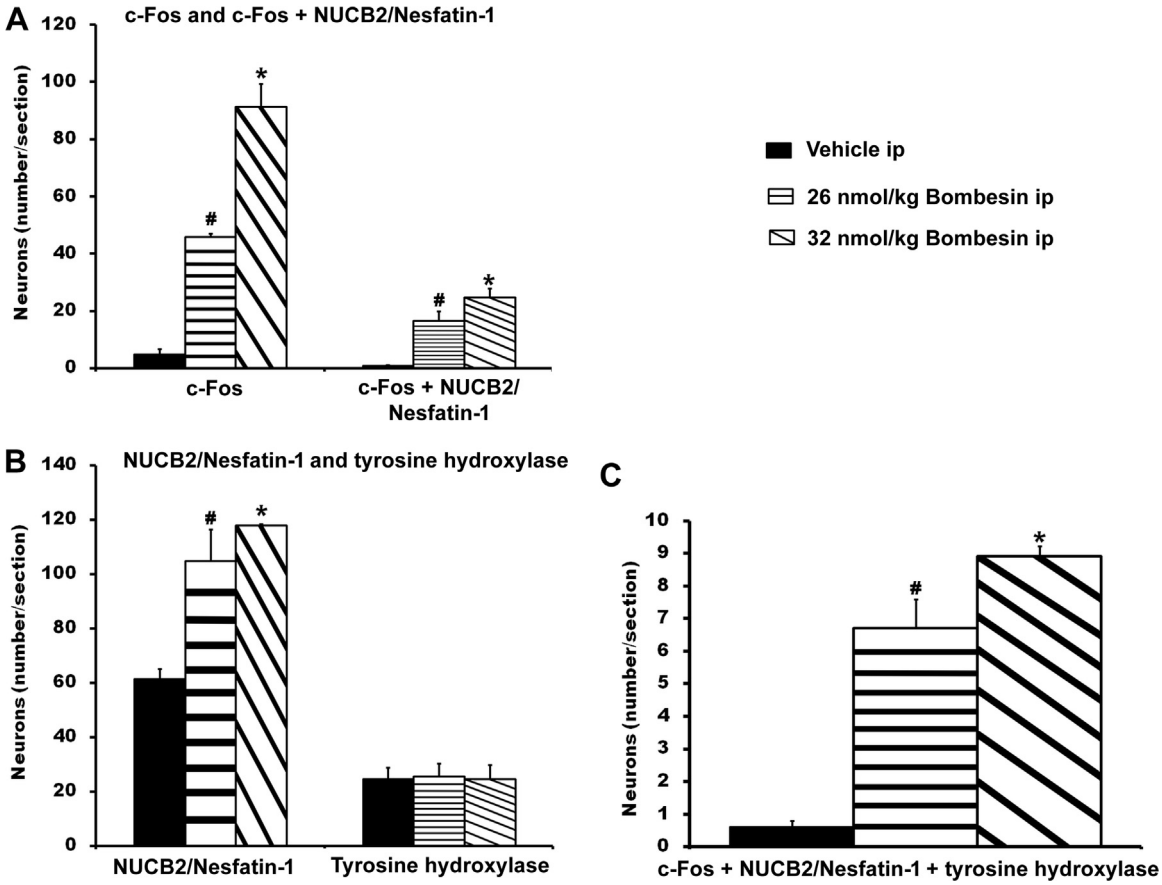

Fig. 4. Quantification of activated neurons in the nucleus of the solitary tract. Bombesin gradually increased the number of c-Fos immunoreactive neurons in the nucleus of the solitary tract. Additionally, the number of c-Fos positive neurons colocalized with NUCB2/nesfatin-1 immunoreactive significantly increased after bombesin injection compared to vehicle treatment (**A**). The number of NUCB2/nesfatin-1 immunoreactive neurons increased after the bombesin injection, while the number of tyrosine-hydroxylase immunoreactive neurons was not affected (**B**). Triple-labeling with c-Fos, NUCB2/nesfatin-1 and tyrosine-hydroxylase antibodies showed an increase of c-Fos positive NUCB2/nesfatin-1 immunoreactive neurons that were co-localized with tyrosine-hydroxylase (**C**). Data are expressed as mean ± SEM of 3 rats/group. $^{*,\#}$ $p < 0.05$ *vs.* vehicle. Abbreviations: ip: intraperitoneal.

matrix. For cryoprotection, blocks were exposed to a sucrose gradient (15% w/v and 27.3% w/v), then shock-frozen in hexane at −70 °C, and finally stored at −80 °C until further processing.

4.3. Immunohistochemistry

4.3.1. Staining for c-Fos immunoreactivity

Initially, 25-μm free-floating brain sections were pretreated with 1% w/v sodium borohydride in phosphate buffered saline (PBS) for 15 min. Thereafter, sections were incubated in a solution containing 1% (w/v) bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, USA), 0.3% (v/v) Triton X-100% and 0.05% (v/v) phenylhydrazine in PBS for 60 min to block unspecific antibody binding. The diluted primary antibody solution (rabbit anti-rat c-Fos, Oncogene Research Products, Boston, USA; 1:4000 in a solution of 5% w/v BSA, 0.3% [v/v] Triton X-100, and 0.1% [w/v] sodium azide in PBS) was applied for 42 h at room temperature. Subsequently, sections were washed with PBS and then incubated with the secondary antibody solution (goat biotin-SP-conjugated anti-rabbit IgG, Jackson ImmunoResearch Laboratories Inc., West Grove, USA; 1:1000 in 1% [w/v] BSA in PBS) for 12 h at room temperature. After rinsing with PBS three times, sections were incubated in avidin-biotin peroxidase complex (ABC; 1:200; Vector Laboratories, Peterborough, UK) in PBS for 6 h. Sections were rinsed with PBS three times again, and were then incubated in TSA™ tetramethyl rhodamine tyramide in amplification solution (PerkinElmer, Waltham, USA) for 10 min at room temperature. After washing with PBS, sections were stained with 4,6-diamidino-2-phenylindole (DAPI) for 15 min to counterstain cell chromatin. DAPI counterstaining was used in order to identify all relevant brain nuclei. Brain sections were finally embedded in 8 μl anti-fading solution (100 mg/ml 1,4-diazabicyclo[2.2.2] octane, Sigma-Aldrich; in 90% [v/v] glycerine, 10% [v/v] PBS, pH 7.4).

4.3.2. Double staining for c-Fos and NUCB2/nesfatin-1 immunoreactivity

Staining for c-Fos immunoreactivity followed the protocol described above. After rinsing with PBS three times, sections were incubated in PBS with 1% (w/v) BSA for 1 h and were then exposed to the second primary antibody solution (rabbit anti-nesfatin-1; Phoenix Pharmaceuticals, Inc; Burlingame, USA; 1:1000 in PBS containing 1% [w/v] BSA) for 24 h at room temperature. Fluorescein isothiocyanate (FITC) conjugated goat anti-rabbit IgG (Sigma-Aldrich) in PBS was applied for 12 h at room temperature after sections were rinsed with PBS three times. Finally, sections were washed, counterstained with DAPI, and embedded in anti-fading solution.

4.3.3. Triple-staining for c-Fos, NUCB2/nesfatin-1 and oxytocin immunoreactivity

For triple staining against c-Fos, NUCB2/nesfatin-1 and oxytocin we used the protocol described above. Additionally, we further incubated sections with the third primary antibody solution (monoclonal mouse anti-oxytocin; Chemicon International, Hofheim am Taunus, Germany; 1:7000 in 1% [w/v] BSA, and 0.1% [v/v] sodium azide in PBS) for 24 h at room temperature. After washing

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with PBS three times, brain sections were incubated overnight with the secondary goat anti-mouse IgG antibody (Alexa Fluor™ 633, Molecular Probes, Leiden, Netherlands; 1:400 in 1% [w/v] BSA). Then, sections were washed, counterstained with DAPI, and embedded with anti-fading solution.

4.3.4. Triple-staining for c-Fos, NUCB2/nesfatin-1 and tyrosine hydroxylase immunoreactivity

Triple staining against c-Fos, NUCB2/nesfatin-1 and tyrosine hydroxylase followed the protocol described above. We then further incubated with the third primary antibody solution (mouse anti-tyrosine hydroxylase; Sigma-Aldrich, 1:6000 in 1% [w/v] BSA, and 0.1% [v/v] sodium azide in PBS) for 24 h at room temperature. Brain sections were rinsed with PBS three times and then incubated overnight with the secondary goat anti-mouse IgG antibody (Alexa Fluor™ 633, Molecular Probes; 1:400 in 1% [w/v] BSA). Finally, sections were washed, counterstained with DAPI, and embedded with anti-fading solution.

4.4. Data and statistical analysis

Neurons with red nuclear staining were considered c-Fos immunoreactive. Every third of all consecutive coronal 25 μm sections was counted for c-Fos staining bilaterally in the PVN (bregma −1.30 to −2.12 mm), bilaterally in the ARC (bregma −2.12 to −3.60 mm) and bilaterally in the NTS (bregma −13.24 to −14.30 mm) according to the coordinates by Paxinos and Watson (1997). The other sections were used for immunohistochemical double and triple staining. All sections were analyzed using confocal laser scanning microscopy (cLSM 760, Carl Zeiss, Jena, Germany).

The average number of c-Fos immunoreactive cells per section for the brain nuclei mentioned above was calculated for three (in the NTS) and four rats (in the PVN and ARC) per experimental group. Also double (c-Fos/NUCB2/nesfatin-1) and triple labeled neurons (c-Fos/NUCB2/nesfatin-1/oxytocin and c-Fos/NUCB2/nesfatin-1/tyrosine hydroxylase) were quantitatively assessed in the PVN and NTS. The investigator was blinded to treatments received by the animals.

All data are presented as mean ± SEM and analyzed by ANOVA or ANOVA on ranks dependent on the distribution of the data. Differences between treatment groups were evaluated with the Holm-Sidak or Student-Newman-Keuls *post hoc* test. $P < 0.05$ was considered significant. All analyses were performed using SigmaStat 3.1 (Systat, San Jose, USA).

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Research report

Cholecystokinin and bombesin activate neuronatin neurons in the nucleus of the solitary tract

Malika Guggenberger[a], Kim-Marie Engster[a], Tobias Hofmann[a], Matthias Rose[a], Andreas Stengel[a,b], Peter Kobelt[a,*]

[a] Charité Center for Internal Medicine and Dermatology, Department for Psychosomatic Medicine, Charité-Medical University in Berlin, Corporate Member of Freie Universität Berlin, Humboldt-University in Berlin, and Berlin Institute of Health, Berlin, Germany
[b] Department of Psychosomatic Medicine and Psychotherapy, Medical University Hospital Tuebingen, Tuebingen, Germany

HIGHLIGHTS

- A large amount of neuronatin neurons can be found in the NTS and the DMV.
- Cholecystokinin and bombesin cause an activation of neuronatin neurons in the NTS.
- The activated neuronatin neurons are partly co-localized with tyrosine hydroxylase.

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ABSTRACT

Neuronatin (Nnat) is involved in the regulation of cellular molecular signaling and appears to be also linked to metabolic processes. The gastrointestinal peptides cholecystokinin (CCK) and bombesin (BN) have an effect on the short-term inhibition of food intake and induce neuronal activation in different brain nuclei, prominently in the nucleus of the solitary tract (NTS) involved in the modulation of food intake. The aim of the study was to examine if Nnat immunoreactivity is detectable in the NTS, and whether peripheral CCK-8S or BN cause c-Fos activation of Nnat neurons. Non-fasted male Sprague-Dawley rats received an intraperitoneal (i.p.) injection of 5.2 or 8.7 nmol CCK-8S/kg or 26 or 32 nmol BN/kg (n = 4 all groups) or vehicle solution (0.15 M NaCl; n = 7). The number of c-Fos neurons was determined 90 min post injection in the NTS and dorsal motor nucleus of the vagus (DMV). We observed Nnat immunoreactive neurons in the NTS and DMV. CCK-8S (25-fold and 51-fold, $p = 0.025$ and $p = 0.001$) and BN (31-fold and 59-fold, $p = 0.007$ and $p = 0.001$) at both doses increased the number of c-Fos positive neurons in the NTS. CCK and BN did not show a significant effect in the DMV. Both doses of CCK-8S (24-fold and 48-fold $p = 0.011$ and $p = 0.001$) and bombesin (31-fold and 56-fold, $p = 0.002$ and $p = 0.001$) increased the number of activated Nnat neurons in the NTS ($p = 0.001$) compared to the vehicle group, while in the DMV no significant increase of c-Fos activation was detected. In conclusion, i.p. injected CCK-8S or BN induce an increased neuronal activity in NTS Nnat neurons, giving rise that Nnat may play a role in the regulation of food intake mediated by peripheral CCK-8S or BN.

1. Introduction

The neuronatin (Nnat) gene is a paternally expressed imprinted gene encoding a small proteolipid, which was primarily known to be a brain-specific protein involved in neuronal differentiation (Dou and Joseph 1996a,b). Later on, it was also shown to be expressed in the pituitary gland, adrenal glands, uterus, skeletal muscles, ovaries, lungs and pancreas (Wijnholds et al., 1995; Niwa et al., 1997; Arava et al., 1999; John et al., 2001).

Previous studies suggested the physiological relevance of Nnat in metabolic regulation. A knockout of the Nnat-gene in mice appears to be correlated with hyperphagia and the development of obesity (Millership et al., 2018). In humans, several SNPs (single nucleotide polymorphisms) are associated with the development of severe juvenile and adult obesity (Vrang et al., 2010a,b,c). Additionally, altered paternal methylation patterns of the Nnat-gene seem to have an impact on

* Corresponding author at: Department of Medicine, Division Psychosomatic Medicine and Psychotherapy, Charité – Universitätsmedizin Berlin, Campus Mitte, Sauerbruchweg 5, 10117 Berlin, Germany.
E-mail address: peter.kobelt@charite.de (P. Kobelt).

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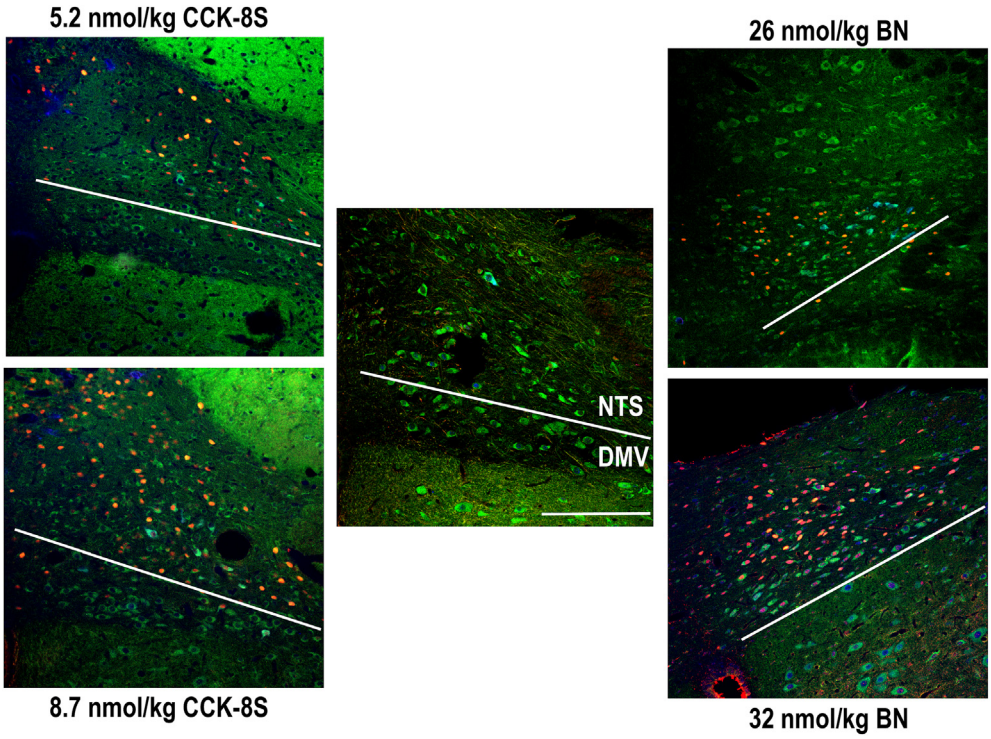

Fig. 1. CCK-8S and bombesin injected intraperitoneally dose-dependently increased the number of c-Fos immunoreactive neurons in the NTS but not in the DMV. While the number of activated (c-Fos; red fluorescence) neuronatin-ir neurons (green fluorescence) increased after bombesin (BN) and sulphated cholecystokinin 8 (CCK-8S) injection, the total number of neuronatin-ir and tyrosine hydroxylase-ir neurons (blue fluorescence) did not change compared to vehicle treat. The white scale bar represents 200 μm. The white line marks the anatomic division between NTS and DMV according to the anatomic landmarks given by the stereotactic atlas of Paxinos and Watson (Paxinos 1997). NTS = nucleus of the solitary tract, DMV = dorsomedial nucleus of the vagus.

genetical determination of obesity (Soubry et al., 2015, 2016; Dalgaard et al., 2016). Nnat mRNA is expressed in different brain nuclei involved in the regulation of food intake (Vrang et al., 2010a,b,c). The NTS is one of the key nuclei for the integration of meal-related and metabolic signals from the periphery and neuronal inputs from other brain regions (Loewy, 1991; Berthoud and Morrison, 2008; Rinaman, 2010; Grill and Hayes, 2012; Wu et al., 2012). Thus, it is also mediating the anorexigenic effects of gastrointestinal peptides like bombesin (BN) and cholecystokinin (CCK) (Crawley and Schwaber, 1983; Moran et al., 1988; Ladenheim and Ritter, 1993; Sutton et al., 2005; Sayegh, 2013). Both peptides reduce the meal size and have an effect on short-term inhibition of food intake (Gibbs et al., 1973, 1979; Kissileff et al., 1981; Johnston and Merali, 1988). The signaling pathways of the NTS mediating the short-term effects are – at least for the effect of CCK – well documented. The viscerosensoric signal of CCK is mediated by the CCK-A-receptor on vagal afferences projecting to the NTS in the brainstem (Gibbs et al., 1973; Raybould et al., 1985; Ritter and Ladenheim, 1985; Day et al. 1994; Baptista et al., 2005). However, the exact mechanisms about how activation of the NTS is leading to termination of a meal are yet to be fully characterized.

BN is a 14-amino acid peptide discovered in 1971 by isolation from the skin of the European frog, Bombina Bombina (Anastasi et al., 1971). After peripheral or central injection into brain regions like hypothalamic nuclei and the NTS, BN causes a reduction of food intake similar to CCK. (Gibbs et al., 1979; Gibbs, 1985; Kyrkouli et al., 1987; Johnston and Merali, 1988). The effects of BN are mediated by the two different G protein-coupled receptors for mammalian BN-like peptides, the neuromedin receptor (BB1) and the GRP receptor (BB2), which are distributed in the GI tract and in CNS regions such as the nucleus of the solitary tract (NTS) of the brainstem (Moran et al., 1988; Wada et al., 1991; Ohki-Hamazaki et al., 1997; Moody and Merali, 2004; Kamichi et al., 2005).

The mapping of brain neuronal circuits recruited by hormone signals has been achieved by evaluating changes of the immediate early gene c-Fos, which allows the identification of activated neurons at the cellular level (Dragunow et al., 1987; Sagar et al., 1988; Krukoff, 1993). According to numerous studies, changes in c-Fos showed that peripheral injection of CCK and BN causes neuronal activation in the NTS (Olson et al., 1992; Bonaz et al., 1993; Chen et al., 1993; Day et al., 1994; Li and Rowland, 1996; Engster et al., 2016).

Since the NTS is necessary for the regulation of food intake and for the effects of CCK and BN, we investigated whether Nnat neurons can also be found in the NTS through immunohistochemical staining. Furthermore, c-Fos activation in Nnat neurons of the brain has not yet been examined. Thus, the aim of the present study was to investigate whether peripheral injection of the peptide hormones CCK and BN is inducing c-Fos activation of Nnat immunoreactive (ir) neurons in the NTS and the dorsal motor nucleus of the vagus (DMV).

Previous studies implicated that the activation of catecholaminergic neurons in the NTS is partially involved in the effects of peripheral CCK and BN on food intake (Babic et al., 2009; Engster et al., 2016), and both peptides induced c-Fos in TH-expressing neurons of the NTS (Luckman, 1992; Travagli et al., 2006; Engster et al., 2016). Therefore, we also examined whether Nnat-ir neurons are co-localized with TH after peripheral injection of CCK or BN.

2. Results

2.1. Effects of i.p. injected CCK and bombesin on c-Fos expression and the number of neuronatin and tyrosine hydroxylase immunoreactive neurons in the nucleus of the solitary tract and the dorsal motor nucleus of the vagus in the brainstem

2.1.1. Nucleus of the solitary tract

Triple immunohistochemical staining showed that CCK-8S (5.2 or 8.7 nmol/kg) induced an increase of c-Fos immunoreactivity in the nucleus of the solitary tract (NTS) (mean ± SEM: 30 ± 15 and 62 ± 14 neurons per section, respectively; Figs. 1 and 2 A) compared to the vehicle group (1.2 ± 0.9 neurons per section, $p = 0.025$ and $p = 0.001$). The higher dose of CCK-8S caused a higher increase in c-Fos signals than the lower dose ($p = 0.027$). I.p. injection of bombesin (BN) (26 nmol/kg and 32 nmol/kg; Figs. 1 and 2 A) also strongly increased the expression of c-Fos positive neurons in the NTS (38 ± 8 and 71 ± 8 neurons per section, respectively) compared to the vehicle

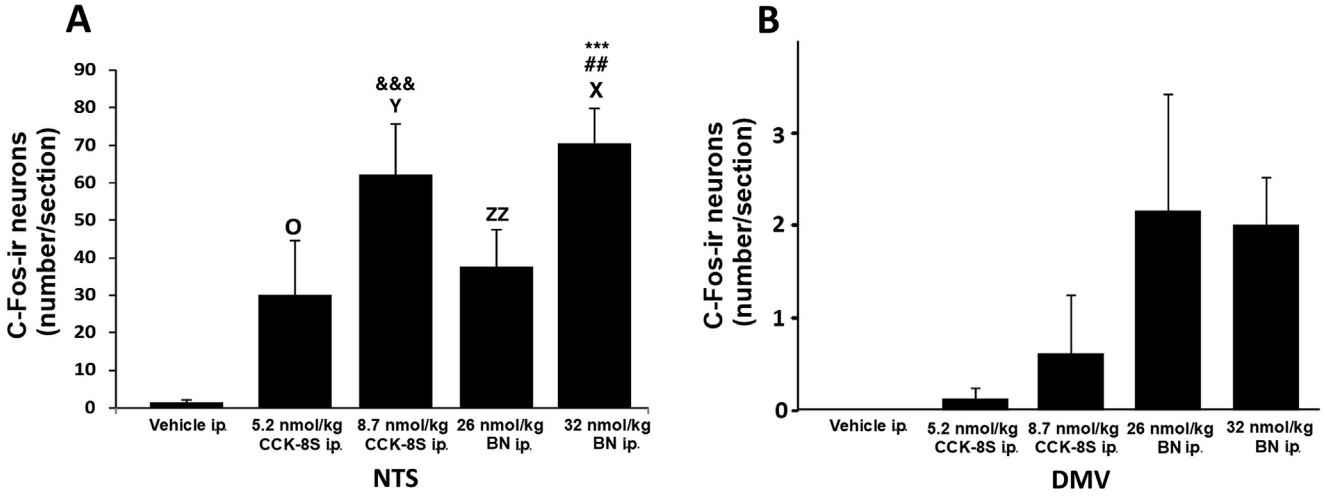

Fig. 2. In the NTS both peptides dose-dependently increased the number of c-Fos neurons compared to vehicle treated rats (A). In the dorsomedial nucleus of the vagus (DMV) CCK-8S and Bombesin (BN) did not increase the number of c-Fos neurons (B). Data are mean ± SEM of 4 (per treatment group) −7 (vehicle group) rats. $^{***}p < 0.001$, $^{\#\#}p < 0.01$, $^{X}p < 0.05$ *vs.* vehicle, *vs.* 5.2 nmol/kg CCK-8S and *vs.* 26 nmol/kg BN; $^{ZZ}p < 0.01$*vs.* vehicle; $^{\&\&\&}p < 0.001$, $^{Y}p < 0.05$ *vs.* vehicle, *vs.* 5.2 nmol/kg CCK-8S; $^{O}p < 0.05$ *vs.* 5.2 nmol/kg CCK-8S. NTS = nucleus of the solitary tract, DMV = dorsomedial nucleus of the vagus.

group (1.2 ± 0.8 neurons per section, $p = 0.007$ and $p = 0.001$; Fig. 2 A). The higher dose of BN caused more c-Fos activation than the lower dose ($p = 0.023$).

We detected a large number of neuronatin (Nnat)-ir neurons in the NTS. Interestingly, we also observed some nerve fibers of NTS neurons that were stained by Nnat antibody (Fig. 11). We also observed that vesicle-like structures were stained by the Nnat antibody (Fig. 11). Compared to vehicle treatment (62 ± 5 neurons per section), the number of Nnat-ir neurons in the NTS did not significantly increase after CCK-8S injection (69 ± 5 and 69 ± 10 neurons per section, $p > 0.05$; Fig. 3 A) or BN injection (71 ± 4 and 58 ± 3 neurons per section, $p > 0.05$; Fig. 3 A).

After CCK-8S injection, the number of c-Fos and Nnat-ir double labeled neurons in the NTS was greatly increased in a dose-dependent manner (19 ± 9 and 38 ± 7 neurons per section; Fig. 4 A) compared to the vehicle group (0.8 ± 0.6 neurons per section, $p = 0.011$ and $p = 0.001$). Also, the injection of BN caused a dose-dependent increase of activation (c-Fos) in Nnat-ir neurons (25 ± 4 and 45 ± 3 neurons per section, respectively; Fig. 4 A) compared to the vehicle group (0.8 ± 0.6 neurons per section, $p = 0.002$ and $p = 0.001$). The higher doses of CCK and BN caused a higher increase in activated Nnat-ir neurons than the lower doses, respectively ($p = 0.017$ and $p = 0.013$).

We also found tyrosine hydroxylase (TH) neurons in the NTS of all treatment groups. The number of TH-ir neurons in the NTS did not significantly increase after CCK-8S (20 ± 3 and 18 ± 3 neurons per section, respectively) or BN injection (19 ± 3 and 24 ± 8 neurons per section, respectively, $p > 0.05$; Fig. 5 A) compared to the vehicle group (10 ± 3 neurons per section), while we observed a significant increase of c-Fos positive TH neurons in the NTS after both doses of CCK-8S (10 ± 6 and 11 ± 3 neurons per section, respectively, $p = 0.026$ and $p = 0.014$) and BN (9 ± 2 and 12 ± 4 neurons per section, respectively; Fig. 6 A) compared to the vehicle group (0.2 ± 0.1 neurons per section, $p = 0.043$ and $p = 0.01$). Furthermore, we detected TH-ir neurons partly co-localized with Nnat. The number of the Nnat-ir neurons co-localized with TH after CCK-8S (19 ± 4 and 16 ± 2 neurons per section, respectively) or BN injection (18 ± 4 and 22 ± 8 neurons per section, respectively; Fig. 7 A) did not significantly increase compared to the vehicle group (9 ± 2 neurons per section, $p > 0.05$).

Triple staining in the NTS for Nnat, TH and c-Fos indicated that

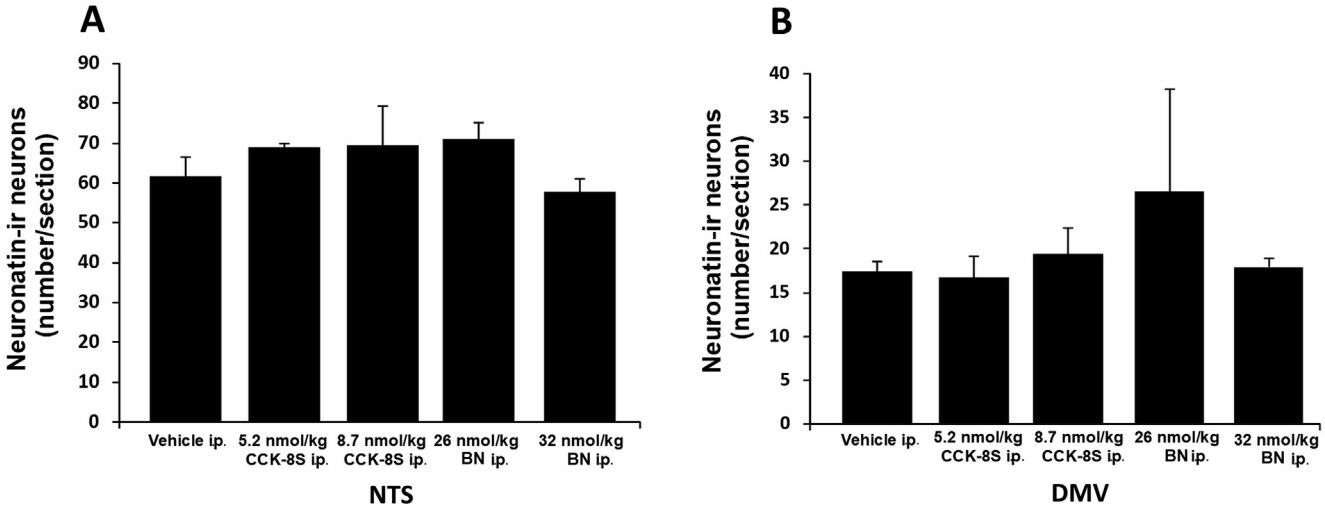

Fig. 3. The number of neuronatin neurons did not increase after i.p. CCK-8S or bombesin administration in the NTS (A) and the DMV (B) compared to the vehicle group. Data are mean ± SEM of 4 (per treatment group) and 7 (vehicle group) rats. NTS = nucleus of the solitary tract, DMV = dorsomedial nucleus of the vagus, BN = bombesin.

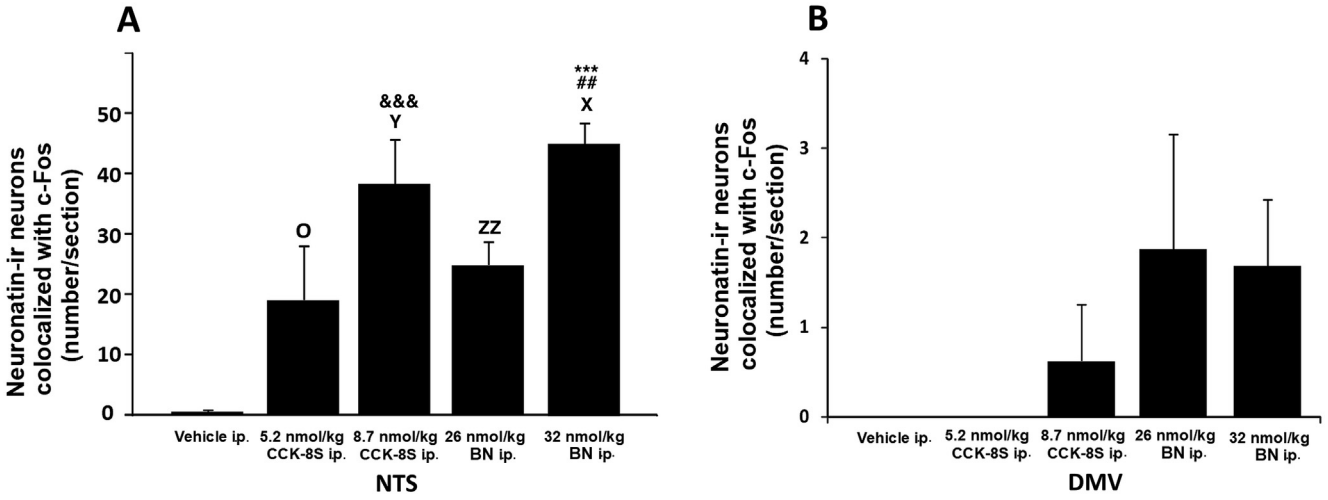

Fig. 4. Peripheral injection of CCK-8S and bombesin dose-dependently increased the number of activated (c-Fos) neuronatin-ir neurons in the NTS compared to the vehicle group (A). In the DMV both peptides did not affect the number of activated neuronatin-ir neurons (B). Data are mean ± SEM of 4 (per treatment group) and 7 (vehicle group) rats. $^{***}p < 0.001$, $^{\#\#}p < 0.01$, $^{x}p < 0.05$ *vs.* vehicle, *vs.* 5.2 nmol/kg CCK-8S, and *vs.* 26 nmol/kg CCK-8S; $^{ZZ}p < 0.01$ *vs.* vehicle, $^{\&\&\&}p < 0.001$, $^{Y}p < 0.05$ *vs.* vehicle and *vs.* 5.2 nmol/kg CCK-8S, $^{O}p < 0.05$ *vs.* vehicle. NTS = nucleus of the solitary tract, DMV = dorsomedial nucleus of the vagus, BN = bombesin.

activated Nnat-ir neurons are partly co-localized with TH. Treatment with BN induced an increase of c-Fos positive Nnat neurons co-localized with TH (9 ± 2 and 11 ± 4 neurons per section, respectively, Figs. 1, 8 A, 10) compared to the vehicle group (0.2 ± 0.1 neurons per section, $p = 0.09$ and $p = 0.001$). We observed no difference between the two doses of BN ($p > 0.05$) (Fig. 8 A). After treatment with CCK-8S (5 ± 3 and 5 ± 1 neurons per section, respectively, Figs. 1, 8 A, 9) we also found c-Fos, Nnat and TH-ir neurons in the NTS but it did not reach statistical significance compared to the vehicle group (0.2 ± 0.1 neurons per section, $p > 0.05$).

2.1.2. Dorsal motor nucleus of the vagus

In contrast to the NTS, in the DMV neither peripheral injection of CCK-8S (0.13 ± 0.13 and 0.63 ± 0.63 neurons per section) nor of BN (2.2 ± 1.3 and 2.01 ± 0.5 neurons per section, respectively) caused an increase of c-Fos positive neurons compared to vehicle treatment (0 ± 0 neurons per section, $p > 0.05$, Figs. 1, 2 B, 4B, 6B, 8B). Similar to the NTS, the number of Nnat-ir neurons in the DMV did, compared to the vehicle treatment (17 ± 1 neurons per section), not significantly increase after ip injection of CCK-8S (17 ± 3 and 19 ± 3 neurons per section, respectively, $p > 0.05$) or BN injection (27 ± 12 and 18 ± 1 neurons per section, $p > 0.05$, Figs. 1, 3 B). Unlike the NTS, there were no TH positive neurons or Nnat neurons co-localized with TH found in the NTS after vehicle treatment or after injection of CCK-8S or BN ($p > 0.05$, Figs. 1, 5, 7).

3. Discussion

In the present study, we observed neuronatin (Nnat) neurons in the nucleus of the solitary tract (NTS) and the dorsal motor nucleus of the vagus (DMV) and showed that peripheral injected CCK-8S and bombesin (BN) are both inducing a dose-dependent c-Fos activation of Nnat-ir NTS neurons in non-fasted rats. The c-Fos activation by higher doses of CCK-8S and BN (8.7 and 32 nmol/kg) and the lower doses of both peptides (5.2 and 26 nmol/kg) in the NTS are comparable. In contrast to the NTS, we observed no c-Fos activation in the DMV after peripheral injection of CCK-8S or BN, which is in line with previous studies (Engster et al., 2014, 2016; Zittel et al., 1999). We did not observe an increase of Nnat positive neurons after CCK-8S or BN injection in the NTS or DMV. Previous studies showed that peripheral CCK and BN

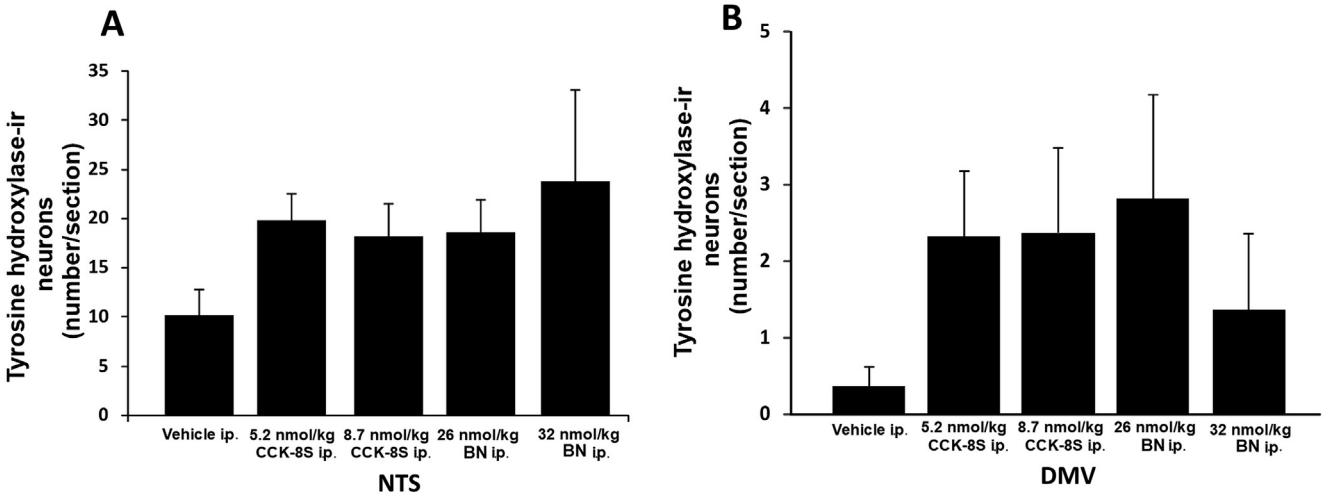

Fig. 5. Both peptides had no effect on the number of tyrosine hydroxylase positive neurons in the NTS (A) and DMV (B) after 90 min post i.p. injection. Data are mean ± SEM of 4 (per treatment group) and 7 (vehicle group) rats. NTS = nucleus of the solitary tract, DMV = dorsomedial nucleus of the vagus, BN = bombesin.

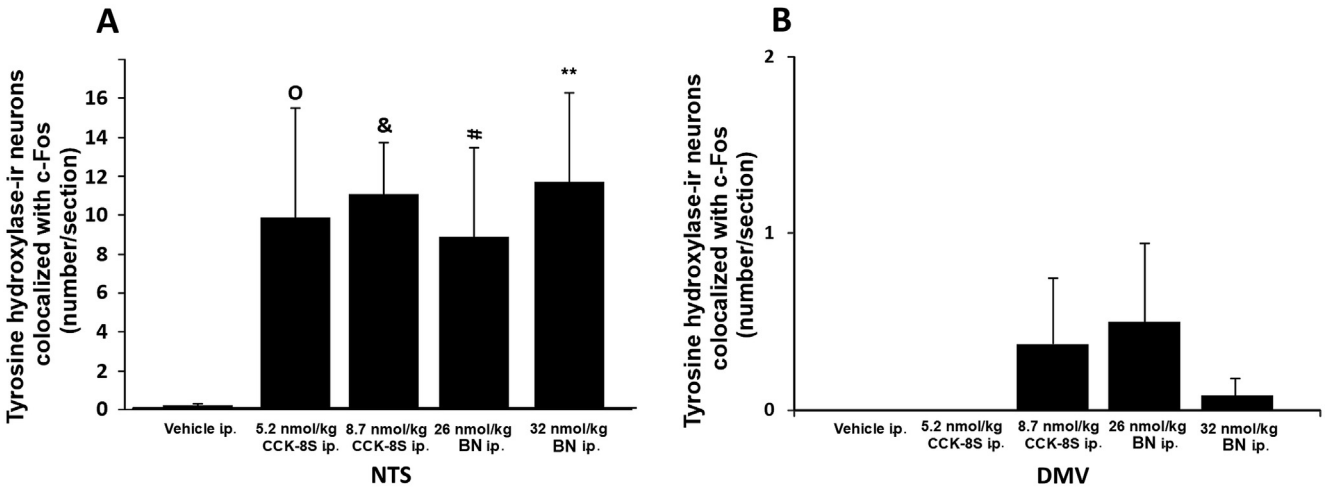

Fig. 6. After CCK-8S and bombesin injection we observed a significant increase of tyrosine hydroxylase positive neurons co-localized with c-Fos in the NTS (A) but not in the DMV (B) compared to the vehicle group. Data are mean ± SEM of 4 (per treatment group) and 7 (vehicle group) rats. $^{**}p = 0.01$, $^{\#}p < 0.05$, $^{\&}p < 0.05$, $^{O}p < 0.05$ *vs*. vehicle. NTS = nucleus of the solitary tract, DMV = dorsomedial nucleus of the vagus, BN = bombesin.

injections induced c-Fos immunoreactivity in TH expressing neurons of the NTS from where catecholaminergic projections are leading to several forebrain nuclei (Luckman, 1992; Travagli et al., 2006). In line with these studies, we found TH positive neurons in the NTS, which were activated by peripheral injection of CCK-8S and BN. These TH positive neurons were partially co-localized with Nnat.

The NTS and the DMV are important nuclei for the regulation of food intake by both CCK and BN. The visceral sensory signal of CCK initiating the short-term effect on appetite is mediated by the CCK-A receptor on vagal afferences leading to the NTS in the brainstem (Gibbs et al., 1973; Raybould et al., 1985; Ritter and Ladenheim, 1985; Day et al., 1994; Baptista et al., 2005). In the NTS, noradrenergic projections from the A2 cell group are activated which has been shown to be critical for the effect of peripheral CCK (Ricardo and Koh 1978; Smith et al., 1981a,b; Rinaman 2003). The resulting neural signal is transmitted from the NTS to the adjacent DMV which contains the preganglionic parasympathetic motoneurons that contribute vagal motor output back to the upper gastrointestinal tract (Travagli et al., 2006).

CCK still shows the same inhibitory effects on food intake in chronically decerebrated rats and these effects are in part inhibited by lesions in the NTS in rats (Crawley and Schwaber, 1984; Edwards et al., 1986; Grill and Smith, 1988). Thus, CCK appears to induce satiety at least partly *via* the brainstem. Unlike CCK, abdominal vagotomy does not impair the effect of BN (Smith et al., 1981a,b). Its inhibitory effect on food intake is mainly mediated by spinal afferent nerve fibers (Michaud et al., 1999). Both peptides, BN and CCK, have neuromodulating functions that are important for autonomic and behavioral effects. These effects are, at least in part, mediated by BN-containing vagal and spinal afferents to the NTS and the DMV (Stuckey et al., 1985; Lynn et al. 1996). The brainstem also appears to play a crucial role for the effects of BN. Lesion studies showed that the satiating effect of BN was impaired by lesions of areas like the area postrema, the NTS or the DMV, while lesions of the forebrain did not cause this effect (West et al., 1982; Bellinger and Bernardis, 1984; Geary et al., 1986). One of the effects of BN in the gastrointestinal tract is the secretion of CCK from the small intestine (Erspamer et al., 1974). Thus, it could be hypothesized that this might be a mechanism mediating the BN-related effects on the short-term termination of food intake. However, it was shown that BN and CCK have different effects on the termination of food intake. Compared to BN, CCK appears to have a stronger effect on the intake of liquid food than on solid food (Gibbs et al., 1979) indicating that the effects of BN on food intake might not be completely dependent

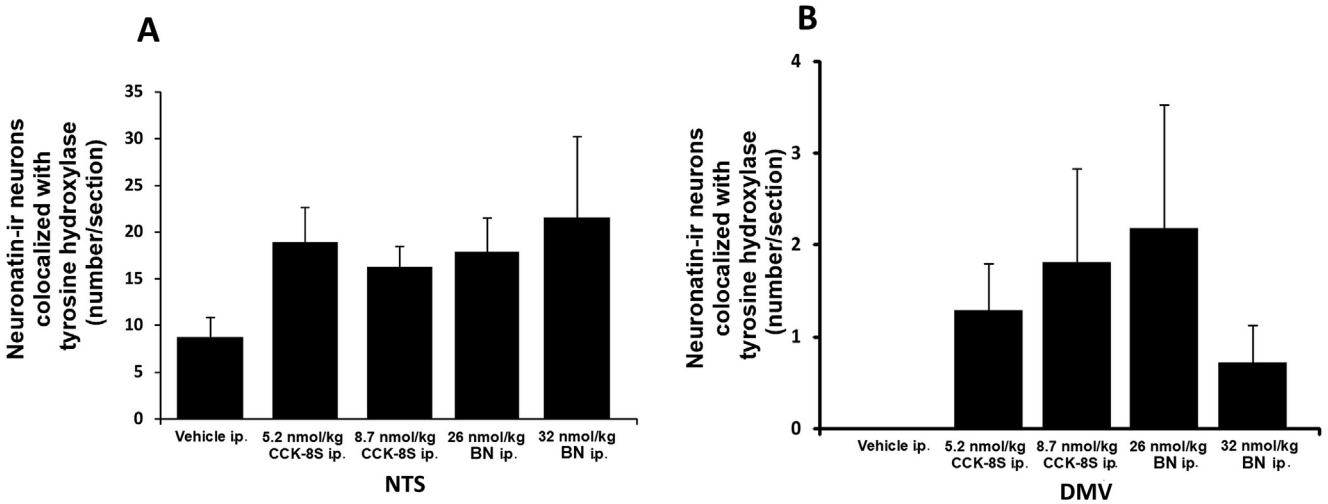

Fig. 7. Both peptides had no effect on the number of neuronatin neurons co-localized with tyrosine hydroxylase in the NTS (A) and in the DMV (B) 90 min after i.p. injection. Data are mean and SEM of 4 (per treatment group) and 7 (vehicle group) rats. NTS = nucleus of the solitary tract, DMV = dorsomedial nucleus of the vagus, BN = bombesin.

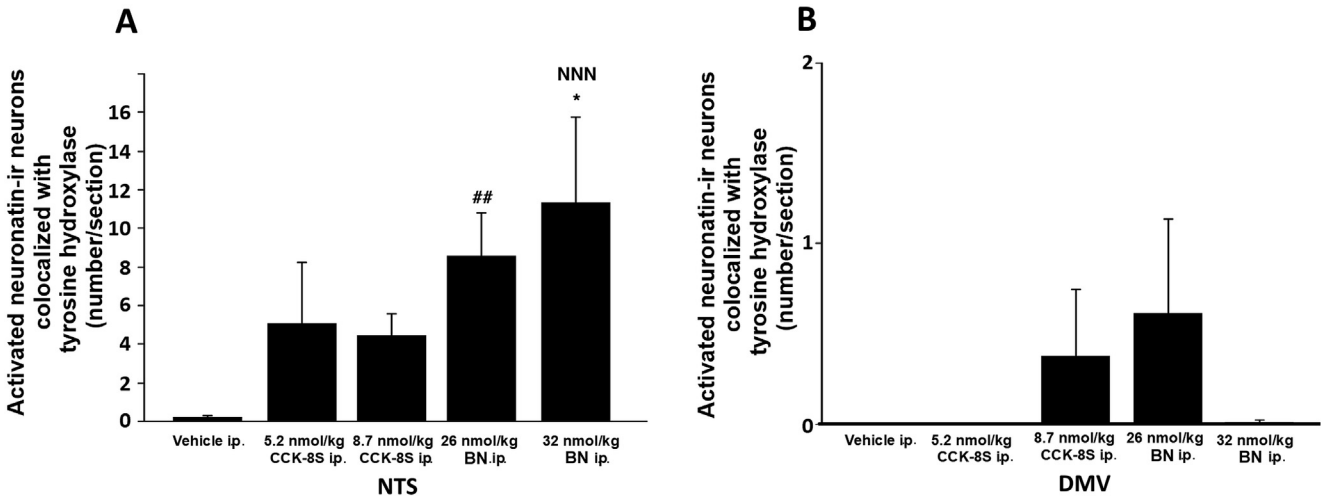

Fig. 8. In the NTS c-Fos positive neuronatin-ir neurons were partly co-localized with tyrosine hydroxylase after CCK-8S and bombesin i.p. injection. Quantification of the neurons showed that the number of triple-labeled neurons significantly increased after bombesin i.p. injection while injection of CCK did not reach a significant increase (A). In the DMV no significant effect was observed after i.p. injection of CCK-8S and bombesin. (B). Data are mean ± SEM of 4 (per treatment group) and 7 (vehicle group) rats. $^{NNN}p < 0.001$, $^{*}p < 0.05$ *vs.* vehicle and *vs.* 8.7 nmol/kg CCK-8S; $^{\#\#}p < 0.01$ *vs.* vehicle. NTS = nucleus of the solitary tract, DMV = dorsomedial nucleus of the vagus, BN = bombesin.

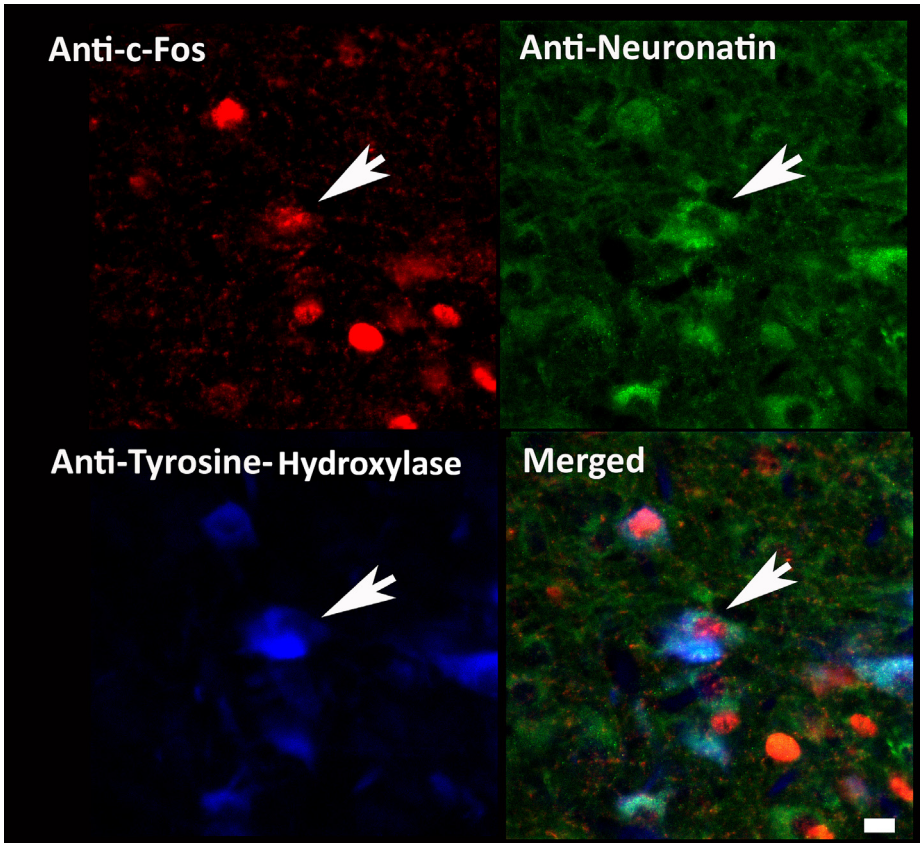

Fig. 9. Higher magnification from the CCK-8S image (8.7 nmol/kg) with triple-labeling against c-Fos (red fluorescence), neuronatin (green fluorescence) and tyrosine hydroxylase (blue fluorescence. Merging the single images resulted in turquoise fluorescent coloring of the cytoplasm of labeled neurons. The white scale bar represents 10 μm.

on CCK.

Previous investigations implicated the role of Nnat neurons in hypothalamic brain regions for the regulation of metabolic processes energy homeostasis and nutrient signaling and showed that Nnat mRNA expression in rodents is responsive to acute signals such as fasting or the peripheral injection of the satiety hormone leptin (Tung et al., 2008; Vrang et al., 2010a,b,c; Scott et al., 2013). Until now, there has been no reported interactions between Nnat and gastrointestinal peptides like CCK and BN. In the current study, we showed that Nnat positive neurons in the NTS are activated by the peripheral injection of CCK-8S and BN. Nnat co-localizes in neurons containing important mediators of appetite control such as cocaine and amphetamine regulated transcript (CART), melanin-concentrating hormone (MCH) and orexin in hypothalamic nuclei (Vrang et al., 2010a,b,c). In previous studies, it was shown that the neurocircuit from the NTS to the hypothalamus is involved in the termination of food intake and decrease of body weight, which is partly mediated by CCK-neurons as well as noradrenergic neurons (Roman et al., 2016, 2017; D'Agostino et al., 2016). It could be

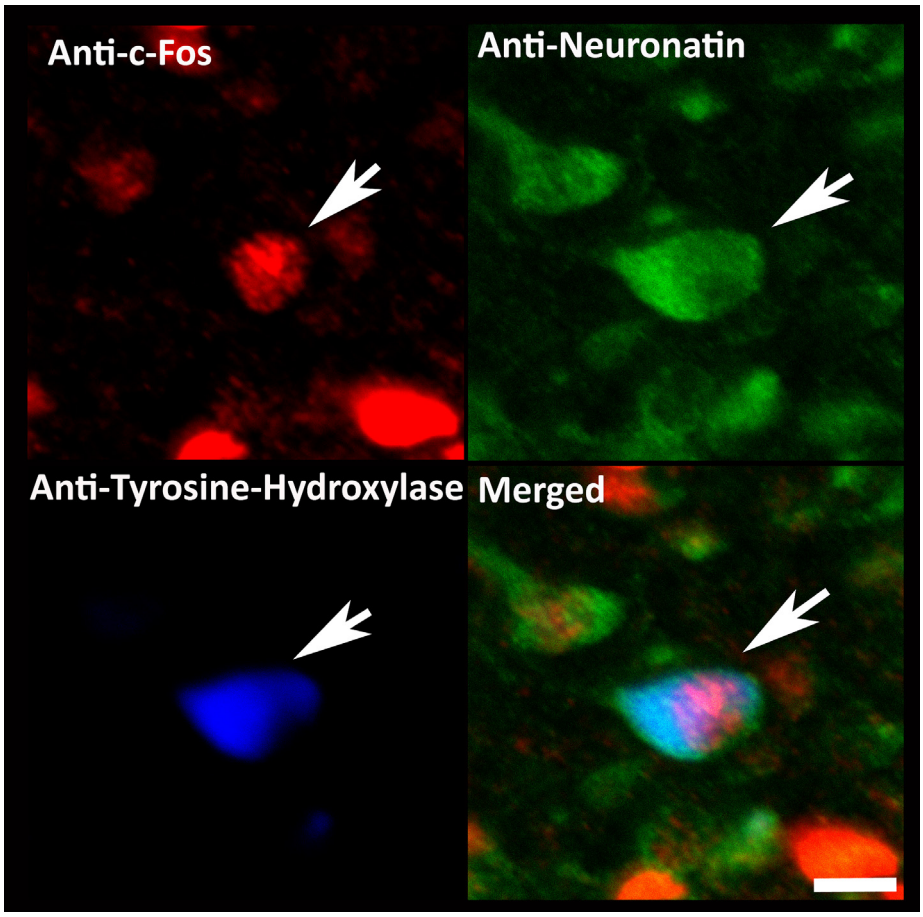

Fig. 10. Higher magnification from the bombesin image (26 nmol/kg) with triple-labeling against c-Fos (red fluorescence), neuronatin (green fluorescence) and tyrosine hydroxylase (blue fluorescence). Merging the single images resulted in turquoise fluorescent coloring of the cytoplasm of labeled neurons. The white scale bar represents 10 μm.

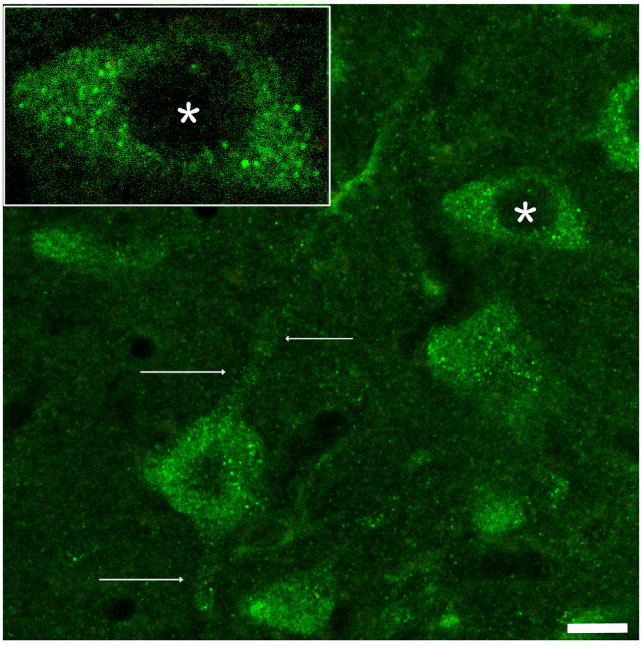

Fig. 11. We observed at higher magnification that some of the NTS neurons have neuronatin- ir nerve fibers (arrows). The insert displays a digital magnification of a neuronatin-ir cell (asterisk); it shows that the neuronatin-ir cell body of the neuron contains vesicle-like structures. The white scale represents 10 μm.

hypothesized that activated Nnat neurons might be involved in the interaction between the NTS and the hypothalamus regulating the energy homeostasis, which is partially mediated by CCK and noradrenergic neurons.

Tyrosine hydroxylase (TH) is the rate-limiting enzyme in catecholamine biosynthesis. Phosphorylation causes an increase in the TH activity which has been shown to regulate catecholamine synthesis (Lindgren et al., 2002). In previous studies, peripheral CCK or BN injections also induced c-Fos immunoreactivity in TH-expressing neurons of the NTS, which is in line with the current findings (Luckman 1992; Travaglin et al., 2006; Engster et al., 2016). Lesions of TH neurons showed to impair CCK-induced satiety (Rinaman et al., 1995; Babic et al., 2009), and the effect of CCK on decreasing food intake is correlated with the number of TH neurons in the NTS (Rinaman, 2003). At least for CCK, it is known that catecholaminergic pathways are crucial for its satiating effect (Ricardo and Koh, 1978; Smith et al., 1981a,b; Rinaman, 2003). In the current study, it has been observed that after the injection of CCK and BN, c-Fos-positive Nnat neurons were partly co-localized with TH. Thus, there might be a connection between catecholaminergic pathways and the activation of Nnat neurons mediating the effects of CCK and BN.

We observed numerous vesicle-like structures in the NTS neurons stained by the Nnat antibody, implicating that the Nnat immunoreactivity takes place in neuronal vesicles. A neuromodulating function could also be hypothesized. Other studies suggested a role of Nnat in cellular signal processing. Nnat has transcription factor binding sites and is involved in several intracellular signaling processes, for example in the regulation of glucose homeostasis and glucose-dependent insulin secretion (Chu and Tsai, 2005; Suh et al., 2005; Joe et al., 2008) or adipocyte differentiation, by increasing the intracellular Ca^{2+} level through antagonizing the Ca^{2+} ATPase in the endoplasmatic reticulum (Klemm et al., 2001). Furthermore, Nnat might also promote inflammatory processes including initiation of the nuclear factor-kb

pathway in response to dietary excess (Mzhavia et al., 2008). A study by Lin et al., showed that Nnat might initiate neural induction by increasing intracellular Ca^{2+} which increases the phosphorylation of ERK 1/2 (Lin et al., 2010). The MAPK/ERK pathway of signal transduction is involved in many physiological regulation processes such as cellular differentiation, embryogenesis or apoptosis (Santarpia et al., 2012). Interestingly, it has also been shown that exogenously injected CCK increases the MAPK/ERK signaling pathway in NTS neurons and that inhibition of this pathway attenuates the effect of CCK on the suppression of food intake in rats (Sutton et al., 2004, 2005). Since it is not clear which pathways are leading to the meal termination mediated by NTS activation, this could provide evidence for a crucial role of changes in intracellular signaling patterns. Therefore, a potential function of Nnat as a mediator of CCK-induced satiety – possibly mediated trough the ERK pathway – can be hypothesized. However, the potential relevance of ERK signaling and Nnat for homeostatic pathways should be part of further investigations. Since the Nnat protein is not only expressed in rat and mice brain nuclei, but also in anatomically equivalent human brain nuclei, Nnat neurons and potentially Nnat modulated neuronal networks could also have a physiological relevance for the regulation of metabolism in humans (Vrang et al., 2010a,b,c).

In summary, we detected Nnat neurons in the NTS and the DMV of the brainstem using immunohistochemical staining. CCK-8S and BN both caused a dose dependent increase of c-Fos in Nnat neurons in the NTS, while the different doses used for CCK-8S and BN had a comparable effect on the increase of c-Fos positive neurons. Furthermore, the activated Nnat neurons were party co-localized with TH. According to these findings, we hypothesize that Nnat is mediating effects on the energy homeostasis/food intake through cellular signaling pathways or neuromodulation. However, the interaction of the gastrointestinal peptides CCK and BN with Nnat could provide evidence that Nnat is involved in the short-term termination of meals mediated by the NTS. Previous studies also suggest a role of Nnat in the long-term regulation of energy homeostasis (Vrang et al., 2010a,b,c; Soubry et al., 2016). Thus, the activation of NTS Nnat neurons in different energy states and body constitutions should be subject to further investigations. In the present study, we detected Nnat neurons in the NTS and the DMV for the first time. Nnat mRNA in the NTS and its potential genetical alternations should be examined in order to provide a better comprehension of the interaction between peripheral, central and genetical mechanisms of energy homeostasis.

4. Experimental procedures

4.1. Animals

Male Sprague-Dawley rats (Harlan-Winkelmann Co., Borchen, Germany) weighing 150–180 g were housed under controlled conditions of illumination (12:12 h light/dark cycle, lights switched on at 6 a.m.), temperature (22 ± 2° C) and humidity, for a minimum of 21 days before the experiments. Previous studies show that elevated ghrelin plasma levels might be a reason for the reduced neuronal responsiveness to cholecystokinin (CCK) under fasting conditions (Maniscalco and Rinaman 2013; Maniscalco et al., 2020). Thus, animals were fed with a standard rat diet (Altromin™, Lage, Germany) and tap water *ad libitum*. For a period of 14 days all rats were accustomed to the experimental conditions by handling them daily and putting them in the position to mimic the procedure of intraperitoneal (i.p.) injection. At the time of the peptide treatment, the rats weighed 250–300 g. Experimental procedures and animal care were performed following institutional ethic guidelines and conformed to the requirements of the state authority for animal research conduct (protocol G 0067/15).

4.2. Peptides

In this study we dissolved sulphated CCK-8 (CCK-8S) and bombesin (BN) (Bachem AG, Heidelberg, Germany) in distilled water with 1% (v/v) 1 N NH4 OH, which was then aliquoted and stored at −20°C. Immediately before the start of the experiments, each peptide was further diluted in vehicle solution consisting of sterile 0.15 M NaCl (Braun, Melsungen, Germany) to achieve the final concentration of 5.2 and 8.7 nmol/kg (6 and 10 μg/kg) for CCK-8S and 26 and 32 nmol/kg (42 and 51 μg/kg) for BN. The doses of CCK-8S and BN were based on previous studies (Scarpignato and Bertaccini 1981; Li and Rowland 1996; Engster et al., 2016). Peptide solutions were stored on ice during the experiments.

4.3. Experimental protocol

All experiments started at the same time of day (between 10:00 a.m. to 10:30 a.m.), i.e. 3.5–4 h after the start of the light phase to achieve maximum consistency. *Ad libitum* fed rats received an ip injection (final volume: 500 μl) of vehicle solution (0.15 M NaCl; n = 7), CCK-8S (5.2 or 8.7 nmol/kg, n = 4/group) or BN (26 or 32 nmol/kg, n = 4/group) at 10 a.m. Animals continued to have free access to food and water. At 90 min after the injection, rats were deeply anesthetized with i.p. 10 mg/kg xylazine (Rompun™ 2%, Bayer, Leverkusen, Germany) and 100 mg/kg ketamine (Ketanest ™, Curamed, Karlsruhe, Germany) and heparinized with 2500 IU heparin (Liquemin™ , Hoffmann-La Roche, Grenzach-Whylen, Germany) injected i.p. Transcardial perfusion was performed as described previously (Kobelt et al., 2004). After dissection brains were kept overnight in a 5% w/v sucrose solution and subsequently cut into 1.0–4.5 mm coronal blocks. Blocks were exposed to a sucrose gradient (15% w/v and 27.3% w/v) for cryoprotection, thereafter shock-frozen in hexane at −70 °C, and finally stored at −80 °C until further processing.

4.4. Immunohistochemistry

4.4.1. Triple staining for c-Fos detection, neuronatin immunoreactivity and tyrosine hydroxylase immunoreactivitiy in the nucleus of the solitary tract and in the dorsal motor nucleus of the vagus in the brainstem

Prior to the staining, free-floating 25 μm brain sections were pretreated for 15 min with 1% (w/v) sodium borohydride in phosphate buffered saline (PBS). Subsequently, sections were incubated for 60 min in a solution containing 1% (w/v) bovine serum albumin (BSA) and 0.3% (v/v) Triton X-100, and 0.05% (v/v) phenylhydrazine in PBS to block unspecific antibody binding. Thereafter, the sections were pre-incubated 60 min in a solution of 0,1% [v/v] sodium azide 0,3% [v/v] Triton X-100 and 1% [w/v] BSA in PBS. After washing three times in PBS containing 0,1% [v/v] sodium azide, the diluted primary antibody solution (rabbit anti-c-Fos, Oncogene Research Products, Boston, MA, USA; 1:4000 in a solution of 5% [w/v] BSA, 0,1% [v/v] sodium azide and 0.3% [v/v] Triton X-100 in PBS) was applied for 24 h at room temperature. The sections were rinsed three times in PBS containing 0,1% [v/v] sodium azide and incubated with the secondary antibody solution (goat biotin-SP-conjugated anti rabbit IgG, Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA; 1:1000 in 1% [w/v] BSA and 0,1% [v/v] sodium azide in PBS) for 12 h at room temperature. After rinsing in PBS three times, sections were incubated for 6 h in avidin–biotin peroxidase complex (ABC; 1:200; Vector Laboratories, Peterborough, UK) in PBS. Thereafter, sections were again washed in PBS three times and then pre-incubated with borate-puffer. Then, they were incubated in TSA™ tetramethyl rhodamine tyramide in amplification solution (PerkinElmer, Waltham, MA, USA) for 10 min at room temperature. After washing three times in PBS, sections were incubated in PBS with 1% (w/v) BSA for 1 h and were then exposed to the second primary antibody solution (rabbit anti-neuronatin; Phoenix Pharmaceuticals, Inc.; Burlingame, USA; 1:400 in PBS containing 1% [w/v] BSA) for 48 h at room temperature. After rinsing them three times in PBS with 0,1% [v/v] sodium azide, sections were pre-incubated in PBS with 1% (w/v) BSA and 0.1% [v/v] sodium azide for

60 min. Thereafter, fluorescein isothiocyanate (FITC) conjugated goat anti-rabbit IgG (Sigma-Aldrich, Taufkirchen, Germany) in PBS containing 1% [w/v] BSA and 0,1% [v/v] sodium azide 0,1% was applied at room temperature for 12 h. Furthermore, we incubated with the third primary antibody solution (mouse anti-tyrosine hydroxylase; Sigma-Aldrich, 1:6000 in 1% [w/v] BSA, and 0.1% [v/v] sodium azide in PBS) for 24 h at room temperature. Brain sections were washed with PBS three times and then incubated for 12 h with the secondary goat anti-mouse IgG anti- body (Alexa Fluor™ 633, Molecular Probes, Inc., Eugene, OR, USA; 1:400 in PBS with 0.1% [v/v] sodium azide). Finally, after rinsing three times in PBS, sections were stained for 15 min with 4′,6-diamidino-2-phenylindole (DAPI) to counterstain cell chromatin. Thereafter, brain sections were embedded in anti-fading solution (100 mg/ml 1,4-diazabicyclo [2.2.2] octane, Sigma; in 90% (v/v) glycerine, 10% (v/v) PBS, pH 7.4).

4.5. Confocal laser scanning microscopy

Every third of all consecutive coronal 25 µm sections were counted for c-Fos staining bilaterally in the nucleus of the solitary tract (NTS) and the dorsomedial nucleus of the vagus (DMV) (bregma 13.24 to 14.30 mm). The distinction between the nuclei was made according to the anatomic landmarks given by the stereotactic atlas of Paxinos and Watson (Paxinos and Watson, 1997). Furthermore, the DMV shows a lower cell density and larger neurons compared to the NTS. To analyze all sections, we used confocal laser scanning microscopy (cLSM 760, Carl Zeiss, Jena, Germany). Neurons with red nuclear staining were considered c-Fos immunoreactive (ir). Neurons with green cytoplasmic staining were considered as neuronatin (Nnat)-ir cells and neurons with blue cytoplasmic staining as tyrosine hydroxylase (TH)-ir cells. The average number of c-Fos, Nnat and TH cells per section for the brain nuclei mentioned above was calculated for each rat. Also, single (c-Fos, Nnat, TH), double (c-Fos/Nnat, c-Fos/TH, Nnat/TH) and triple labeled neurons (c-Fos/Nnat/TH) were quantitatively assessed in the NTS and DMV. The investigator was blinded to injections received by the animals.

4.6. Statistical analysis

The software, SigmaStat 3.1., was used for statistical analysis. All data are presented as mean ± SEM and analyzed by ANOVA or ANOVA on ranks based on the distribution of the data. Differences between treatment groups were assessed with the Fischer LSD or Dunn's test. The level of significance was set at $p < 0.05$.

CRediT authorship contribution statement

Malika Guggenberger: Investigation, Conceptualization, Visualization, Writing - original draft, Formal analysis, Data curation. **Kim-Marie Engster:** Investigation. **Tobias Hofmann:** Resources. **Matthias Rose:** Resources. **Andreas Stengel:** Resources, Writing - review & editing. **Peter Kobelt:** Conceptualization, Methodology, Resources, Project administration, Validation, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Lebenslauf

"Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht."

Vollständige Publikationsliste

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