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Neurochemical mechanisms mediating LPS-induced anorexia

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1 Summary

Anorexia is a prominent component of the innate immune response during bacterial infections or other immune challenges. Over the last years substantial progress has been made in identifying the contributions of several peripheral components of the immune reaction to illness anorexia. However, neither how these peripheral events communicate with the brain nor which central neural mechanisms they activate to produce illness anorexia is clear. Several lines of evidence suggest that illness anorexia arises from altered neural processing within the same networks that mediate normal eating. For example, prostaglandin E_2 (PGE_2), brain serotonin (5-HT) and catecholamines (CA) all seem to play an important role in both normal eating and illness anorexia. A common experimental model of anorexia following gram-negative bacterial infection is bacterial lipopolysaccharide (LPS) administration. This thesis addresses the possible neurochemical mechanisms of LPS anorexia. We wanted to identify brain sites involved in the initial phase of LPS anorexia, which might also be necessary for the subsequent phases, and to identify possible neurotransmitters or receptors involved. Brain areas that have been implicated in the control of normal eating are the paraventricular nucleus (PVN), arcuate nucleus (Arc) and the central nucleus of the amygdala (CeA) in the forebrain, the hindbrain areas nucleus tractus solitarii (NTS), A1 area of noradrenergic ventrolateral medulla (A1), area postrema (AP) and the raphe nuclei such as the midbrain dorsal and median raphe nuclei (DR and MnR) and the hindbrain raphe pallidus nucleus (RPa) and raphe magnus nucleus (RMg).

In the first study, we further investigated the role of the 5-HT2C receptor (2CR) in LPS anorexia. Anatomically, we focused on the PVN, Arc and CeA because these areas receive 5-HT projections from the midbrain raphe. In particular, ascending 5-HT fibers contact hypothalamic neuropeptide Y and pro-

opiomelanocortin neurons via 5-HT1B receptors (5-HT_{1B}R) or 2CR and could therefore be the morphological substrate mediating the effect of 5-HT on eating.

In Experiment 1, we intraperitoneally (ip) injected male Long Evans rats with the potent and selective 2CR antagonist SB 242084 or vehicle 1 h before dark onset and with LPS or vehicle at dark onset, and we measured the effects on food intake for 23 h. Because our goal was to identify sites involved in the initial phase of LPS anorexia, we treated another group of rats identically, but sacrified them and measured expression of c-Fos protein 90 min after LPS injection. c-Fos protein was measured immunocytochemically, and the numbers of neurons expressing c-Fos was counted in all brain areas mentioned above. LPS alone reduced the nocturnal food intake starting 2 hours after ip injection and it increased the number of c-Fos expressing cells in all brain areas examined. Pretreatment with SB 242084 completely reversed LPS anorexia and, most relevant to our hypothesis that 5-HT connections from the midbrain raphe to the hypothalamus are crucial for LPS anorexia, pretreatment with SB 242084 completely reversed the LPS-induced increases in c-Fos expression in the PVN and CeA and tended to do so in the Arc. Besides that SB 242084 also reversed LPS-induced c-Fos increases in the DR, MnR, RPa, NTS and A1 and tended to do so in the RMg.

These findings indicate that 5-HT signaling via 2CR is necessary for LPS anorexia under our conditions and for much of LPS-induced neuronal activation. Because the reversal of LPS-induced c-Fos by SB 242084 was widespread, however, we could not identify a single likely pathway for the mediation of LPS anorexia.

As previous studies also implicated PGE_2 in LPS anorexia [1, 2] we performed a second series of studies to better understand the role of PGE_2 in LPS anorexia and especially to identify the brain areas where it may act to elicit LPS anorexia. The cyclooxygenase-2 (COX-2) antagonist NS-398 or vehicle was administered ip to rats 1 h before dark onset and 1 h later they were injected ip with LPS or vehicle. We examined first the effects on food intake within the first 23 hours and mapped brain regions in which PGE_2 was necessary for neuronal

activation, indicated again by c-Fos immunocytochemistry measured 90 min after ip LPS. Second, we investigated whether PGE_2 action in the midbrain raphe nuclei plays a role in LPS anorexia. In one set of rats, PGE_2 was microinjected directly into the DR and the effect on food intake was measured. In another set of rats, food intake in response to LPS was measured after NS-398 administration in the DR and MnR.

Pretreatment with NS-398 completely reversed LPS anorexia, suggesting that reduction of PGE_2 synthesis blocked LPS anorexia under our conditions, and reduced or eliminated LPS-induced c-Fos expression in several brain areas. indicating that PGE_2 is necessary for some or all LPS-induced neural activation. Intracranial injections of PGE_2 into the DR reduced food intake whereas intracranial injections of NS-398 into the DR and MnR reduced LPS anorexia. Therefore, the action of PGE_2 in the midbrain raphe might be required for ip LPS anorexia.

In the last study we investigated whether a graded anorectic effect of LPS could be observed using two different doses of ip LPS, and whether such a graded anorectic effect would be associated with a graded neural activation. Since our previous studies indicated the midbrain raphe to play an important role in LPS anorexia and given that this area is rich in 5-HT neurons we also performed a co-localization study of c-Fos and tryptophan-hydroxylase (TPH), a marker of 5-HT-ergic neurons in the DR, MnR and RPa. Additionally the NTS and ventrolateral medulla innervate the PVN with CA projections which are predominantly involved in the immune-responsive input to the PVN. Therefore, in this last study we also analyzed the co-localization of c-Fos and tyrosine-hydroxylase (TH), a marker of CA neurons, in the A1, PVN and NTS.

We observed a dose-related reduction in food intake in the initial 1-2 h after injection which was also associated with a dose-related increase in neural activation in the DR, PVN and Arc. Both doses significantly increased the c-Fos expression in the other brain areas examined, but these effects were not dose-related. These findings suggest that the graded LPS-induced anorectic effect is associated with a dose-related increase in neural activation in the DR, PVN and

Arc. We also observed significant increases in c-Fos expression in the DR, MnR and RPa neurons that were TPH-expressing neurons. Finally, the increased activation in the A1 by both doses of LPS may be CA-mediated because the c-Fos expression was significantly co-localized with TH positive neurons in this area, but not in the NTS or PVN.

In summary these findings support the hypothesis that several brain areas such as the midbrain raphe, PVN, CeA and Arc are involved in the initial phase of LPS anorexia and that 5-HT, PGE_2 and CA play a necessary role in LPS anorexia.

2 Zusammenfassung

Anorexie ist ein wichtiger Bestandteil der Reaktion des angeborenen Immunsystems während bakteriellen Infektionen oder bei anderen Herausforderungen des Immunsystems. In den letzten Jahren wurden wesentliche Fortschritte bei der Identifizierung der verschiedenen peripheren Komponenten des Immunsystems identifiziert, die zur Entstehung von Anorexie führen. Weitgehend unklar ist jedoch, wie periphere Signale mit dem Gehirn kommunizieren und welche zentralen Mechanismen sie aktivieren, um Anorexie auszulösen. Erkenntnisse aus unterschiedlichen Studien lassen vermuten, dass Anorexie bei Krankheit durch Aktivitätsveränderungen im gleichen neuronalen Netzwerk im Gehirn entsteht, welches normalerweise die Nahrungsaufnahme steuert.

Zum Beispiel scheinen Prostaglandin-E2 (PGE_2), Serotonin (5-HT), aber auch Katecholamine (CA) bei der normalen Steuerung der Nahrungsaufnahme, wie auch bei der Anorexie, eine wichtige Rolle zu spielen. Ein bekanntes experimentelles Model zur Untersuchung der bakteriell induzierten Anorexie ist die Verabreichung von Lipopolysacchariden (LPS).

Die vorliegende Dissertation befasst sich mit den möglichen neurochemischen Mechanismen der durch LPS induzierten Anorexie. Ziel war es, insbesondere diejenigen Hirnregionen zu identifizieren, welche in der Anfangsphase der LPS-induzierten Anorexie eine entscheidende Rolle spielen, sowie herauszufinden, welche Neurotransmitter und Rezeptoren involviert sein könnten. Hirnregionen, welche in die Steuerung der normalen Nahrungsaufnahme involviert sind, sind im Vorderhirn der hypothalamische Nucleus paraventricularis (PVN), der Nucleus arcuatus (Arc) und die zentralen Bereiche der Amygdala (CeA). Im Hinterhirn sind es unter anderem der Nucleus tractus solitarii (NTS), die A1-Region der norardrenergen, ventrolateralen Medulla (A1), die Area postrema (AP) und die Raphe-Kerne wie zum Beispiel im Mittelhirn der dorsale und mittlere Raphe-Kern (DR und MnR) sowie im Hinterhirn der Raphe pallidus-Kern (RPa) und der Raphe magnus-Kern (RMg).

In der ersten Studie untersuchten wir die Rolle der 5-HT-2C Rezeptoren (2CR) bei der LPS-Anorexie. Anatomisch konzentrierten wir uns dabei auf den PVN, den Arc und die CeA, da diese Hirnregionen von 5-HT-Fasern aus der Mittelhirnregion innerviert werden. Im Besonderen innervieren diese 5-HT-Fasern die hypothalamischen Neuropeptid Y- und Pro-opiomelanokortin-Neurone über 5-HT1B Rezeptoren (5-HT_{1B}R) oder 2CR. Diese Verbindungen könnten daher das morphologische Substrat sein, welches die Wirkung von 5-HT auf die Nahrungsaufnahme vermittelt.

Im ersten Experiment erhielten Long Evans-Ratten vor Beginn der Dunkelphase eine intraperitoneale (ip) Injektion des potenten 2CR-Antagonisten SB 242084 oder eine Kontrollinjektion. Zu Beginn der Dunkelphase erhielten sie eine zweite ip Injektion von LPS oder Kontrolllösung. Daraufhin wurde die Nahrungsaufnahme für 23 Stunden gemessen. Unser Ziel war es, Hirnregionen zu identifizieren, welche bei der durch LPS induzierten Anorexie eine entscheidende Rolle spielen. Daher wurde eine zweite Gruppe von Long Evans-Ratten identisch behandelt wie die erste Gruppe, mit dem Unterschied, dass diese Tiere 90 Minuten nach der LPS-Injektion getötet wurden, um die Expression des c-Fos-Proteins in den jeweiligen Hirnregionen zu messen. Das c-Fos Protein wurde immunozytologisch gemessen und die Anzahl der Neurone, die c-Fos exprimieren, wurde in den oben erwähnten Hirnregionen gezählt. LPS reduzierte die Futteraufnahme innerhalb der ersten 2 Stunden der Dunkelphase und erhöhte die Anzahl der c-Fos exprimierenden Neurone in allen untersuchten Hirnregionen. Die Vorbehandlung mit SB 242084 verhinderte die LPS-induzierte Anorexie vollständig. Im Einklang mit unserer Hypothese, dass 5-HT-Verbindungen vom Mittelhirn zum Hypothalamus für die LPS-induzierte Anorexie eine entscheidende Rolle spielen, führte die Vorbehandlung mit SB 242084 zu einer völligen Blockade der LPS-induzierten c-Fos-Expression innerhalb des PVN und des CeA. Derselbe Trend wurde auch im Arc beobachtet. Zudem reduzierte SB 242084 auch die LPS-induzierte c-Fos-Expression in DR, MnR, RPa, NTS und A1. Im RMg war der Trend ebenfalls vorhanden.

Diese Ergebnisse legen nahe, dass 5-HT, welches via 2CR Signale weiterleitet, unter unseren Bedingungen bei der durch LPS induzieren neuralen Aktivierung notwendig ist. Da aber die Reduktion der LPS-induzierten neuralen Aktivität durch SB 242084 nicht auf einzelne Hirnregionen beschränkt war, können wir aus unseren Ergebnissen nicht auf einen spezifischen Signalisationsweg schliessen.

Früheren Studien zufolge spielt auch PGE_2 eine wichtige Rolle bei der durch LPS induzierten Anorexie. Deswegen führten wir eine zweite Studie durch, um die Rolle von PGE_2 bei der LPS-induzierten Anorexie besser zu verstehen und ebenfalls insbesondere die dabei involvierten Hirnregionen zu identifizieren. Eine Stunde vor Beginn der Dunkelphase wurde einer Gruppe von Ratten der Cyclooxygenase-2 (COX-2)-Antagonist NS-398 oder entsprechende Kontrolllösung ip injiziert. Zu Beginn der Dunkelphase erhielten die Ratten eine zweite ip Injektion von LPS oder Kontrolllösung. Zuerst wurde der Effekt dieser Behandlungen auf die Nahrungsaufnahme innerhalb der ersten 23 Stunden gemessen; danach wurde wie bereits oben erwähnt 90 Minuten nach der 2. Injektion die neuronale Aktivität innerhalb der spezifischen Hirnregionen gemessen. Des Weiteren untersucht wir, ob PGE_2 in den Raphe-Kernen des Mittelhirns für die LPS-Anorexie eine Rolle spielt. Daher wurde bei einer weiteren Gruppe von Ratten der Effekt einer Mikroinjektion von PGE_2 in den DR auf die Nahrungsaufnahme untersucht. Eine zusätzliche Gruppe von Ratten erhielt schliesslich eine Mikroinjektion von NS-398 in den DR und MnR und der Effekt von LPS auf die Nahrungsaufnahme wurde ebenfalls untersucht.

Die Vorbehandlung mit NS-398 führte zu einer kompletten Blockierung der LPS-induzierten Anorexie. Dieses Ergebnis spricht dafür, dass die PGE_2-Synthese für die LPS-Anorexie unter unseren Bedingungen unabdingbar ist. Des Weiteren führte NS-398 zu einer totalen Eliminierung oder zumindest zu einer Reduktion der durch LPS induzierten c-Fos-Expression in mehreren Hirnregionen. Nach diesen Resultaten ist PGE_2 für die durch LPS ausgelöste neurale Aktivierung notwendig oder zumindest daran beteiligt. Die direkte Injektion von PGE_2 in den DR führte zu einer Reduktion der Nahrungsaufnahme und die Injektion von NS-

398 in den DR und MnR zu einer Reduktion der LPS-induzierten Anorexie. Dies weist darauf hin, dass PGE_2 an der Induktion der durch ip LPS ausgelösten Anorexie in den Raphekernen des Mittelhirns beteiligt ist.

In der letzten Studie wurde untersucht, ob zwei verschiedene ip verabreichte Dosen von LPS (12.5 und 100 μg/kg) auch zu einem unterschiedlich stark ausgeprägten anorektischen Effekt führen und ob ein derartiger Unterschied gegebenenfalls auch in einer unterschiedlichen neuronalen Aktivierung zu sehen ist. Unsere vorhergehenden Studien liessen eine wichtige Rolle der Raphekerne des Mittelhirns für die LPS-Anorexie vermuten. Diese Hirnregion ist reich an 5-HT-Neuronen. Daher führten wir in den Raphe-Regionen DR, MnR und RPa auch eine Kolokalisationsstudie mit c-Fos und Tryptophanhydroxylase (TPH) durch. Letzteres ist ein Indikator für 5-HT-Neurone, Zudem zeigten frühere Studien, dass der NTS und die ventrolaterale Medulla den PVN mit CA-Fasern innervieren und dass diese CA-Projektionen überwiegend in die Weiterleitung von Immunsignalen an den PVN involviert sind. Deswegen wurde innerhalb des A1, NTS und PVN eine weitere Kolokalisationsstudie mit c-Fos und Tyrosine-Hydroxylase (TH) durchgeführt, einem Indikator für CA-Neurone.

Wir beobachteten eine von der LPS-Dosis abhängige Reduktion der Nahrungsaufnahme innerhalb der ersten 1-2 Stunden nach der Injektion. Diese dosisabhängige Verzehrsreduktion ging mit einer dosisabhängigen neuralen Aktivierung innerhalb des DR, PVN und Arc einher. Beide LPS-Dosierungen erhöhten die c-Fos-Expression auch in allen anderen untersuchten Hirnregionen, aber diese Effekte waren nicht dosisabhängig. Diese Befunde weisen darauf hin, dass der dosisabhängige verzehrsreduzierende Effekt von LPS mit einer gleichzeitigen dosisabhängigen neuronalen Aktivierung in den Hirnregionen DR, PVN und Arc einhergeht. Wir beobachteten auch eine Zunahme der c-Fos Expression innerhalb der TPH-positivrn Neurone des DR, MnR und RPa.

Die Kolokalisationsstudie mit c-Fos und TH zeigte, dass die erhöhte neuronale Aktivierung innerhalb der A1-Region durch beide LPS Dosierungen CA-Neurone betrifft, denn die c-Fos exprimierenden Neurone waren auch TH-positiv. Im NTS oder PVN wurde jedoch keine Kolokalisation zwischen c-Fos und TH gefunden.

Insgesamt unterstützen diese Resultate die Vermutung, dass unterschiedliche Hirnareale wie die Raphe-Kerne des Mittelhirns, PVN, CeA und Arc in die initiale Phase der LPS-Anorexie involviert sind und dass 5-HT, PGE_2 sowie CA bei der LPS-Anorexie eine wichtige Rolle spielen.

3 General Introduction

3.1 The Innate Immune System

Infectious microorganisms entering the body activate innate immune system responses. The innate immune system uses four strategies in host defense: (1) Anatomic barriers, such as the skin and mucous membranes; (2) physiologic responses, such as fever, low pH and release of chemical mediators; (3) specialized immune cells (monocytes, neutrophils, macrophages, etc.), which internalize, kill, and digest whole microorganisms and (4) inflammatory responses, such as tissue damage or infection-induced leakage of vascular fluid containing serum proteins with antibacterial activity [3].

The macrophages, natural killer cells, neutrophils and mast cells are cells of the innate immune system which provide the primary defense against common bacterial infections. These cells can combat many different pathogens without requiring prior exposure to them. They eliminate pathogens by ingesting and digesting exogenous antigens, including whole microorganisms and insoluble particles as well as infected, injured and dead host cells. This process of ingestion is called phagocytosis.

Not all pathogens, however, are recognized and eliminated by the innate immune response. Some are eliminated by the lymphocytes of the acquired immune system. The two major populations of lymphocytes are the B lymphocytes (B cells) and T lymphocytes (T cells). B cells mature in the bone marrow. The antigen-binding or B cell receptor they express when they leave the bone marrow is a membrane-bound antibody molecule. When a naive B cell first encounters an antigen that matches its membrane-bound antibody, the B cell divides rapidly. The progeny further differentiate into memory B cells and effector, or plasma cells. Memory B cells express the same membrane-bound antibody as the parent B cell. Plasma cells, on the other hand, secrete newly formed antibodies and have little, or no membrane-bound antibodies [3].

T cells also arise from the bone marrow, but, unlike B cells, they migrate to the thymus gland for maturation. The T cells express a T-cell receptor on their membranes, but the recognition of an antigen by a T cell is only possible if the antigen is bound to a cell membrane protein called major histocompatibility complex (MHC). The class I MHC molecules are expressed by nearly all nucleated cells and class II MHC molecules only by antigen-presenting cells. Furthermore, there are two groups of T cells, the T helper (T_H) cells and the T cytotoxic (T_C) cells. After recognizing the antigen-MHC class II molecule complex, T_H cells are activated to become effector cells, which secrete various cytokines. These cytokines play an important role in the additional activation of B cells, T_C cells, macrophages, and various other cells that participate in the immune response.

As it takes about 4-7 days until the acquired immune response is effective, the innate immune response is essential for the initial response to infections.

3.2 The acute-phase response and anorexia

Homeostasis is defined as the maintenance of a variety of vital physiological parameters within a relatively narrow range that is optimal for function. Homeostatic regulation is the organizing principle of many behavioral and physiological responses. Therefore, if an organism is faced with an infectious microorganism or other immune challenges, it responds with a graded defense reaction, often starting as a local inflammation. Independent of whether bacteria (e.g., bacterial cell wall components, bacterial toxins, and DNA) or viral particles (e.g., viral RNA or glycoproteins) cause it, the organism responds by stimulating the release of immune factors that contribute to neutralizing or removing the invading pathogens. If the local inflammation fails to contain the pathogen, the defense reaction develops into the systemic acute phase response (APR). The APR is characterized by physiological changes including fever, increased slow-wave sleep, and increased hypothalamic-pituitary-adrenal (HPA) axis activity, and

by behavioral changes including anorexia, adipsia, depression and lethargy [4]. At least initially the APR is beneficial for the organism because it facilitates containment and elimination of the pathogen and eventually promotes tissue regeneration and healing [5]. The APR can become harmful, however, if its intensity or duration is uncontrolled.

Anorexia is an integral element of the APR. It is a complex response resulting from a number of signals derived from the immune, nervous, and endocrine systems. It accompanies acute and chronic infections, cancer and other diseases. During chronic diseases, the wasting often exceeds the effect of starvation because energy expenditure is increased in addition to anorexia. Moreover, the anorexia is accompanied by catabolic mechanisms which lead to tissue breakdown.

3.3 The LPS model of infection anorexia

Bacterial lipopolysaccharide (LPS) is a structural component of the cell wall of gram-negative bacteria, such as E. coli, and is a special target for the immune system. As most clinical signs of gram-negative bacterial infections in animals and humans, including the anorexia, are mimicked by LPS, LPS administration is a commonly used model for the study of innate immune responses. LPS has a core carbohydrate linked to a phospholipid (lipid A) anchored in the outer bilayer membrane of the intact bacteria. It is released during bacterial proliferation or lysis [6]. The lipid A portion of the LPS molecule is exposed [7] once the LPS is shed and can elicit a variety of biological responses by binding to two serum proteins, LPS-binding protein (LBP) and soluble CD14. LBP is a hepatic acute-phase protein which circulates in the blood, where it recognizes LPS and forms a high-affinity complex with it. Also, LBP seems to aid LPS in docking at the receptor complex, which is composed of the toll-like receptor-4 (TLR-4) and the

myeloid differentiation protein-2, by initially binding LPS and then forming a complex with CD14. Membrane bound CD14 (mCD14) is expressed by many immune cells and endothelial cells (EC). If mCD14 is absent, the circulating, soluble form of CD14 is involved in the response [8, 9]. Downstream of TLR-4 the myeloid adapter protein MyD88 initiates an intracellular signaling cascade which ultimately activates the transcription factors nuclear factor kappa beta (NFkB) and activating protein-1 to trigger the production of pro-inflammatory cytokines, prostanoids, and other mediators [10, 11]. The activation of these pathways is involved in LPS anorexia because interference with CD14, TLR4 [12] and MyD88 [13] signaling and with NFkB activation [14] all reduced the eating-inhibitory effect of LPS. In addition, the absence of MyD88 signaling completely eliminated anorexia in response to interleukin-1 beta (IL-1β) [13].

3.4 Other forms of illness anorexia

Another clinically important form of illness anorexia occurs during cancer, when metabolic actions of cytokines that are significant in chronic diseases and other tumor-derived substances also lead to cachexia [15]. The anorexia-cachexia syndrome during cancer is a complex metabolic condition affecting carbohydrate, protein, and fat metabolism. It results in anorexia, weight loss, wasting of skeletal muscle and negative nitrogen balance [16, 17]. In some cancers, cytokines produced by the tumor add to the cytokine response resulting from immune activation [18-21].

Anorexia also occurs in other diseases, such as inflammatory bowel diseases, ulcerative colitis and Crohns' disease [22]. In most of these diseases cytokines seem to play an important role in mediating the anorexia [23]. Therefore, the underlying mechanisms might overlap with those mediating LPS-induced anorexia.

3.5 Peripheral – central communication in APR

3.5.1 Cytokines as mediators of LPS anorexia

Most cytokines are glycoproteins. Their main role is in cell-to-cell signaling, via autocrine, paracrine, or endocrine (hormonal) modes of action. Cytokines are categorized as being pro-inflammatory (stimulatory) or anti-inflammatory (inhibitory).

Pro-inflammatory cytokines are the major endogenous mediators of local inflammation and the systemic APR, including anorexia [24]. Several pro-inflammatory cytokines, including IL-1, IL-2, IL-6, IL-8, IL-18, tumor necrosis factor alpha (TNF-α), interferon-γ (IFNγ), and ciliary neurotrophic factor (CNTF), have been shown to inhibit eating after peripheral or central administration [15, 25-27], in some instances synergistically [28, 29]. The activation of the immune cells and EC by LPS leads to the release of the pro-inflammatory cytokines. These and other findings suggest that pro-inflammatory cytokines contribute to LPS anorexia. Acute pharmacological or immunological antagonism of cytokines attenuates peripheral LPS-induced anorexia [30-35] more efficiently than genetic ablation of a particular cytokine or its receptor [11]. These discrepancies may be related to the overlapping and redundant actions of the cytokines and suggest a developmental compensation.

IFN-γ has been suggested to play a special role in LPS anorexia [36]. In contrast to mice with a genetic lack of other pro-inflammatory cytokines, IFN-γ knockout mice are insensitive to the anorectic effect of LPS [37], suggesting that IFN-γ is necessary for LPS anorexia. IFN-γ is mainly produced in natural killer cells and T-cells [38], neither of which express TLR-4 [39]. Thus, LPS presumably indirectly stimulates IFN-γ production. This is thought to be mediated by macrophage-derived IL-12, IL-18, and TNF-α [38, 40]. The main function of IFNy is to activate macrophages and EC [38], therefore it may function as a positive

feedback signal that enhances the signaling cascade that ultimately leads to LPS anorexia.

Most evidence indicates that endocrine signaling mediates peripheral LPS-induced anorexia. Ip injected LPS or IL-1β reduced eating in rats similarly after subdiaphragmatic vagal deafferentation (SDA) or sham vagotomy [41, 42]. As SDA is the most selective and specific method available to lesion abdominal vagal afferents, these data demonstrate that vagal afferents are not necessary for the eating-inhibitory effects of ip injections of LPS or IL-1β [43-45]. Also, celiac superior ganglionectomy did not alter the eating-inhibitory effects of LPS and IL-1β [41, 42], suggesting that splanchnic afferents are also not involved. Similar anorectic effects were observed after administration of LPS into the hepatic portal vein or vena cava, suggesting that the liver does not play a major role in LPS-anorexia [41]. On the other hand, the cytokine-induced activation of vagal afferents [46, 47] is involved in LPS-induced fever [48]. More specifically, experiments employing selective lesions of individual abdominal vagal branches have shown that the integrity of the hepatic vagal branch, but not of the celiac or gastric branches [49], is necessary for the development of fever in response to intravenous (iv) LPS [50]. Together, these findings suggest that the vagus is an important route of communication through which peripheral cytokines signal the brain in the mediation of peripheral LPS-induced fever, but not anorexia.

3.6 Other endocrine mediators: Leptin and Ghrelin

3.6.1 Leptin

Leptin is an adipokine produced by white adipocytes that is thought to participate in the normal control of appetite and energy expenditure [51-53]. LPS and pro-inflammatory cytokines increase the expression and production of leptin in adipose tissue [54-58]. Neutralization of circulating leptin by a leptin antiserum

reversed the eating-inhibitory effect of LPS [59]. This suggests that leptin contributes to LPS anorexia.

As is the case with most pro-inflammatory cytokines, leptin signaling is not necessary for LPS anorexia because obese Zucker rats, which have largely dysfunctional leptin receptors, showed similar LPS anorexia as lean Zucker rats did [60]. Also, leptin-deficient ob/ob mice and leptin receptor-deficient db/db mice exhibit LPS-induced anorexia, although the db/db mice appear to be less sensitive to this effect [61], which may reflect a contribution of leptin receptor signaling.

3.6.2 Ghrelin

Ghrelin is a peptide hormone secreted from gastric endocrine cells. It stimulates growth hormone and eating and is considered a candidate meal initiation signal in rodents and humans [62, 63]. LPS has been reported to decrease circulating ghrelin levels [64-66]. IL-1 receptor antagonism as well as the cyclooxygenase (COX) antagonist indomethacin prevented this decrease [66], suggesting that the phenomenon is mediated by IL-1β and prostaglandin (PG) - dependent pathways. Administration of ghrelin antagonizes the inhibition of eating and of gastric emptying induced by LPS. These findings suggest that reductions in ghrelin secretion might be involved in LPS anorexia, although it is unclear how LPS and cytokines might do this.

3.7 Blood-brain barrier mechanisms

Pro-inflammatory cytokines secreted in response to LPS are actively transported across the blood-brain barrier (BBB) and thus may act directly on neurons in the brain to elicit anorexia. [49]. Alternatively, cytokines can reach these neurons via the circumventricular organs (CVO) where the BBB is leaky (e.g., the organum vasculosum laminae terminalis, the subfornical organ, the AP

and the median eminence) [67]. Bacterial products and proinflammatory cytokines also increase BBB permeability [68]. Binding of cytokines and bacterial products to receptors on BBB EC causes activation of the transcription factor NF-kB [69] and triggers the release of nitric oxide (NO), prostanoids or eicosanoids [70-75], which, by acting on BBB EC, perivascular microglia or astrocytes, weaken the vascular tight junctions [76] and allow proinflammatory cytokines more direct access to the brain parenchyma. Finally, eicosanoids and PGE_2, in particular, may also act as downstream mediators of pro-inflammatory cytokines modulating neuronal function.

3.8 Downstream mediators of LPS and cytokine actions: Prostaglandins

Several cytokines (e.g., IL-1β, TNF-α, and IFN-γ) synergistically increase COX-2 mRNA expression in BBB EC [71, 77-80] and potently stimulate prostanoid production [73, 81]. COX and microsomal prostaglandin synthase (mPGEs) catalyze the transformation of arachidonic acid into PGE_2, which diffuses into the neuropil and activates its receptors (EPR). PGE_2 is the primary prostanoid produced by BBB EC in response to LPS, IL-1β, and IL-6 [82], but iv LPS also induces COX-2 like immunoreactivity (IR) in perivascular microglia and in meningeal macrophages throughout the brain [83].

PGE_2 is a key neuromodulator responsible for the induction of fever during infection [84-87]. The primary brain site in which body temperature is regulated is the preoptic area (POA) of the hypothalamus [88-90], where PGE_2 is thought to act directly or indirectly on thermoregulatory neurons [91]. PGE_2 is also involved in LPS anorexia. Non-specific COX inhibitors and the specific COX-2 inhibitor NS-398, but not the COX-1 inhibitor resveratrol, have been shown to attenuate the anorectic effects of LPS and IL-1β [2, 26, 92], indicating that COX-2 activation contributes to LPS anorexia. Concomitant with its eating-inhibitory effect, ip LPS

markedly increased PGE_2 concentration in the cerebrospinal fluid (CSF), but not in the plasma [2], and NS-398, but not resveratrol, blocked this increase.

Where PGE_2 might act to mediate LPS anorexia is unclear. Intracerebroventricular (icv) [93] as well as direct administration of PGE_2 into the lateral hypothalamus [94] has been shown to reduce eating in rats, but the doses used were high, so that the specificity of the observed effects is questionable. Interestingly, ip or icv administered IL-1β induced a robust up-regulation of mPGEs-1 in the brain [95]. mPGEs-1 catalyses the last step of PGE_2 biosynthesis. IL-1β failed to decrease food intake in mPGEs-1 knock out mice [95], but these animals still reduced eating in response to PGE_2. These findings suggest that mPGEs-1 is essential for IL-1β-induced anorexia.

All four PGE_2 receptor subtypes (EP1-4) are widely distributed in the brain. Somatodendritic EP3R in the POA are supposed to mediate fever because mice lacking this receptor failed to show a febrile response to PGE_2, IL-1β, or LPS [96]. EP3R expressing neurons in the POA project directly to the raphe pallidus nucleus (RPa) [97], which contains premotor neurons controlling sympathetic drive to brown adipose tissue (BAT) [98]. BAT is the major organ for experimentally induced fever in the rat [99]. Other brain areas may be involved in the induction of fever as well. The paraventricular nucleus (PVN) for instance is the major site of autonomic control, and lesions of the PVN reduced the febrile responses to icv PGE_2 or ip LPS administration [100]. Lacroix and Rivest [101] reported that PGE_2 activates PVN neurons that express corticotrophin-releasing hormone (CRH) and that this activation is reversed by indomethacin. More specifically, Zhang et al. [102] demonstrated that iv LPS or IL-1β induced expression of EP4R mRNA in PVN CRH neurons in rats. It is currently unknown, however, which EPRs are involved in the eating-inhibitory effect of LPS.

3.9 Neuroanatomy and neurochemistry of LPS anorexia

Several brain areas and neurotransmitters are involved in the control of normal eating. Ip injection of LPS elicits expression of c-Fos protein in many of these brain areas, for example, in the forebrain, the central nucleus of the amygdala (CeA), in the hypothalamus, the PVN and arcuate nucleus (Arc) [101, 103-105], and in the hindbrain nucleus tractus solitarii (NTS) [101, 103, 105-107], area postrema (AP) [103, 105, 107], and A1area of the ventrolateral medulla [101, 103, 105]. LPS also elicits c-Fos expression in several raphe nuclei, including the midbrain dorsal and median raphe nuclei (DR and MnR) [105, 108]. At present, however, neither the brain sites that are initially activated by LPS-induced signals nor the relative contribution of specific brain areas to different elements of the APR is well understood.

3.9.1 PVN

Systemic LPS administration causes a strong and prolonged activation of the HPA-axis [109]. LPS activates transcription and synthesis of CRH in the PVN [104], and inhibition of CRH release by lesion of this hypothalamic area abolished the effects of LPS on the activation of the HPA-axis [109]. This effect may be mediated by PGE_2 acting through EP4R on PVN CRH neurons [102] because central administration of PGE_2 induced c-Fos expression in PVN CRH neurons [110] and because indomethacin blocked the neuronal activation in the PVN induced by LPS [101, 111] or IL-1β [112-114]. CRH might also play a role in LPS anorexia because central CRH antagonist administration attenuated IL-1β induced anorexia [115]. The PVN receives input from several other brain sites including the Arc [116-118], the CeA [119], the A1 and NTS [120] and the midbrain raphe nuclei [121-124].

3.9.2 Arc

It has been reported that LPS induces neural activation in the Arc, as measured by c-Fos mRNA and c-Fos protein [101, 103-105]. One population of Arc neurons expresses anorexigenic pro-opiomelanocortin (POMC). Activation of some of these neurons causes release of the neurotransmitter α-melanocyte stimulating hormone (α-MSH), which in turn activates melanocortin (MC) 3/4-receptors (MC3/4-R). The POMC neurons of the Arc project to neurons in the PVN [116-118] and other areas that express MC3/4-R. Localized PVN injections of the MC3/4-R agonist MTII inhibited eating, whereas injections of MC3/4-R antagonist SHU9119 or MC4-R antagonist HS014 increased eating [125-127]. MC also appears to be involved in LPS anorexia because exogenous α-MSH enhanced [128], whereas disruption of MC signaling by central administration of SHU-9119 [128] or agouti-related protein (AgRP) [129] inhibited LPS-induced anorexia. AgRP is a potent endogenous orexigen that stimulates eating mainly through competitive antagonism of central MC3/4 receptors [130]. The eating-inhibitory effect of LPS is also attenuated in MC4-R knock-out mice [129].

3.9.3 CeA

The CeA is robustly activated by systemic IL-1β [112, 113, 120], and it plays a role in the activation of the HPA axis. There is a direct neural projection from the CeA to the PVN, but this involves only a small number of cell bodies [119, 131-134], and IL-1β-activated neurons in the CeA do not correspond to those that project directly to the PVN [120]. This suggests that the CeA contributes to the HPA axis response to immune challenges via an indirect pathway. However, LPS and IL-1β may also activate neurons in the lateral part of the parabrachial nucleus (PBN). Retrograde tracer studies indicate that the lateral PBN has an important

influence on the CeA c-Fos response to immune activation [135-137]. Interestingly, the PBN may be indirectly activated by Arc POMC neurons that project to the PBN [138] and activate MC4-R on neurons which express calcitonin gene-related peptide (CGRP) [139, 140]. These CGRP neurons, in turn, project to the CeA [140] and express EP3R [141, 142]. Thus, infection-related signals also may affect the central circuitries controlling food intake and energy balance through PGE_2 and EP3R in the PBN.

3.9.4 Midbrain raphe nuclei

The midbrain DR and MnR are of interest because they are rich in serotonergic (5-HT) neurons and because the role of brain 5-HT in the inhibitory control of eating is well established. For example, an increase in 5-HT activity or postsynaptic 5-HT receptor stimulation reduces eating [143, 144]. 5-HT is one of the possible neurochemical mediators of LPS anorexia. Peripheral administration of LPS or IL-1β increased the neurochemical response of 5-HT neurons, as indicated by increased tryptophan or 5-hydroxyindoleacetic acid (5-HIAA) concentrations [145, 146]. LPS anorexia was attenuated after midbrain raphe injections of the 5-HT1A autoreceptor agonist ($5\text{-}HT_{1A}R$) 8-OH-DPAT [147]. These findings implicate midbrain raphe 5-HT neurons in LPS anorexia because the $5\text{-}HT_{1A}R$ function mainly as negative-feedback, somatodendritic autoreceptors [148]. LPS anorexia was also attenuated by the specific 5-HT2C receptor (2CR) antagonist SB 242084 [149] in rats [150, 151] and mice [152]. 5-HT signaling via the 2C and 1A receptors seems to be sufficient for the role of 5-HT in LPS anorexia because antagonism of other 5-HT receptors such as 1B, 2A or 3 receptors ($5\text{-}HT_{1B}R$, $5\text{-}HT_{2A}R$ or $5\text{-}HT_3R$) failed to do so [151].

The mechanism via which 5-HT neurons are activated during LPS anorexia is not clear yet. One possible mediator is PGE_2. As discussed above, PGE_2 appears important in LPS anorexia because raphe 5-HT neurons express PGE_2 EP3R [153] and are activated by PGE_2 [114, 154].

Forebrain areas that are known to be involved in the normal control of eating, such as the PVN, Arc and CeA, are innervated by 5-HT fibers from the midbrain raphe [121-124, 155]. These fibers synapse on hypothalamic Arc and PVN neurons that express 2CR and 5-HT_{1B}R [156, 157]. The 2CR in the PVN [158-161] appear to be necessary for LPS-induced HPA-axis activation [162] because the activation of magnocellular neurons is prevented by 5-HT depletion with the irreversible inhibitor of tryptophane-5-hydroxylase para-chlorophenylalanine [103].

In summary, several studies implicate 5-HT in LPS anorexia and suggest that 5-HT neurons of the midbrain raphe nuclei and their interconnections with the forebrain via 5-HT_{1B}R or 2CR may be involved in the mediation of LPS-induced anorexia, although the mechanisms of these interactions are not clear yet. Aside from the role of 5-HT in LPS anorexia there is evidence for a relation between cancer anorexia and increased hypothalamic 5-HT neurotransmission [163]. The synthesis of 5-HT depends on the availability of the amino acid precursor tryptophan [164], and tryptophan levels increase in the plasma and brain parenchyma during growth of some tumors in animals and man [165, 166]. This increase is proposed to lead to an increase in 5-HT synthesis and release. In line with this idea, an increase in hypothalamic 5-HT concentrations was observed in anorectic, tumor bearing rats [164, 167, 168]. Interestingly, tumor removal normalized the hypothalamic 5-HT levels and improved their food intake [168]. Finally, intrahypothalamic injection of the 2CR antagonist mianserin reduced tumor anorexia [169]. All in all, these data strongly support a role of hypothalamic 5-HT in cancer anorexia.

3.9.5 NTS and A1

Brainstem (NTS and A1) catecholamines (CA) are also implicated in the neuroendocrine response to peripheral immune activation, and they are integral to the stimulation of the HPA [170, 171]. For example, discrete lesions of medullary

CA pathways projecting to the PVN prevent c-Fos induction in the PVN following acute IL-1β challenge. [120]. Via direct, predominantly CA projections, the ventrolateral medulla (VLM) also provides input to the PVN after LPS administration [136].

In the NTS, LPS activates mainly non-CA neurons [101]. These neurons also project to the PVN [172] and express several neuropeptides, including glucagon-like peptide-1 (GLP-1) [173-175]. This is interesting because these GLP-1 neurons are activated after systemic administration of LPS [176] and appear to be involved in LPS anorexia. For example, GLP-1 R antagonist delivered to the fourth ventricle substantially reduced the anorexic response to LPS [177]. Neurons upstream of GLP-1 neurons in this circuit may include POMC and CRH neurons, which have been shown to be involved in LPS anorexia as well [115, 128].

The NTS is also a primary target for viscerosensory inputs transmitted via the vagal and glossopharyngeal nerves [178]. As reviewed above, however, although vagal afferents appear to be involved in LPS-induced fever [48], they are not necessary for LPS anorexia because the eating-inhibitory effects of low ip doses of LPS or IL-1β were not affected by SDA [43-45]. The NTS also receives fibers from several brainstem areas such as the DR [179], the raphe magnus nucleus (RMg) [179, 180] and the RPa [180], which could also be involved in its activation.

3.9.6 AP

The AP does not seem to play an important role in mediating LPS anorexia or immune activation of the hypothalamus because lesions of the AP and the adjacent part of the NTS did not eliminate the anorectic effects of ip IL1β [181] or ip LPS [182], and did not reduce hypothalamic c-Fos expression in response to iv IL-1β [114]. However, a role for the AP in regulating the HPA responses was suggested by the finding that AP lesion blocked the IL-1-induced elevation of

plasma corticotrophin and corticosterone as well as c-Fos expression in the PVN [183].

3.9.7 Hindbrain raphe nuclei

A contribution of the RPa to LPS anorexia has not been proposed previously, but can not be excluded. Neurons of the RPa can be activated by ip LPS as well as other inflammatory mediators [103, 105]. This area, however, is thought to be primarily involved in the hyperthermic and gastrointestinal effects of LPS. The RPa receives descending projections from the POA, which contribute importantly to the induction of fever [88-90] (see above). Nakamura et al. demonstrated that the rostral RPa relays neural signals from the POA to the peripheral sympathetic effectors contributing to fever development after admistration of PGE_2 into the POA [97]. Similarly, the RMg is mainly implicated in the modulation of pain perception. Whether it may also be involved in mediating LPS-induced anorexia, remains to be investigated.

In summary, PGE_2, 5-HT, and other neurotransmitters and neuropeptides which control normal eating and energy balance are apparently also involved in signaling LPS anorexia. This is consistent with the idea that LPS anorexia arises from altered neuronal processing within the same network that is involved in the control of normal eating. At present, however, neither the brain site(s) that are initially activated by LPS-induced signals nor the relative contribution of specific brain areas or neurochemicals to the anorexia and other elements of the APR is well understood.

4 Thesis aims

The overall aim of the present work was to further investigate the neural mechanisms of LPS anorexia. We used immunocytochemical methods to identify the brain areas involved in mediating anorexia elicited by ip injections of bacterial LPS. Neurons that are activated in response to a variety of stimuli can be identified by immunocytochemical detection of expression of c-Fos protein, the product of the immediate-early gene *c-fos*, which is expressed beginning about one hour after electrophysiological activation of some populations of neurons [184]. c-Fos expression is often used to characterize the distribution and number of individual neurons activated during eating and numerous other functional states. We here used this technique to identify the brain areas activated by LPS.

The doses of LPS we used in our studies have been shown to mimic the clinical features of gram-negative bacterial infection or septicemia [185-187] and to reduce food intake in rats [92, 186, 188], but to not produce indications of endotoxin shock [185]. It is therefore reasonable to relate the doses that we used in our study to normal pathophysiological responses to moderate gram-negative bacterial infections and generalized inflammation [189], including the anorexia. This is important because many previous studies used much higher doses of LPS to investigate the effect on neuronal activation in the brain [101, 103, 105, 107, 190]. Also, we focused on brain areas that are related to autonomic and endocrine control and to the control of normal eating, such as PVN, Arc, CeA [101, 103-105], NTS [101, 103, 105-107], AP [103, 105, 107], A1 noradrenergic neurons in the VLM [101, 103, 105] and the raphe nuclei such as DR [105, 108], MnR, RPa [103, 105] and RMg.

The first study was performed to further confirm and investigate the role of 5-HT and specifically of 2CR signaling in LPS-induced anorexia in rats using the 2CR antagonist SB 242084 [149]. The first experiment characterized the effects of SB 242084 pretreatment on LPS anorexia. In the second experiment we used immunocytochemical techniques to assay the numbers of cells expressing c-Fos

in brain sites where altered neuronal activity, either in 5-HT neurons or in their projection sites, might cause anorexia. We measured c-Fos expression 90 min after LPS injection in order to assess brain areas with increased electrophysiological activity at about the time anorexia begins.

To better understand the role of PGE_2 in LPS anorexia, and more specifically to identify the areas of the brain in which PGE_2 may act to elicit anorexia following ip injections of LPS, we performed a second series of experiments. First, we identified a situation in which pretreatment with the COX-2 inhibitor NS-398 effectively reduced LPS anorexia and then mapped brain regions in which PGE_2 was necessary for neuronal activation. Again we measured c-Fos expression 90 min after LPS injection. To investigate more specifically whether PGE_2 action in the midbrain raphe nuclei might play an important role in mediating LPS anorexia, we performed a third experiment in which PGE_2 was microinjected directly into the DR and measured the effects on food intake. In addition, we also performed a study in which NS-398 was administered directly into the DR and MnR at the same time when rats received ip LPS injections, to investigate whether there is a specific role of PGE_2 in the midbrain raphe nuclei in LPS anorexia.

In the last study we employed two different doses of ip LPS to investigate whether a graded anorectic effect of LPS could be observed, and if so, whether there is also a graded LPS effect on neural activation, which might help to identify crucial brain sites involved in LPS anorexia. To examine whether the midbrain raphe neurons activated by LPS are 5-HT neurons, we also performed a co-localization study using double immunofluorescence techniques. We investigated the co-localization within these areas of c-Fos and tryptophan-hydroxylase (TPH), a marker of 5-HT neurons. Finally, we also performed another co-localization study to investigate the role of CA neurons by using double immunostaining for c-Fos and tyrosine hydroxylase (TH) in the A1 area as well as in the PVN and the NTS. The CA neurons in the NTS and A1 play a critical role in the induction of the HPA response to immune related stimuli [120]. Further, the NTS and the A1 provide immune-responsive input to the PVN after LPS administration via direct, predominantly CA projections [136].

5 Serotonin-2C receptors are necessary for LPS-induced anorexia and c-Fos expression

5.1 Introduction

Anorexia is a prominent component of the innate immune response during bacterial infection and other immune challenges. Although substantial progress has been made in identifying the contributions of several components of the peripheral immune system to illness anorexia (reviewed in [191, 192]), it is not yet clear how such peripheral events are communicated to the brain or what central neural mechanisms underlie illness anorexia (reviewed in [191, 192]).

Several lines of evidence suggest that illness anorexia arises from altered neuronal processing within the same networks that mediate normal eating [23, 191-193]. For example, brain serotonin (5-HT) appears to play an important role in both normal eating [194] and illness anorexia [23, 191-193]. In healthy animals, agonists to the 5-HT1B (5-HT_{1B}R) and 5-HT2C receptors (2CR) reduce food intake [195] and antagonists to these receptors attenuate the anorectic effect of the 5-HT agonist d-fenfluramine [156], a drug that blocks the reuptake of 5-HT and stimulates its release [196, 197]. The paraventricular (PVN) and arcuate (Arc) hypothalamic nuclei and the central nucleus of the amygdala (CeA), all of which are implicated in the normal control of eating, are innervated by 5-HT fibers from the midbrain raphe [121-124]. These fibers connect to hypothalamic pro-opiomelanocortin (POMC) and neuropeptide Y (NPY) neurons by 2CR and 5-HT_{1B}R, respectively [156, 157], and could therefore provide the anatomical substrate for the effect of 5-HT on eating. 2CR knockout mice are hyperphagic and obese compared to wild-type mice [198] and display blunted responses to anorectic properties of d-fenfluramine.

A number of studies implicate 5-HT, and in particular the 2CR, in illness anorexia. There is strong evidence for a role of increased hypothalamic 5-HT neurotransmission in cancer anorexia [163]: Tumor growth in animals and man is accompanied by an increase in the amino acid tryptophan in plasma and brain [165, 166], and this increase is expected to lead to an increase in 5-HT synthesis because tryptophan is the precursor for 5-HT synthesis [164]. An increase in hypothalamic 5-HT concentrations was observed with the onset of anorexia in tumor bearing rats [164, 167, 168]. Tumor removal normalized the hypothalamic 5-HT levels and improved food intake [168]. Moreover, intrahypothalamic injection of the 5-HT2R antagonist mianserin reduced tumor anorexia [169]. Also, administration of 2CR antagonists attenuates the anorexia induced by intraperitoneal (ip) injections of lipopolysaccharide (LPS) [150, 151], a gram-negative bacterial endotoxin, whereas administration of the $5HT_{1B}R$ antagonist cyanopindolol did not [151]. Furthermore, direct injection of the 5-HT1A receptor ($5\text{-}HT_{1A}R$) agonist 8-OH-DPAT into the midbrain raphe also attenuated LPS anorexia [150]. Because $5\text{-}HT_{1A}R$ are thought to function mainly as negative-feedback, somatodendritic autoreceptors [148], this result suggests that increased activity of 5-HT neurons in the midbrain raphe is involved in the mediation of LPS anorexia.

The present studies were undertaken to further investigate the role of 2CR signaling in LPS-induced anorexia in rats using the specific 2CR antagonist SB 242084 [149]. Whereas in previous studies [150, 151] SB 242084 has been shown to attenuate LPS anorexia starting at 4 hours after LPS injection, our focus was on the initial phase of LPS anorexia. Therefore, in contrast to these previous studies [150, 151] we injected SB 242084 prior to LPS. The first experiment characterized the effects of SB 242084 pretreatment on LPS anorexia. In the second experiment, we used immunocytochemical techniques to assay the numbers of cells expressing c-Fos, the product of the immediate early gene c-Fos, in brain sites where altered neuronal activity, either in 5-HT neurons or in their projection sites, might cause anorexia. In some populations of neurons, c-Fos is expressed beginning about one hour following electrophysiological activation [184]. Therefore, to identify sites potentially mediating the early phase of LPS

anorexia and perhaps necessary for the subsequent phases, we collected brain tissue 90 min after LPS injection. We examined the dorsal (DR) and median (MnR) midbrain raphe nuclei and the PVN, Arc and CeA for the reasons described above. We also examined the raphe magnus (RMg) and raphe pallidus (RPa) hindbrain raphe nuclei because they are interconnected with the midbrain raphe nuclei and because each expresses 2CR [158-161]. Finally, we examined the nucleus tractus solitarii (NTS) and the A1 area of the ventrolateral medulla (A1) because several studies implicate these areas in LPS anorexia [101, 103-105].

5.2 Materials and Methods

5.2.1 Animals and housing

Male Long-Evans rats bred on site were used. At age of 5-6 weeks, they were housed individually in rooms maintained at 22 ± 2 ℃ with 12:12-h light-dark cycles (lights on: 2300) and offered chow (Provimi Kliba NAFAG #3433, Kaiseraugst, Switzerland) and water ad libitum except as noted below. All procedures were approved by the Veterinary Office of the Canton of Zurich.

5.2.2 LPS-induced anorexia

Twenty four rats (body weight range 220-270 g) were individually housed in stainless-steel hanging cages with wire-mesh bottoms (33x18x20 cm). Food cups were removed from cages at 1000 h (1 h prior to lights out) daily and filled with fresh food. Beginning about 2 weeks before the experiments, rats were ip injected with 1 ml/kg 0.9% sterile saline (B. Braun Medical, Emmenbrücke, Switzerland) at 1000 h and again at 1100 h daily. Food was returned at 1100 h, and intakes corrected for spillage were measured 1, 2, 4 and 23 h later. The effects on

spontaneous eating of LPS (*Escherichia coli*, serotype 0111:B4; # L-2630, Sigma, Buchs, Switzerland) and SB 242084 (1*H*-indole-1-carboxyamide,6-chloro-2,3-dihydro-5-methyl-*N*-[6-[(2-methyl-3-pyridinyl)oxyl]-3- pyridinyl]-6-chloro-5-methyl-1-[(2-[2-methylpyrid-3-yloxy]pyrid-5-yl)-carbamoyl] indoline; Sigma # S-8061) were then tested. LPS and SB 202484 solutions were freshly prepared in 0.9% saline daily, with concentrations adjusted so that injection volume was 1 ml/kg. Saline or 0.3 mg/kg SB 202484 was injected at 1000 h, and saline or 100 µg/kg LPS was injected at 1100 h. These doses were selected on the basis of prior studies [150, 188, 199]. Rats were randomly assigned to one of four treatment groups: (1) saline/saline, (2) SB 242084/saline, (3) saline/LPS, (4) SB 242084/LPS.

5.2.3 LPS-induced c-Fos expression

Twenty six rats (body weight range 160-250 g) were individually housed in acrylic cages (16 x 55 x 22 cm) with wood-chip bedding (Rettenmaier & Söhne, Rosenberg, Germany) and adapted to the procedure described above, except that food was not returned until 4 h after dark onset daily; this was done to adapt the rats to the food deprivation before sacrifice. On the experimental day, the 4 treatments described above were administered, and 90 min later the rats were anesthetized with ip injections (1 ml/kg) of 50 mg/kg sodium pentobarbital (Nembutal, Abbott Laboratories, Abbott Park, USA), thoracotomized, and transcardially perfused (20 ml/min) with 100 ml 0.1 M phosphate buffer (PB, pH 7.4); followed by 300 ml of 4 % paraformaldehyde in 0.1 M PB. The brains were removed, postfixed in 4% paraformaldehyde solution for 2 h at room temperature and then in 20% sucrose in 0.1 M PB for 2 days at 4 °C. Forty-micron thick sections of hindbrain (14.3 -7.3 mm posterior to bregma [200]) and forebrain (1.8 -2.7 mm posterior to bregma) were cut on a freezing sliding microtome, collected in six serial ordered sets, and stored in a 40% 0.1M PB, 30% ethylene glycol, and 30% glycerol solution at –20 °C.

One set each of hindbrain and forebrain sections was processed for c-Fos expression using 2 % nickel-diaminobenzidine (Ni-DAB; Chemie Brunschwieg, Basel, Switzerland). Sections were rinsed in 0.1 M PB buffer for 10 min, incubated in 1% sodium borohydride for 30 min, rinsed in PB, incubated in 0.5 % H_2O_2 in 0.1 M PB for 10 min, rinsed in PB, incubated in 1 % normal goat serum (NGS, Vector Laboratories, Burlingame, CA, USA) solution in 0.1 M PB with 0.3 % Triton-X (0.3 % PBTx) for 1 h, and then incubated in primary antibody solution (1:10,000 rabbit polyclonal c-Fos antibody, Ab-5 Calbiochem, Cat#PC38, La Jolla, CA, USA, in 0.3 % PBTx and 1% NGS) overnight. The next day, sections were incubated with biotinylated anti-rabbit goat IgG (1:300; BA-1000, Vector Laboratories) for 1 h and stained with streptavidin-Ni-DAB-peroxidase complex reaction (ABC, 1:300, Vector Laboratories, Reactolab SA, Servion, Switzerland). Sections then were mounted on gelatinized microscope slides, dehydrated in an increasing series of alcohols, defatted in xylene, and cover slipped with Permount (Fisher Scientific, Wohlen, Switzerland).

The number of cells expressing c-Fos was quantified using Image Pro software (Version 6.0, MediaCybernetics, Silver Spring, MD, USA) in the following regions (locations are mm caudal to bregma as per [4]): DR (mean of 4 sections, 9.30 – 7.80 mm), MnR (mean of 3-4 sections, 8.3-7.3 mm), PVN (one section, 1.80 mm), Arc (one section, 2.65mm) and CeA (one section, 2.65mm), RMg (mean of 3-5 sections, 11.60 - 9.08 mm), RPa (mean of 10-14 sections, 10.52 - 14.08 mm), NTS (mean of 3-5 sections, 13.65 - 14.30 mm) and A1 (one section, 13.65 mm). Digital images of each section were made on Olympus AX70 microscope (Center Valley, PA, USA). Bilateral counts in each area were done using Image Pro software (Version 6.0, MediaCybernetics, Silver Spring, USA). Cells were considered c-Fos positive if their nuclei contained dark, punctate blue-black immunolabeling and were counted using constant minimum and maximum optical densities and object size criteria, which were based on the group means and validated with visual counts. Mean count/section were analyzed and bilateral counts are presented for midline structures and the mean of the left and right sections for others.

5.2.4 Statistical Analyses

A robust statistical approach was adopted to increase statistical power [201, 202]. Logarithmic or inverse transformations were used as required to improve normality. In addition, to reduce the influence of extreme values, data were converted to standard scores using the median absolute deviate method, and standard scores with absolute values > 1.96 (i.e., $P < 0.05$) were excluded. Data were then analyzed with one-way ANOVA (Sigma stat version 3.5, Systat Software, San Jose, CA, USA) or, if the prerequisites for ANOVA were not met, by Friedman's ANOVA on ranks, followed by Bonferroni-Holm or Mann-Whitney U tests, respectively. Food intakes at the four measurement times were analyzed separately. Four post-hoc comparisons were tested: (1) saline/saline versus saline/LPS, (2) saline/saline versus SB 242084/saline, (3) SB 242084/saline versus SB 242084/LPS, and (4) difference (1) versus difference (3). Data are reported as means ± standard error of the mean (SEM). In the case of parametric analyses, the standard error of the difference (SED) is given to indicate experiment-wide residual variability.

5.3 Results

5.3.1 Food Intake

LPS significantly reduced cumulative food intake at 2, 4 and 23 h after injection (Figure 1; Friedman $H(3) = 7.94$, $P < 0.05$; $F(3,17) = 12.44$, $P < 0.001$, SED = 1.2 g; and $H(3) = 13.54$, $P < 0.05$; for 2, 4 and 23 h, respectively). SB 242084 by itself had no detectable effect on food intake, but completely reversed LPS anorexia at each time. That is, at 2, 4 and 23 h, first, the (saline/saline versus saline/LPS) differences and the [(saline/saline - saline/LPS) versus (SB 242084/saline - SB 242084/LPS)] differences were significant, and second, the (SB 242084/saline) versus (SB 242084/LPS) differences were not significant.

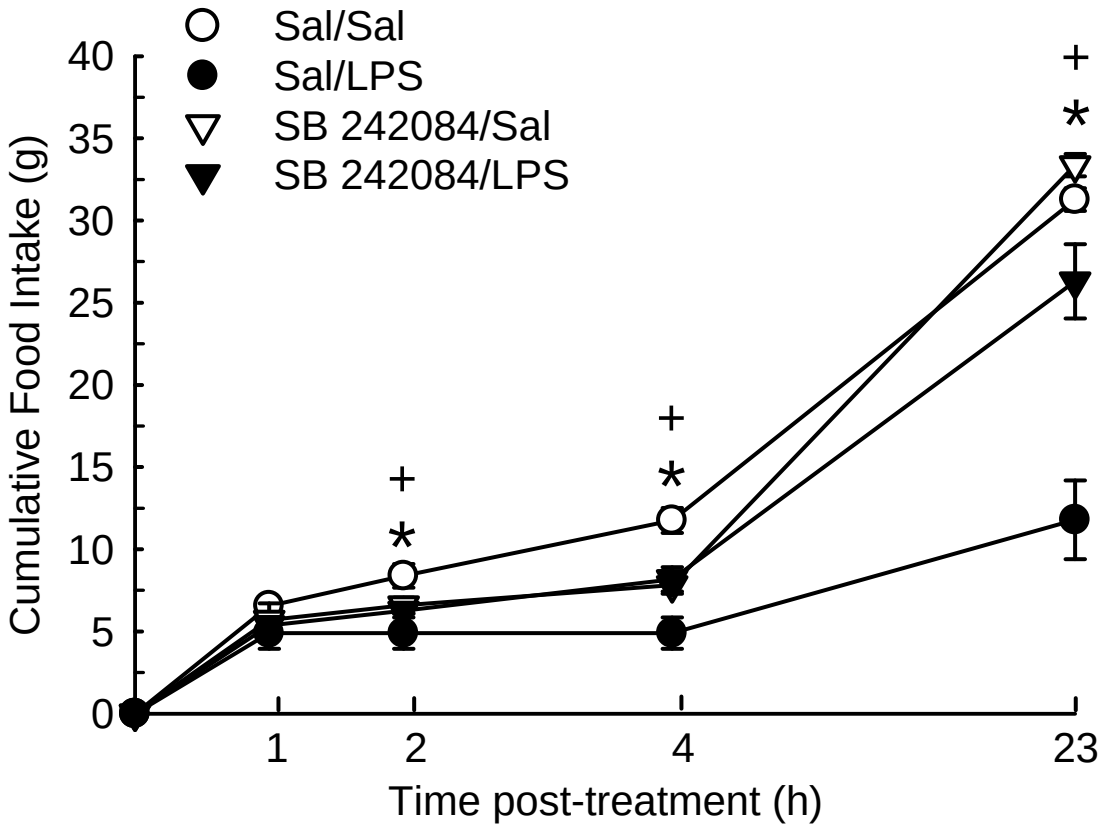

Figure 1. Reversal of LPS anorexia by SB 242084. Rats were ip injected with saline or 0.3 mg/kg SB 242084 1 h before dark onset and saline or 100 µg/kg LPS at dark onset. Data are cumulative food intakes (mean ± SEM). 4-5 rats/group.

*saline/LPS significantly different from saline/saline, Bonferroni-Holm test after significant ANOVA, $P < 0.05$.

+(saline/saline - saline/LPS) significantly different from (SB 242084/saline - SB 242084/LPS), Bonferroni-Holm test after significant ANOVA, $P < 0.05$.

5.3.2 c-Fos Expression

LPS increased c-Fos expression significantly in all nine brain regions examined. In seven of these areas (MnR, DR, PVN, CeA, RPa, NTS, A1), SB 242084 eliminated LPS-induced c-Fos according to the two criteria described above; in two areas (Arc, RMg), LPS did not significantly increase c-Fos in the presence of SB 242084, but the increase was not significantly diminished by SB 242084 either. Figure 2 shows these effects in the DR, and MnR (Figure 2, $F(3,18) = 13.86$, $P < 0.001$, SED = 0.1 cells/section; and $F(3,17) = 60.14$, $P < 0.001$, SED = 1.3 cells/section respectively); Figure 3 shows them in PVN, Arc and CeA (Figure 3, $F(3,18) = 61.1$, $P < 0.001$, SED= 5.1 cells/section; $F(3,20) = 9.85$, $P < 0.05$, SED = 1.9 cells/section; and $F(3,19) = 14.03$, $P < 0.001$, SED = 8.0 cells/section) and Figure 4 shows them in the RMg, RPa, NTS and A1 (Figure 4, $H(3) = 8.37$, $P < 0.05$; $F(3,18) = 19.37$, $P < 0.001$, SED = 0.9 cells/section; $F(3,17) = 30.92$, $P < 0.001$, SED = 0.1 cells/section; and $F(3,18) = 20.97$, $P < 0.001$, SED = 0.8 cells/section, respectively).

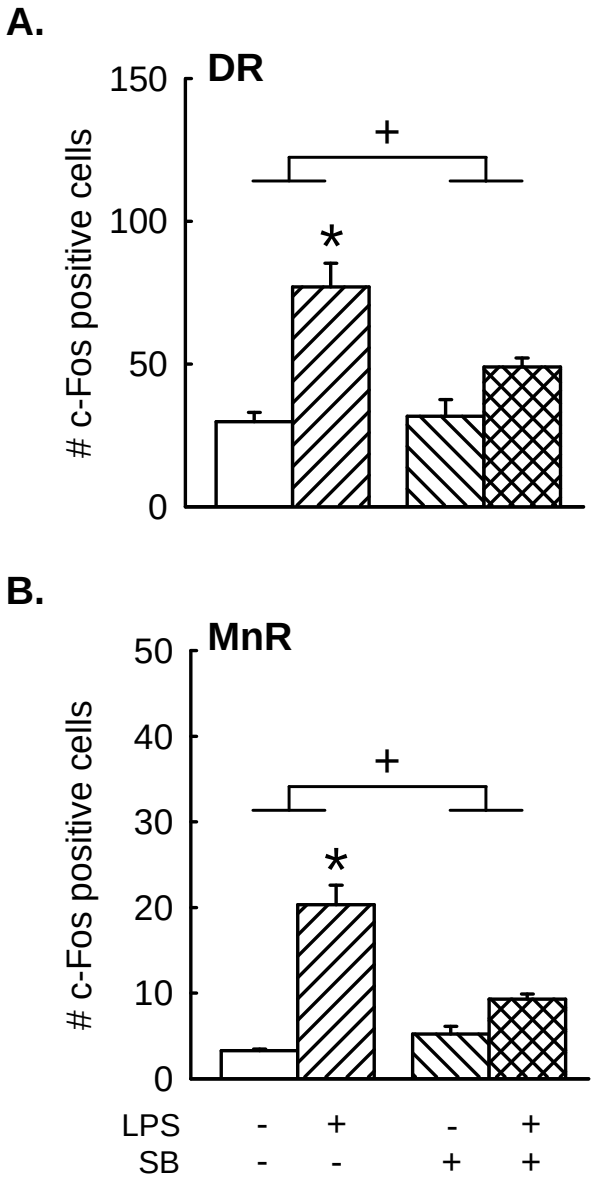

Figure 2a. Reversal of LPS-induced c-Fos expression in the DR (A) and MnR (B) by SB 242084. Rats were ip injected with saline or 0.3 mg/kg SB 242084 1 h before dark onset and saline or 100 µg/kg LPS at dark onset. Data are numbers of cells/section expressing c-Fos (mean ± SEM), 4-8 rats/group.

*saline/LPS significantly different from saline/saline, Bonferroni-Holm test after significant ANOVA or Mann-Whitney U test after significant Friedman's ANOVA on ranks, as described in text, $P < 0.05$.

+(saline/saline - saline/LPS) significantly different from (SB 242084/saline - SB 242084/LPS), Bonferroni-Holm test after significant ANOVA or Mann-Whitney U test after significant Friedman's ANOVA on ranks, as described in text, $P < 0.05$.

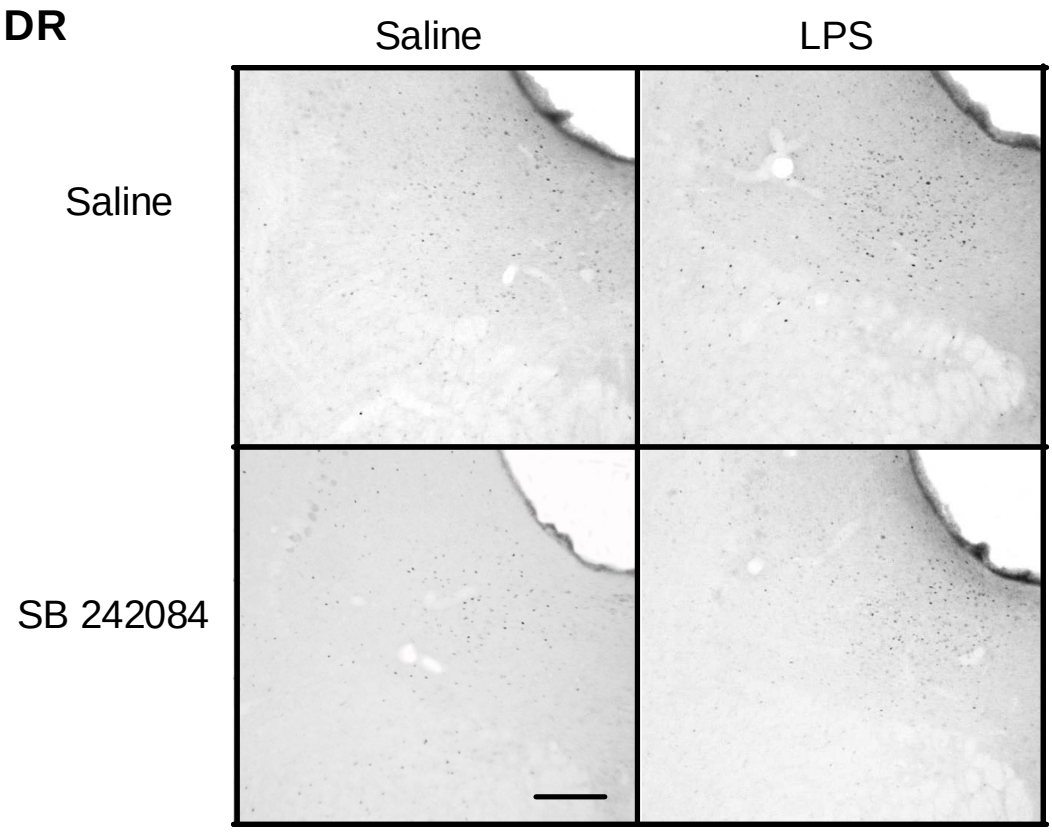

Figure 2b. Photomicrograph depicting c-Fos immunoreactivity in the DR (scalebar 100 µm)

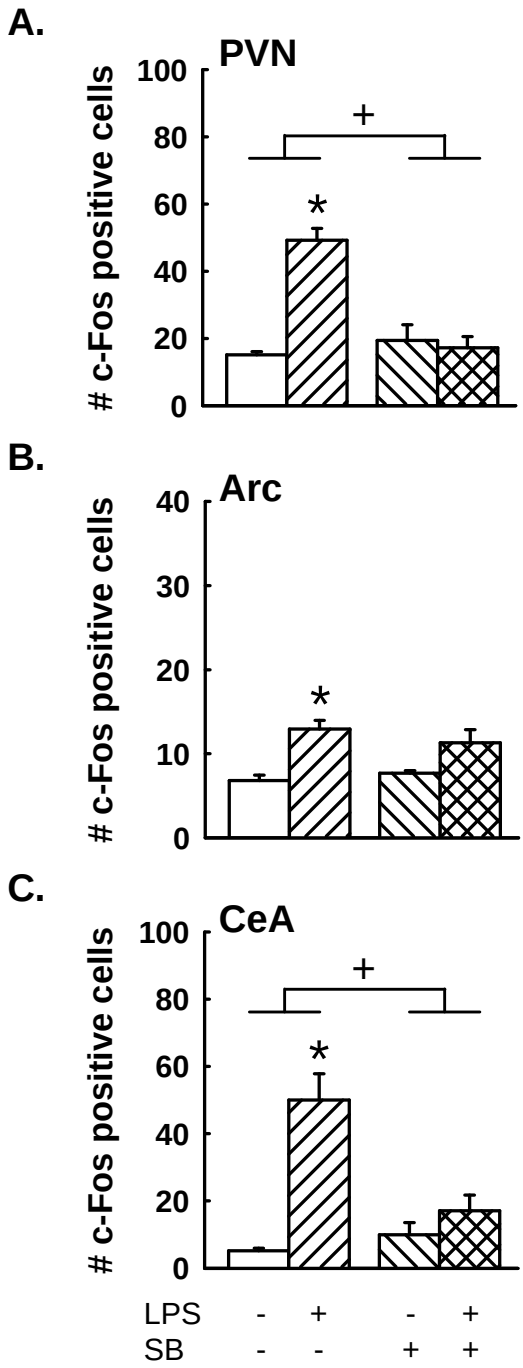

Figure 3a. Effects of SB 242084 LPS-induced c-Fos expression in the PVN (A), Arc (B) and CeA (C). Rats were ip injected with saline or 0.3 mg/kg SB 242084 1 h before dark onset and saline or 100 µg/kg LPS at dark onset. Data are numbers of cells/section expressing c-Fos (mean ± SEM), 4-8 rats/group.

*saline/LPS significantly different from saline/saline, Bonferroni-Holm test after significant ANOVA or Mann-Whitney U test after significant Friedman's ANOVA on ranks, as described in text, $P < 0.05$.

+(saline/saline - saline/LPS) significantly different from (SB 242084/saline - SB 242084/LPS), Bonferroni-Holm test after significant ANOVA or Mann-Whitney U test after significant Friedman's ANOVA on ranks, as described in text, $P < 0.05$.

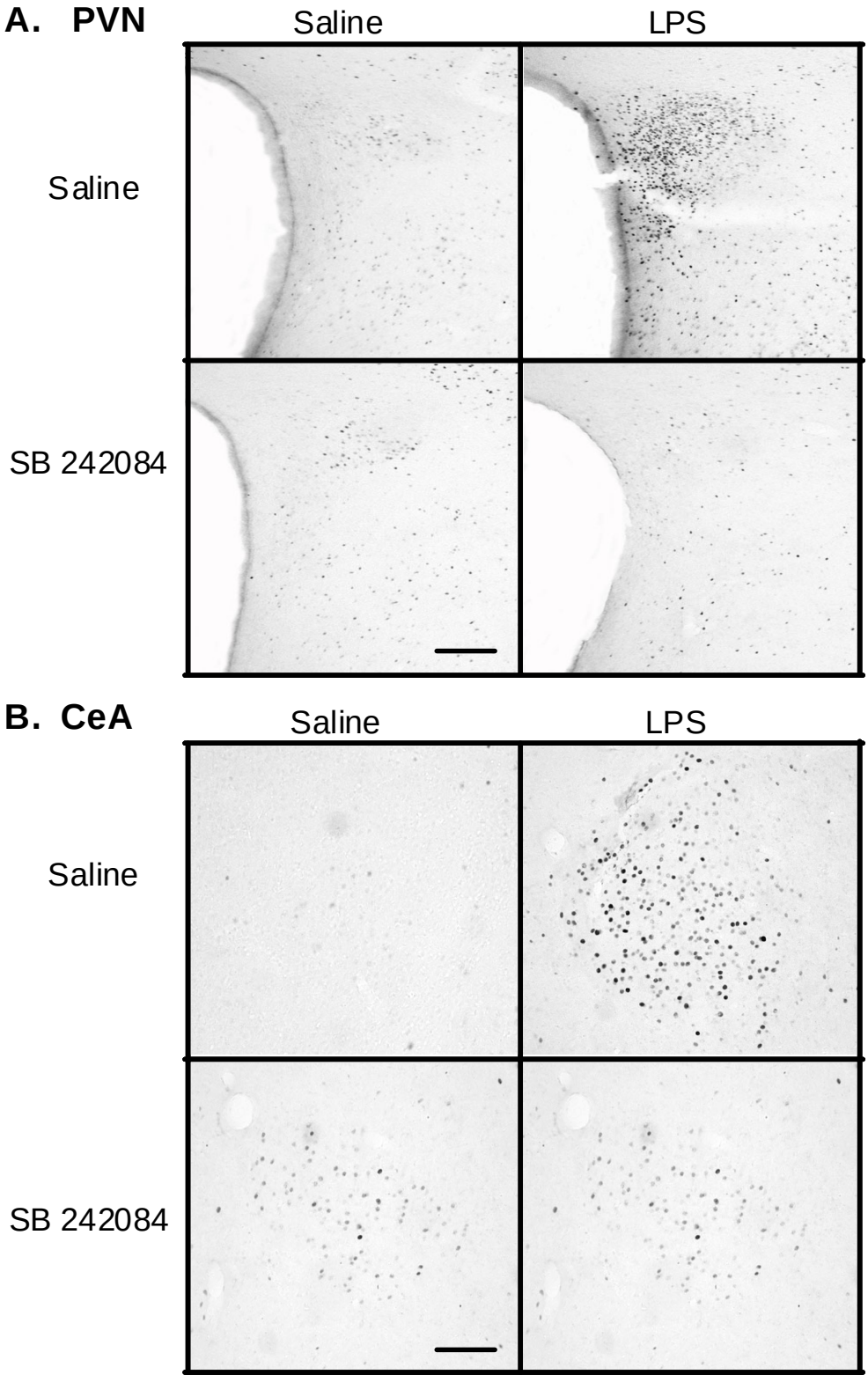

Figure 3b. A: Photomicrograph depicting c-Fos immunoreactivity in the PVN (scalebar 100 µm)

B: Photomicrograph depicting c-Fos immunoreactivity in the CeA (scalebar 50 µm)

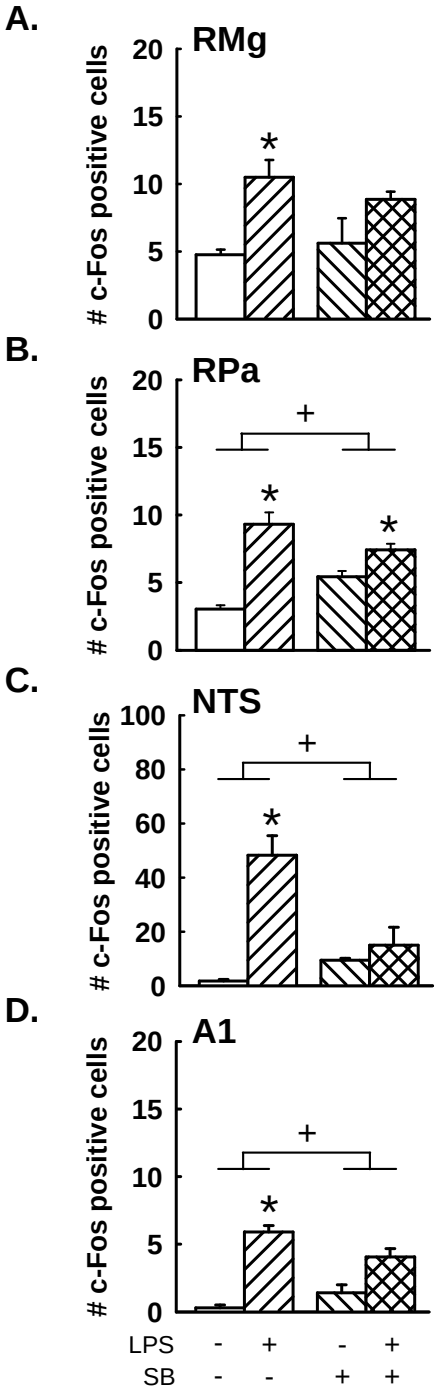

Figure 4. Effects of SB 242084 LPS-induced c-Fos expression in the RPa (A), RMg (B), NTS (C), and A1 (D). Rats were ip injected with saline or 0.3 mg/kg SB 242084 1 h before dark onset and saline or 100 µg/kg LPS at dark onset. Data are numbers of cells/section expressing c-Fos (mean ± SEM), 4-8 rats/group.

*significantly different from control, Bonferroni-Holm test after significant ANOVA or Mann-Whitney U test after significant Friedman's ANOVA on ranks, as described in text, $P < 0.05$.

+(saline/saline - saline/LPS) significantly different from (SB 242084/saline - SB 242084/LPS), Bonferroni-Holm test after significant ANOVA or Mann-Whitney U test after significant Friedman's ANOVA on ranks, as described in text, $P < 0.05$.

5.4 Discussion

We used ip pretreatment of the selective and potent 2CR antagonist SB 242084 in rats to investigate the role of 2CR signaling in LPS-induced anorexia and activation of neural pathways likely to mediate LPS anorexia. As described in the introduction, 5-HT signaling via 2CR, especially 5-HT projections from the DR and MnR to the Arc, PVN and CeA [123, 124], have been implicated in the control of both normal eating and illness anorexia [163, 166, 167, 203, 204]. Because our goal was to identify sites involved in the initial phase of illness anorexia, which also may be necessary for the subsequent phases, we measured c-Fos 90 min after LPS injection.

There were three main findings. First, pretreatment with SB 242084 completely reversed LPS anorexia. Second, in each of the brain sites tested, LPS increased the number of cells expressing c-Fos 90 min later. Third, and most relevant to our hypothesis that 5-HT connections from the midbrain raphe to the hypothalamus are crucial for LPS anorexia, pretreatment with SB 242084 completely reversed the LPS-induced increases in c-Fos expression in the PVN and CeA and tended to do so in the Arc. SB 242084 also reversed LPS-induced c-Fos increases in the DR, MnR, RPa, NTS and A1 and tended to do so in the RMg. We discuss the implications of these data in turn in the next sections.

5.4.1 2CR and LPS anorexia

The role of brain 5-HT in the inhibitory control of eating is well established [203-205]. For example, eating is associated with 5-HT release in the hypothalamus [206] and 5-HT agonists and antagonists influence eating in the expected way [150, 151]. 2CR seem especially important in this context. Transgenic mice lacking 2CR are hyperphagic and obese [198], and have deficits in cholecystokinin- and glucagon-like peptide-1 (GLP-1) -mediated satiation [207].

Some of these effects appear to be mediated by 2CR in the hypothalamic neuronal network thought to underlie homeostatic eating [162].

5-HT seems also to be involved in LPS anorexia. For example, hypothalamic 5-HT1AA/5-HT ratios were elevated 120 min after ip LPS injections, indicating increased 5-HT release, whereas DOPAC/DA ratios, which are indicative of dopaminergic activity, were not [145, 146]. In addition, LPS anorexia was attenuated in rats [150, 151] and mice [198] by ip injection of SB 242084 [150, 151]. 5-HT signaling via 2CR seems to be sufficient for 5-HT's role in LPS anorexia because under conditions in which 2CR antagonism attenuated LPS anorexia, antagonism of 5-HT_{1B}R, 5-HT_{2A} or 5-HT_{3} receptors failed to do so [147, 151]. In apparent contrast to these studies, however, LPS anorexia was not reduced in transgenic mice lacking 2CR [198]. This paradox might be explained by secondary effects, especially developmental changes, arising from indirect actions of the mutation that may mask the primary cell- or tissue-specific intended effect of the knockout [208], but this has not been tested.

Our test, in which the 2CR antagonist SB 242084 was injected prior to LPS administration, is the first to demonstrate a complete statistical reversal of LPS-anorexia from its onset, here between 1 and 2 h, through at least 24 h. Thus, we conclude that 5-HT signaling via 2CR is necessary for LPS anorexia (and perhaps other brain-mediated elements of the APR not measured here) under the conditions tested.

5.4.2 2CR and c-Fos expression

The moderate dose of 100 µg/kg LPS increased the numbers of cells expressing c-Fos in all nine brain areas tested. Although somewhat similar patterns of c-Fos expression in response to LPS have been reported previously [104, 209], three distinctive features of our study are worth noting. First, previously rats received higher doses of LPS (250-1000 µg/kg) [101, 103-105].

Such doses are relatively less selective for anorexia than the 100 µg/kg dose we used. Second, in previous studies, c-Fos was assayed later, 3-6 h following LPS treatment. These data may be more relevant to the later events in the cascade of consequences of innate immune activation. Our findings at 90 min post-LPS, in contrast, reflect neural activation approximately at the same time that the inhibition of eating began (i.e., between 1-2 h post-LPS) and, therefore, may reveal sites causing the anorexia. Third, SB 242084 pretreatment affected LPS-induced c-Fos in a wide range of brain areas. That is, the LPS-induced increase in c-Fos was statistically eliminated in seven of nine areas tested (DR, MnR, PVN, CeA, RPa, NTS and A1) and was no longer significant, although not entirely eliminated according to our statistical criteria, in the Arc and RMg. Thus, 2CR signaling appears necessary for much of LPS-induced neuronal activation, and the ability of SB 242084 to decrease LPS-induced c-Fos expression suggests that SB 242084 may reverse LPS anorexia by decreasing neural signaling in one or more of these areas. At the same time, however, the widespread reversal of LPS-induced c-Fos by SB 242084 does not identify a single likely pathway that mediates LPS anorexia.

5.4.3 DR and MnR

SB 242084 pretreatment eliminated the c-Fos expression in the MnR and the DR. This suggests that 2CR signaling between these sites may contribute to LPS anorexia. As our study is only associational, however, direct evidence that the effective blockade of 2CR produced by SB 242084 occurred in the DR or MnR is still required.

5.4.4 PVN

The LPS-induced increase of c-Fos expression in the PVN is consistent with the LPS-induced increases in c-Fos mRNA and c-Fos protein in this area that have been reported previously to occur under different conditions [101, 103-105]. This similarity indicates that the effect of LPS on PVN c-Fos is robust and largely independent of the experimental conditions. The LPS-induced activation of the PVN has been implicated in stimulation of fever and of the hypothalamic-pituitary-adrenal (HPA)-axis [109, 210, 211]. Because the PVN is also a key site in the hypothalamic neural networks mediating eating [212], these c-Fos data may also be relevant to LPS anorexia. We also demonstrated that LPS-induced PVN c-Fos was reversed by SB 242084.

Together with the antagonism of LPS anorexia by SB 242084 this supports the hypothesis that PVN 2CR signaling is involved in LPS anorexia. Consistent with this assumption, 2CR mRNA is expressed in the PVN [158-161] and Heisler et al [162] reported that the 2CR in the PVN are necessary for 5-HT induced HPA-axis activation.

The LPS-induced c-Fos in the PVN and its reversal by SB 242084 is also consistent with our hypothesis that this neural activation was caused by 5-HT neurons that project from the DR and MnR to the PVN [121, 122]. The PVN, however, also receives non-5-HT inputs from several other structures in which we observed c-Fos, such as A1, NTS [120], and CeA [119]. In addition, PVN neurons may have been non-neuronally stimulated, for example by PGE_2 released from capillaries in the PVN as a consequence of peripheral immune responses to LPS. All in all, our data suggest that further work is warranted to determine, first, the relevance of the effects that we observed on PVN c-Fos expression to LPS anorexia and other elements of the APR, and, second, the exact proximal stimulus activating the PVN.

5.4.5 Arc

LPS significantly increased the c-Fos expression 90 min post ip in the Arc. Previous studies reported an increase in ip LPS-induced c-Fos mRNA or Fos protein [106, 209]in the Arc of rats but at later timepoints and with different doses of LPS than in our study. Although there was no longer a significant increase in LPS-induced Arc c-Fos after SB 242084 pretreatment, this apparent attenuation did not meet our statistical criteria for significance. Therefore, our data do not support a clear conclusion regarding the relationship of Arc c-Fos expression to 2CR signaling and LPS anorexia. This was unexpected given the extensive data relating Arc α-melanocyte stimulating hormone (α-MSH) neurons to LPS anorexia. Arc neurons expressing POMC, the precursor of α-MSH, project to neurons in the PVN [116-118], and other areas, that express the cognate receptors to α-MSH, melanocortin-3 and 4 receptors (MC3/4R), which are also sensitive to agouti-related protein, and these neurons are implicated in LPS anorexia [129, 213]. Furthermore, the Arc receives a strong 5-HT input, in part mediated by 2CR [160, 161, 214]. For example, Heisler et al. [215] reported 2CR were co-expressed with POMC in Arc neurons and that 2CR agonists dose-dependently depolarized these neurons. Thus, further research is required to clarify the role of the Arc in LPS anorexia.

5.4.6 CeA

Previous studies have also demonstrated that LPS increases c-Fos mRNA [101, 103-105, 216] or protein [106, 209, 217] in the CeA. Our SB 242084 data indicate that 2CR signaling is necessary for this increased activation. The CeA also receives 5-HT innervation from the DR and MnR [123, 124] and expresses 2CR [158, 160, 161, 218, 219], consistent with our hypothesis that 2CR projections from the DR and MnR are crucial mediators of LPS anorexia. Again, however, we can not exclude the possibility that SB 242084 affected LPS-induced

CeA c-Fos expression indirectly. For example, retrograde tracer studies indicate that the lateral parabrachial nucleus (PBN) has an important influence on the CeA c-Fos response to immune activation [135-137]. The PBN, in turn, may be indirectly activated by Arc POMC neurons that project to the PBN [138] and activate MC4R on neurons expressing calcitonin gene-related peptide (CGRP) [139, 140] that project to the CeA [140]. Where in this circuit 2CR may be present is still unknown.

5.4.7 RPa and RMg

Our data extend previous reports that RPa neurons express c-Fos 3-4 h after ip injections of 250 µg and 1 mg/kg LPS doses [103, 105] by showing that similar c-Fos responses occur sooner, after smaller LPS doses, and, at least for the RPa, are reversed by 2CR antagonism. Because RPa neurons express 2CR [158, 160, 161], c-Fos expression in these neurons is likely to be a direct effect of 2CR signaling, although indirect stimulation from descending projections downstream from 2CR in the midbrain raphe or other 5-HT areas can not be excluded. It is likely that activation of the RPa and RMg is involved in the hyperthermic or visceral effects of LPS. For example, a projection from the medial preoptic area (POA) to the RPa is involved in induction of fever [71].

5.4.8 NTS and A1

As in the case of the hindbrain raphe nuclei described above, our data extend previous reports [101, 103-105, 216] by showing that similar c-Fos responses in these areas occur sooner, occur after smaller LPS doses, and are reversed by 2CR antagonism. Whether the influence of 2CR is direct or indirect is unclear. 2CR expression has been reported in the NTS [158, 161, 220], but not to our knowledge in the A1. LPS was previously reported to activate tyrosine-

hydroxylase (TH)-immunoreactive cells in the ventrolateral medulla, indicating that catecholaminergic (CA) neurons are part of the mechanism mediating the response to LPS [17].

5.4.9 Implications for Future Research

The studies reported here raise several questions for future studies of the mechanisms mediating LPS anorexia. The most important of these are: (1) Which, if any, of the sites in which SB 242084 eliminated c-Fos activation are necessary for LPS anorexia (or other elements of the acute-phase-response)? (2) Where are the crucial 2CR mediating the effects on LPS-induced anorexia or c-Fos expression that we obtained? (3) If there is more than one necessary site, how are they linked? In particular, is our hypothesis that 5-HT projections from the midbrain raphe to the 2CR in hypothalamus and CeA are crucial for LPS anorexia correct? (4) Are other 5-HT receptor subtypes also involved in these effects? (5) What is the relationship of the effects we observed to other neuronal signaling mechanisms that have been implicated in LPS anorexia, such as GLP-1 [221], α-MSH [222], dopamine [212], CGRP [138, 223], and CA [101] signaling?

6 Evidence for a role of PGE_2 and the dorsal raphe nucleus in LPS-induced anorexia

6.1 Introduction

Bacterial infections and other immune challenges are met by the organism with a complex immune reaction called the acute phase response (APR). Among the elements of the APR are physiological changes including fever, release of acute phase proteins from the liver, increased slow-wave sleep, and increased hypothalamic-pituitary-adrenal axis (HPA) activity as well as behavioral changes including anorexia, adipsia, and inactivity (reviewed in [4]). These active and adaptive responses temporarily suspend normal homeostasis in order to facilitate clearance of pathogens and ultimately to return to the normal physiological state.

Bacterial lipopolysaccharide (LPS) administration is a widely used experimental model of gram-negative bacterial infection. LPS is a component of the outer lipid layer of gram-negative bacteria that is released into the circulation upon bacterial reproduction or lysis. Circulating LPS forms a complex with LPS-binding protein and membrane-bound or soluble CD14. This complex interacts with Toll-like receptor-4 and activates an intracellular signaling pathway that results in pro-inflammatory gene expression leading to the synthesis and release of pro-inflammatory signaling molecules, such as cytokines and prostaglandins [11], which are the first steps of the immune-neuro-endocrine cascade that ultimately leads to the APR (reviewed in [191]).

Prostaglandin E_2 (PGE_2) appears to be an important mediator of LPS anorexia. The synthesis of PGE_2 is catalyzed by cyclooxygenases (COX), which act on arachidonic acid [224]. COX-1, the constitutive form of the enzyme, is involved in a variety of homeostatic processes [225]. COX-2, the inducible form of the enzyme, produces PGE_2 in response to immune stimulation. For example,

COX-2 mRNA and protein are markedly induced in rat brain perivascular microglia and endothelial cells after LPS administration [1, 83, 226-228].

The relative roles of COX-1 and COX-2 in LPS-anorexia have been investigated in both pharmacological and genetic rodent models. Initial studies in male rats showed that indomethacin, a non-specific inhibitor of COX, attenuated LPS-anorexia [229, 230]. Subsequent tests with selective COX-1 and -2 inhibitors indicated that COX-2 is crucial for this effect in both rats and mice [2, 231]. That is, pretreatment of rats with a COX-1 inhibitor did not affect LPS anorexia, whereas pretreatment with the COX-2 inhibitor NS-398 did [2]. Similarly, LPS anorexia was reversed in mice with genetic deletions of COX-2, but not COX-1 isoforms [232]. Furthermore, LPS anorexia was associated with an increase in COX-2 immunoreactivity in endothelial cells throughout the brain [1, 233].

How PGE_2 acts in the brain to elicit LPS-anorexia is still unknown. One possibility is that it acts on serotonin (5-HT) neurons. The evidence for this is that 5-HT is an important neurochemical mediator of LPS anorexia [147, 151] and that 5-HT neurons express EP3 PGE_2 receptors [153].

Here we (1) further tested the hypothesis that brain PGE_2 mediates the anorexia produced by intraperitoneal (ip) injections of LPS in rats using ip injections of the potent and specific COX-2 antagonist NS-398, (2) attempted to identify where in the brain this might occur using c-Fos immunocytochemistry, an indirect measure of neuronal activity [234], (3) tested whether PGE_2 injections directly into the dorsal raphe nucleus (DR), a main site of 5-HT neurons that project into the forebrain, are sufficient to reduce food intake, and (4) tested whether COX-2 antagonism in the DR/median raphe nucleus (MnR) is sufficient to reduce LPS anorexia.

6.2 Materials and Methods

6.2.1 Animals and housing

Male Long-Evans rats, bred in our colony, were housed individually in a room maintained at 22 ± 2 ℃, with a 12:12-h light- dark cycle (lights on: 2300 h). Rats were housed in hanging stainless-steel wire mesh cages (33x18x20 cm) and offered ground chow (Provimi Kliba NAFAG #3433, Kaiseraugst, Switzerland) and water ad libitum, except as noted below. All procedures were approved by the Veterinary Office of the Canton of Zurich.

6.2.2 Experiment 1: LPS anorexia following peripheral COX-2 antagonism

Twenty-three rats were used. One hour prior to dark onset, the rats were weighed and received ip saline injections (1 ml/kg 0.9% saline, Braun Medical, Emmenbrücke, Switzerland). Food cups were removed, filled with fresh chow and weighed. Just prior to dark onset, the rats received a second ip saline injection. Food was then returned and intake was measured 1, 2, 4 and 23 h later. After approximately two weeks of adaptation to these procedures, the rats were divided in 4 groups, and food intake was measured after the following sets of injections, administered one hour before dark onset and just before dark onset, respectively: vehicle followed by saline (n=5), 10 mg/kg *N*-[2-(Cyclohexyloxy)-4-nitrophenyl] methanesulfonamide (NS-398, # N-194, Sigma, Buchs, Switzerland) followed by saline, (n=5), vehicle followed by 100 µg/kg LPS (from *Escherichia coli*, serotype 0111:B4; # L-2630, Sigma) (n=5) and NS-398 followed by LPS (n=7). NS-398 was dissolved in a vehicle of saline plus 0.5% carboxymethyl cellulose (CMC; Bio Concept, Allschwil, Switzerland). LPS was dissolved in saline. All solutions were freshly prepared on the experimental day, with the concentrations adjusted so that injection volume was 1 ml/kg. Doses were selected on the basis of prior studies [151, 188, 199]. This dose of LPS was shown to mimic the clinical features of

gram-negative bacterial infection or septicemia [185-187] and to reduce eating [92, 186], but to not produce systemic hypotension, hyperglycemia or hyperlactacidemia or indications of endotoxin shock [185].

6.2.3 Experiment 2: LPS-induced c-Fos expression following peripheral COX-2 antagonism

Twenty-four rats were adapted to the procedure described above with the exception that food was presented 4 h after the dark onset in order to adapt the rats to the 90-min food deprivation before sacrificing for c-Fos expression. On the experimental day, the same four treatments as above were administered, and 90 minutes after the second injections (i.e., LPS or saline injection) the rats were anesthetized with ip injections (1 ml/kg) of 50 mg/kg sodium pentobarbital (Nembutal, Abbott Laboratories, Abbott Park, IL, USA) and transcardially perfused with 100 ml phosphate buffer (PB, 0.1 M, pH 7.4), followed by 300 ml 4 % paraformaldehyde in 0.1 M PB using a peristaltic pump (Cole-Parmer, Vernon Hills, IL, USA) at a rate of 21 ml/min. The brains were removed, postfixed at room temperature in 4% paraformaldehyde solution for 2 h and in 20% sucrose in 0.1 M PB for 2 days at 4 °C. Six serial sets of 40 µm thick sections of hindbrain were cut beginning at the obex and moving anteriorly (i.e., about 14.3 - 7.3 mm posterior to bregma [200]). Forebrain sections were collected similarly from the optic chiasm through the median eminence (i.e., about 0.1 - 4.1 mm posterior to bregma). Sections were stored in 0.1 M PB with 30% ethylene glycol and 30% glycerol at –20 °C until processing.

One set of hindbrain and forebrain sections were processed for c-Fos expression using diaminobenzidine (DAB; Chemie Brunschwieg, Basel, Switzerland) standard immunocytochemistry technique. Sections were rinsed in 0.1 M PB for 10 min followed by 30 min incubation in 1% sodium borohydride. After another PB rinse, a 10 min-incubation in 0.5 % H_2O_2 in 0.1 M PB, and another PB rinse, tissue sections were incubated for 1 h in 1 % normal goat serum

(NGS, Vector Laboratories, Burlingame, CA, USA) solution in 0.1 M PB with 0.3 % Triton-X (0.3 % PBTx), and then overnight in primary antibody solution (1:10,000 rabbit polyclonal c-Fos antibody, Ab-5 Calbiochem, Cat#PC38, La Jolla, CA, USA, in 0.3 % PBTx and 1% NGS). The next day, sections were incubated for 1h with biotinylated anti-rabbit goat IgG (1:300; BA-1000, Vector Laboratories). Finally, the antibody complexes were visualized using the streptavidin-diaminobenzidine-peroxidase complex reaction (ABC, 1:300, Vector Laboratories, Reactolab SA, Servion, Switzerland) and DAB. Sections then were mounted on gelatinized microscope slides, dehydrated in an increasing series of alcohols, defatted in xylene, and cover slipped with Permount (Fisher Scientific, Wohlen, Switzerland).

The number of cells expressing c-Fos was quantified using Image Pro software (Version 6.0, MediaCybernetics, Silver Spring, MD, USA) in the following regions (locations are mm caudal to bregma as per [4]): paraventricular nucleus (PVN) (one section, 1.80 mm), central nucleus of the amygdala (CeA) (one section, 2.65mm) and arcuate nucleus (Arc) (one section, 2.65mm), nucleus tractus solitarii (NTS) (mean of 3-5 sections, 13.65 - 14.30 mm), A1 region of the ventrolateral medulla (A1) (one section, 13.65 mm), raphe magnus nucleus (RMg) (mean of 3-5 sections, 11.60 - 9.08 mm), MnR (mean of 3-4 sections, 8.3-7.3 mm), DR (mean of 4 sections, 9.30 – 7.80 mm) and the raphe pallidus nucleus (RPa) which was divided into caudal RPa (cRPa) (mean of 10-12 sections, -11.30 - 14.08 mm), and rostral RPa (rRPa) (mean of 3-4 sections, 10.52 – 11.00 mm) subareas.

Digital images of each section were made on Olympus AX70 microscope (Center Valley, PA, USA). Bilateral counts in each area were done using Image Pro software (Version 6.0, MediaCybernetics, Silver Spring, USA). Cells were considered c-Fos positive if their nuclei contained dark, punctate blue-black immunolabeling and were counted using constant minimum and maximum optical densities and object size criteria, which were based on the group means and validated with visual counts. Mean count/section were analyzed and bilateral counts are presented for midline structures and the mean of the left and right sections for others.

6.2.4 Experiment 3: Anorexia following DR administration of PGE_2

Thirty rats were allowed to acclimate to the experimental room for at least 1 week prior to surgery and were then food deprived overnight and anesthetized with 80 mg/kg ketamine HCl (Narketan 10, Chassot, Bern, Switzerland) and 4 mg/kg xylazine (Rompun, Bayer, Leverskusen, Germany). Stainless steel cannulae (24-gauge) were implanted at an angle of 18° ventrally to the vertical AP +1.2 mm, L – 1.2 mm and V +5.5 mm relative to interaural zero. Guide cannulae were secured with acrylic cement and three stainless steel screws that penetrated the skull. Stainless-steel inner stylets were inserted to maintain catheter patency. Stylets were cleaned daily for 2 days post-surgery and every other day thereafter. Rats were allowed to recover 10 d, after which 31 rats that were healthy and ate normally were tested. After 16 h food deprivation and 30 min prior to dark onset, 0 (n = 8), 1 (n = 7), 10 (n = 7), or 100 (n = 8) ng PGE_2 (Sigma) in 0.4 µl aCSF with 5 % dimethyl sulfoxide (DMSO) (# D-8779, Sigma) as vehicle, was infused via a 31-ga stainless steel injection needle that extended 4 mm past the tip of the guide cannulae over a period on 2 min Food was returned at dark onset, and intakes were measured 30 min later. Injection needles were left in place for 30 seconds after infusions to prevent reflux. All solutions were freshly prepared on the experimental day. For verification of the cannula placement the sections were stained with cresyl violet and underwent histological verification.

6.2.5 Experiment 4: LPS anorexia following DR/MnR COX-2 antagonism

Seventeen rats were implanted with DR and MnR cannulas as described above. Rats were food deprived 3 h before dark onset. Immediately thereafter, rats received ip injections (1 ml/kg) of 100 µg/kg LPS or saline as a control. Just prior to dark onset, rats received simultaneous DR and MnR infusions of either 0 (n = 8) or 1 ng (n = 9) NS-398 in 0.4 µl aCSF with 10% DMSO. Food was then returned, and intakes were measured 1, 2, 4 and 23 h later.

6.2.6 Statistical Analyses

A robust statistical approach was adopted to increase statistical power [201, 202]. Logarithmic or inverse transformations were used as required to improve normality. In addition, to reduce the influence of extreme values, data were converted to standard scores using the median absolute deviate method, and standard scores with absolute values > 1.96 (i.e., $P < 0.05$) were excluded. Data from experiments 1-3 were then analyzed with one-way ANOVA (Sigmastat version 3.5, Systat Software, San Jose, CA, USA) or, if the prerequisites for ANOVA were not met, by Friedman's ANOVA on ranks, followed by Bonferroni-Holm or Mann-Whitney U tests, respectively.

Data from Experiment 4 were analyzed with unpaired t-tests. Data that were parametrically analyzed are reported as mean ± standard error of the mean (SEM), with the standard error of the difference (SED) given to indicate experiment-wide residual variability. Data that were non-parametrically analyzed are reported as median ± semi-interquartile range.

6.3 Results

6.3.1 Experiment 1: LPS anorexia following peripheral COX-2 antagonism

LPS reduced cumulative food intake 2, 4 and 23 h after injection (Figure 1; $F(3, 21) = 6.63$, $P < 0.01$, SED = 0.08 g; $H(3) = 14.70$, $p < 0.01$; and $F(3, 16) = 177.05$, $P < 0.001$; SED = 1.40 g, for 2, 4 and 23 h, respectively). NS-398 pre-treatment completely reversed LPS anorexia at each of these times. That is, at each time the difference between (saline/saline versus saline/LPS) and (NS-398/saline versus NS-398/LPS) was statistically significant and the difference between NS-398/saline and NS-398/LPS was not. NS-398 alone had no detectable effect on food intake.

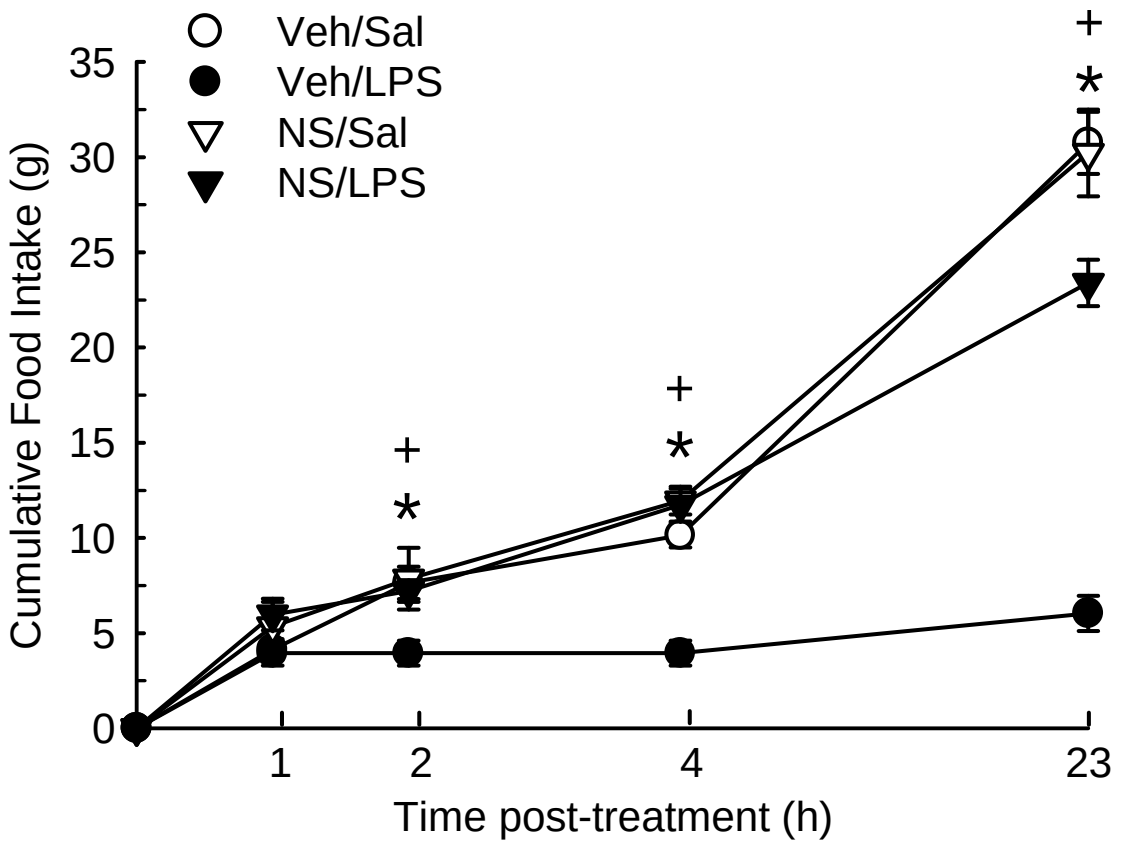

Figure 1. Reversal of LPS anorexia by NS-398. Rats were ip injected with vehicle

or 10 mg/kg NS-398 1 h before dark onset and saline or 100 µg/kg LPS at dark onset. Data are cumulative food intakes (mean ± SEM). 5-6 rats/group.

*vehicle/LPS significantly different from vehicle/saline, Bonferroni-Holm test after significant ANOVA or Mann-Whitney U test after significant Friedman's ANOVA on ranks, as described in text, $P < 0.05$.

+(vehicle/saline – vehicle/LPS) significantly different from (NS-398/saline - NS-398/LPS), Bonferroni-Holm test after significant ANOVA or Mann-Whitney U test after significant Friedman's ANOVA on ranks, as described in text, $P < 0.05$.

6.3.2 Experiment 2: LPS-induced c-Fos expression following peripheral COX-2 antagonism

In the midbrain and hindbrain, LPS increased c-Fos expression in all the regions examined, i.e., each raphe region (Figures 2, 3, 4, ($F(3, 20) = 9.60$, $P < 0.01$, SED = 0.1 cells, $F(3, 20) = 10.90$, $P < 0.01$, SED = 2.0 cells, $F(3, 19) = 12.90$, $P < 0.01$, SED = 1.60 cells, $F(3, 15) = 23.3$, $p < 0.01$, SED = 0.4 cells, $F(3, 17) = 4.09$, $p < 0.05$, SED = 0.6 cells for DR, MnR, cRPa and rRPa, respectively), the NTS (Figure 3, $H(3) = 15.46$, $P < 0.01$) and A1 (Figure 3, $F(3, 19) = 21.00$, $P < 0.01$, SED = 0.1 cells)). NS-398 completely blocked LPS induced c-Fos expression in all areas except the A1 where it markedly reduced it, and the rRPa, where it had no detectable effect.

In the forebrain, LPS significantly increased c-Fos expression in the PVN (Figure 5, $F(3, 22) = 16.20$, $P < 0.01$, SED = 0.2 cells/section) and the CeA ($F(3, 21) = 13.50$, $P < 0.01$, SED = 0.2 cells/section), but not in the Arc ($H(3) = 5.50$, $P > 0.05$). According to the criteria above, NS-398 completely reversed c-Fos expression in the PVN, but had no effect in the CeA ($F(3, 21) = 13.50$, $P < 0.01$, SED = 0.2 cells/section). NS-398 alone did not affect c-Fos expression in the PVN or Arc but did in the CeA ($F(3, 21) = 13.50$, $P < 0.01$, SED= 0.2 cells/section).

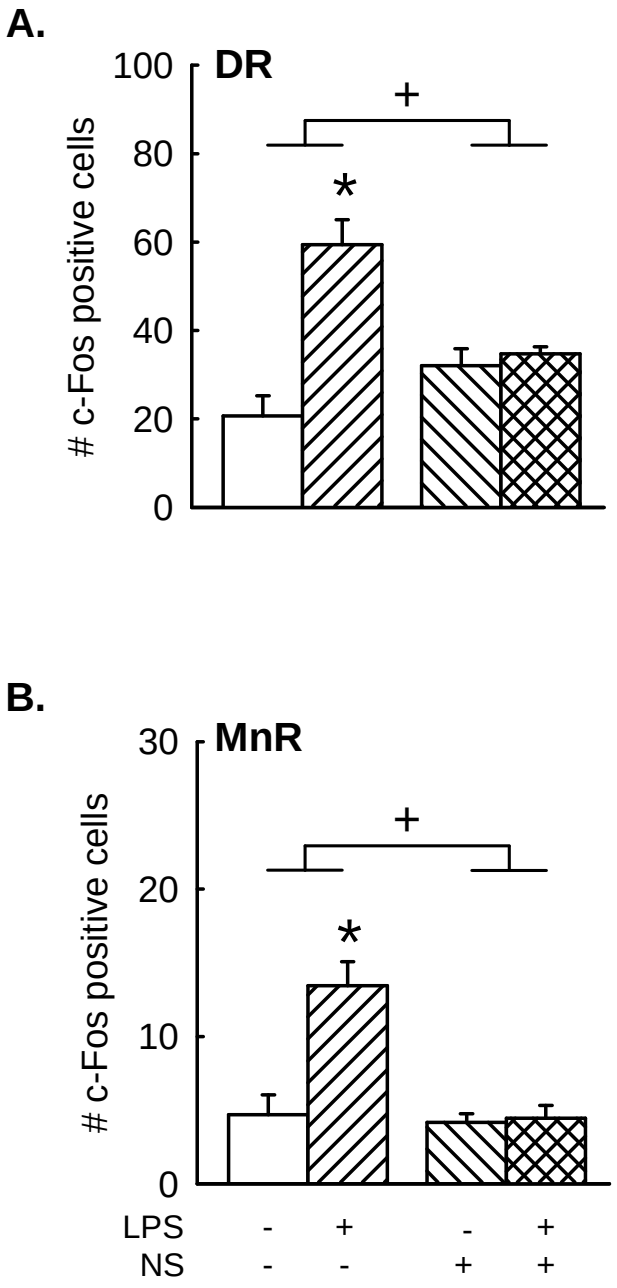

Figure 2a. Reversal of LPS-induced c-Fos expression in the DR (A) and MnR (B) by NS-398. Rats were ip injected with vehicle or 10 mg/kg NS-398 1 h before dark onset and saline or 100 µg/kg LPS at dark onset. Data are numbers of cells/section expressing c-Fos (mean ± SEM), 5-7 rats/group.

*vehicle/LPS significantly different from vehicle/saline, Bonferroni-Holm test after significant ANOVA or Mann-Whitney U test after significant Friedman's ANOVA on ranks, as described in text, $P < 0.05$.

+(vehicle/saline – vehicle/LPS) significantly different from (NS-398/saline - NS-398/LPS), Bonferroni-Holm test after significant ANOVA or Mann-Whitney U test after significant Friedman's ANOVA on ranks, as described in text, $P < 0.05$.

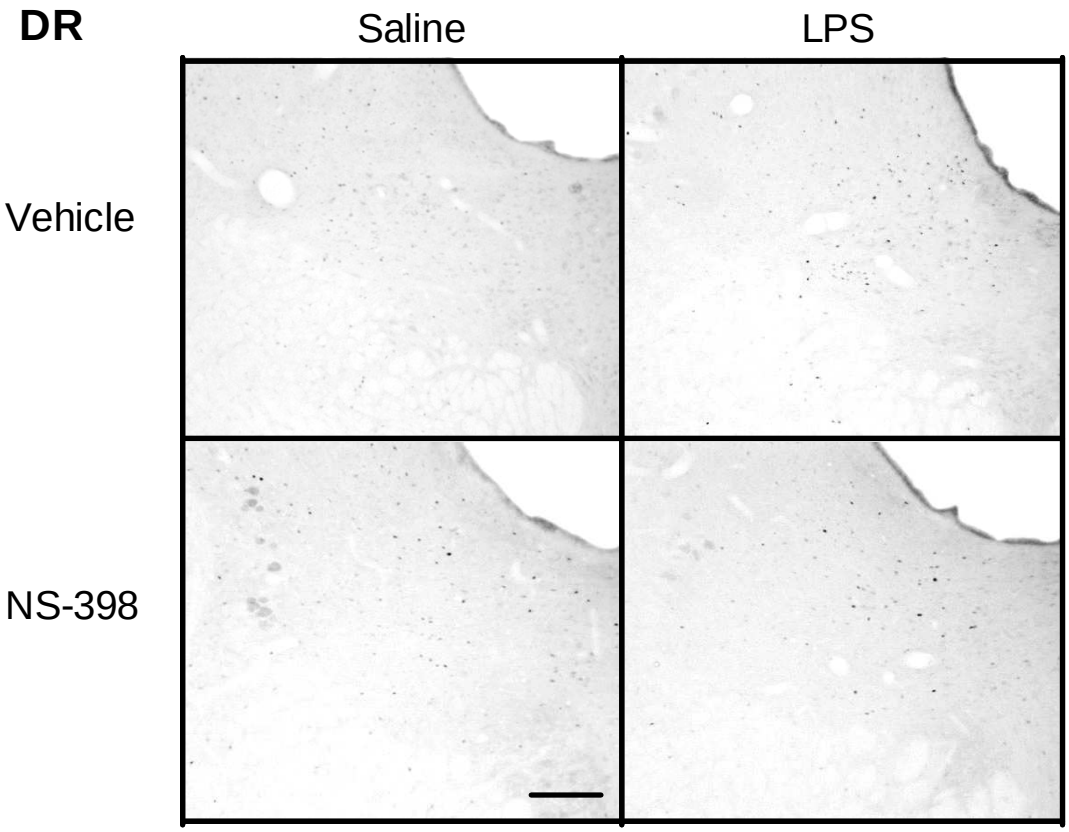

Figure 2b. Photomicrograph depicting c-Fos immunoreactivity in the DR (scalebar 100 µm)

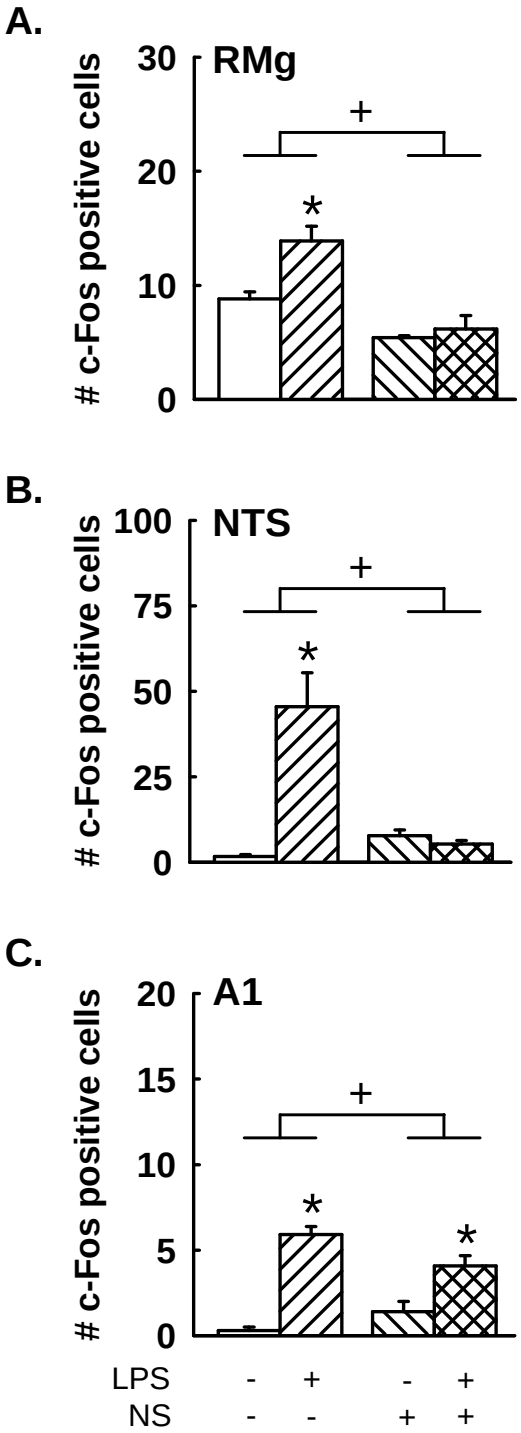

Figure 3. Effects of NS-398 on LPS-induced c-Fos expression in the RMg (A), NTS (B) and A1 (C) by NS-398. Rats were ip injected with vehicle or 10 mg/kg NS-398 1 h before dark onset and saline or 100 µg/kg LPS at dark onset. Data are numbers of cells/section expressing c-Fos (mean ± SEM), 5-7 rats/group.

*significantly different from control, Bonferroni-Holm test after significant ANOVA or Mann-Whitney U test after significant Friedman's ANOVA on ranks, as described in text, $P < 0.05$.

+(vehicle/saline – vehicle/LPS) significantly different from (NS-398/saline - NS-398/LPS), Bonferroni-Holm test after significant ANOVA or Mann-Whitney U test after significant Friedman's ANOVA on ranks, as described in text, $P < 0.05$.

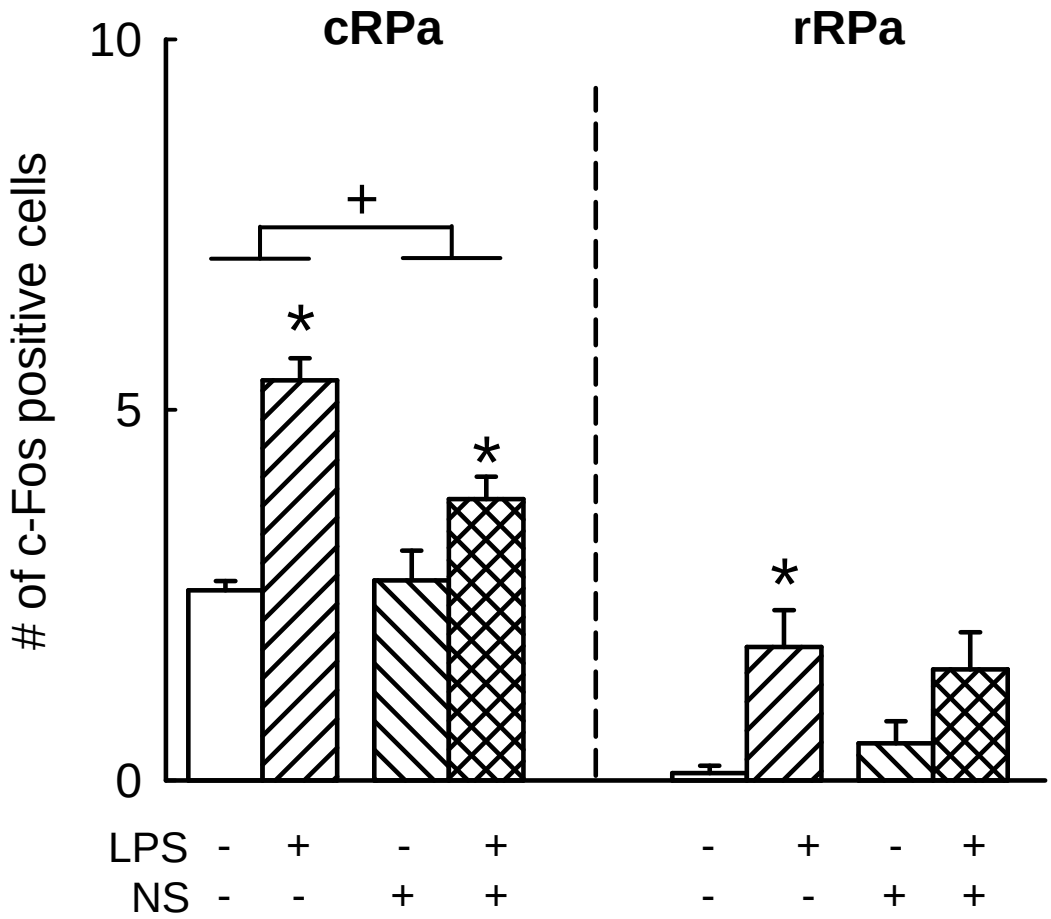

Figure 4. Effects of NS-398 on LPS-induced c-Fos expression in the cRPa and rRPa by NS-398. Rats were ip injected with vehicle or 10 mg/kg NS-398 1 h before dark onset and saline or 100 µg/kg LPS at dark onset. Data are numbers of cells/section expressing c-Fos (mean ± SEM), 5-7 rats/group.

*significantly different from control, Bonferroni-Holm test after significant ANOVA or Mann-Whitney U test after significant Friedman's ANOVA on ranks, as described in text, $P < 0.05$.

+(vehicle/saline – vehicle/LPS) significantly different from (NS-398/saline - NS-398/LPS), Bonferroni-Holm test after significant ANOVA or Mann-Whitney U test after significant Friedman's ANOVA on ranks, as described in text, $P < 0.05$.

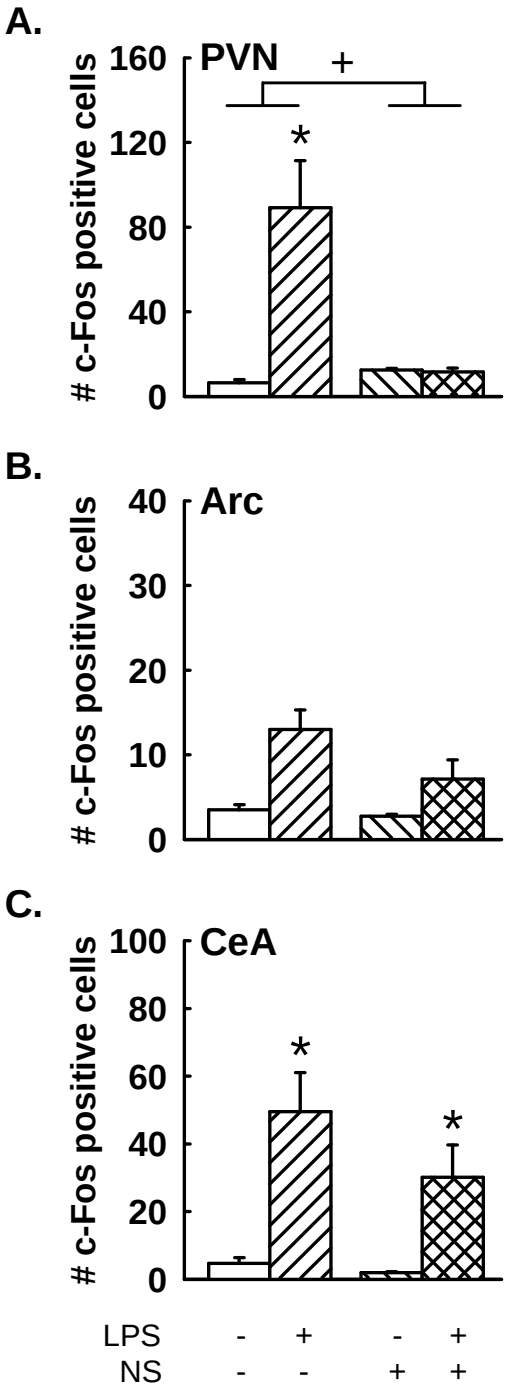

Figure 5a. Effects of NS-398 on LPS-induced c-Fos expression in the PVN (A), Arc (B) and CeA (C) by NS-398. Rats were ip injected with vehicle or 10 mg/kg NS-398 1 h before dark onset and saline or 100 µg/kg LPS at dark onset. Data are numbers of cells/section expressing c-Fos (mean ± SEM), 5-7 rats/group.

*significantly different from control, Bonferroni-Holm test after significant ANOVA or Mann-Whitney U test after significant Friedman's ANOVA on ranks, as described in text, $P < 0.05$.

+(vehicle/saline – vehicle/LPS) significantly different from (NS-398/saline - NS-398/LPS), Bonferroni-Holm test after significant ANOVA or Mann-Whitney U test after significant Friedman's ANOVA on ranks, as described in text, $P < 0.05$.

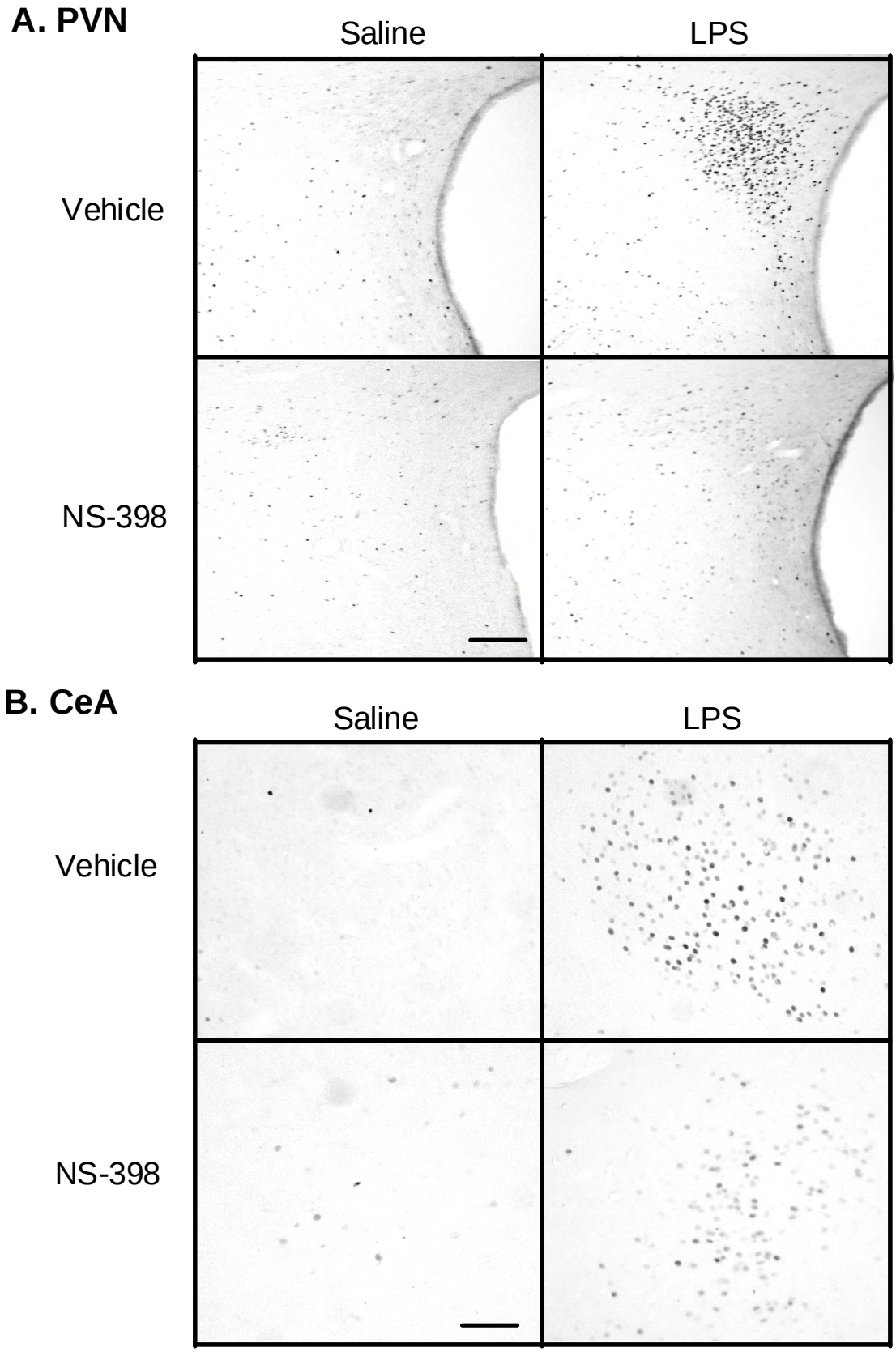

Figure 5b. A: Photomicrograph depicting c-Fos immunoreactivity in the PVN (scalebar 100 µm)

B: Photomicrograph depicting c-Fos immunoreactivity in the CeA (scalebar 50 µm)

6.3.3 Experiment 3: Anorexia following DR administration of PGE_2

16-hr food deprived rats receiving DR injections of PGE_2 ate significantly less than vehicle injected rats during the first 30 min of food access. Specifically, injections of 10 or 100 ng/rat PGE_2 directly into the DR significantly decreased 30 min food intake by more than 50% when compared to vehicle (Figure 6, $F(3, 18) = 6.07$, $P < 0.004$, SED = 0.6 g). Due to the short half-life of PGE_2, food intake at 1 and 2 hr post-injection were not significantly different between PGE_2 and vehicle-injected rats.

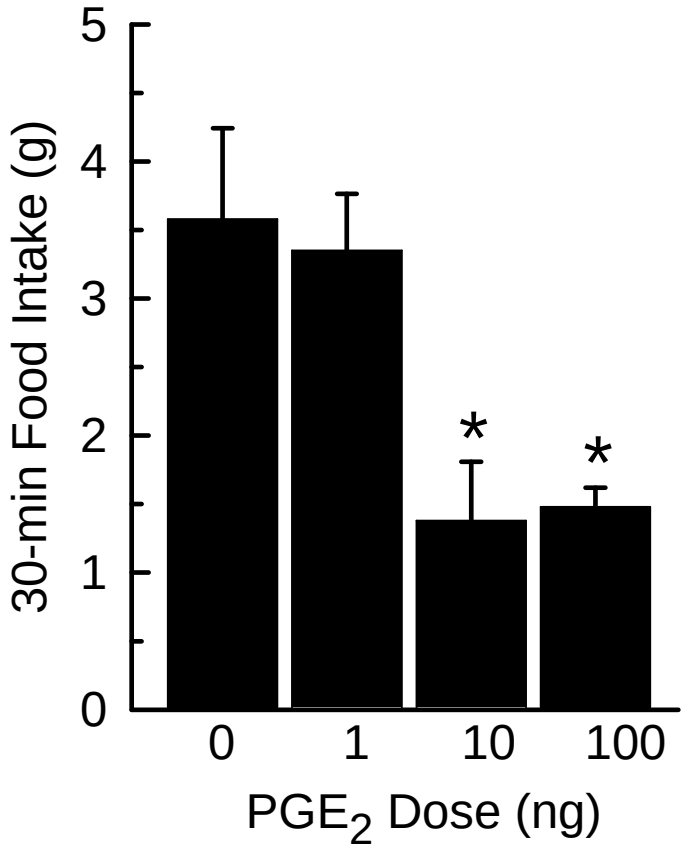

Figure 6. Icv administration of PGE_2 into the DR reduces food intake

Rats were food deprived for 16 h and infused icv with 0, 10, or 100 ng PGE_2 30 min before dark onset. Food was returned and food intake was measured for 30 min. Data are cumulative food intakes (mean ± SEM). 7-8rats/group

*significantly different from control, Bonferroni-Holm test after significant ANOVA or Mann-Whitney U test after significant Friedman's ANOVA on ranks, as described in text, $P < 0.05$.

6.3.4 Experiment 4: LPS-anorexia following DR/MnR COX-2 antagonism

DR/MnR injections of NS-398 attenuated the LPS-induced anorexia at 1, 4, 7, 12 and 24 h after LPS injection (Figure 7, $t(12) = 12.9$, $P < 0.01$, SED = 0.7 g; $t(11) = 2.25$, $P < 0.04$, SED = 1.3 g; $t(12) = 2.9$, $P < 0.01$, SED = 0.7 g; $t(13) = 3.15$, $P < 0.007$, SED = 2.12 and $t(11) = 5.15$, $P < 0.003$, SED = 2.6 g, for 1, 4, 7, 12 and 24 h, respectively). NS-398 did not decrease LPS anorexia at 2 h ($t(9) = 1.41$, $P > 0.05$, SED = 0.6 g).

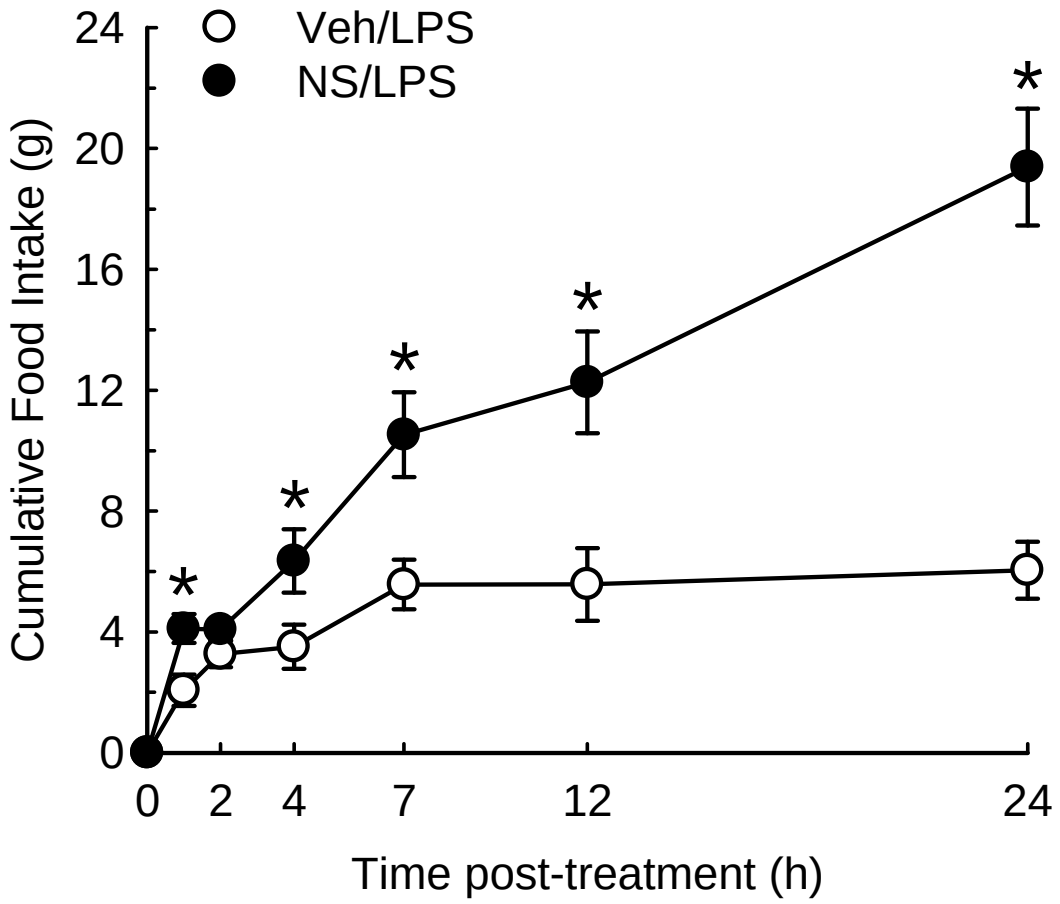

Figure 7. Reversal of LPS anorexia by icv NS-398 administration into the DR/MnR.

Rats were food deprived for 3 h before dark and at the same time injected ip with 100 µg/kg LPS or saline and infused icv with 0 or 1 ng NS-398 at dark onset. Food was returned and food intake was measured for 1, 2, 4 and 23 h . Data are cumulative food intakes (mean ± SEM). 8-9 rats/group

*significantly different from control, Bonferroni-Holm test after significant ANOVA or Mann-Whitney U test after significant Friedman's ANOVA on ranks, as described in text, $P < 0.05$.

6.4 Discussion

Here we present behavioral and cellular evidence that advances the understanding of the role of brain PGE_2 signaling in LPS anorexia. We report four principal results. First, ip injection of the specific COX-2 antagonist NS-398 1 h before ip injection of 100 µg/kg LPS completely eliminated LPS anorexia for the ensuing 23 h. This extends previous reports indicating that reductions in PGE_2 synthesis partially block LPS anorexia under slightly different conditions [2]. Second, ip NS-398 reduced or eliminated LPS-induced c-Fos expression measured 90 min after LPS injection in several brain areas, ranging from the RPa in the caudal medulla to the CeA in the forebrain. These data indicate that PGE_2 is necessary for some or all LPS-induced neural activation in numerous brain areas. Third, intracranial injections of PGE_2 targeted to the DR, a principal site of ascending 5-HT projections, was sufficient to reduce food intake. Direct [94] as well as intracerebroventricular (icv) [93] administration of PGE_2 into the lateral hypothalamus has already been shown to reduce eating in rats, but the administered doses were high and the specificity of the observed effects is questionable. Fourth and finally, injection of NS-398 targeted to the median raphe (DR and MnR) significantly reduced ip LPS anorexia. Thus, the midbrain raphe is one area in which increased PGE_2 is required for ip LPS anorexia.

The effects of ip NS-398 injection on LPS anorexia extend several previous reports implicating PGE_2 as a mediator of illness anorexia [2, 92, 230, 231]. Lugarini et al. [2] showed that ip injection of NS-398 attenuated, but did not eliminate, the anorectic effect of ip LPS in rats [2]. Here, in contrast, ip NS-398 completely reversed LPS anorexia for at least 23 h. This difference in potency may be due to the different vehicle (i.e., CMC vs. a DMSO-saline mixture), timing (i.e., 1 h prior to LPS injection vs. 2 h post or simultaneously with LPS injection), or other differences. We conclude that, at least under some conditions, PGE_2 signaling is necessary for ip LPS anorexia. Our result also appears to extend previous dissociations of illness anorexia and fever [186, 235]. This is because, in contrast to the prolonged reversal of LPS anorexia we report, LPS-induced fever

was reversed for only 3 h after NS-398 injection under conditions similar to ours [1]. As described below, our c-Fos data suggest another dissociation between the mechanisms of illness anorexia and fever.

Ip NS-398 also markedly reduced LPS-induced c-Fos expression measured 90 min after LPS injection. LPS-induced c-Fos expression was statistically eliminated by NS-398 in the DR, MnR, RMg, cRPa, NTS, A1 and PVN. C-Fos expression 90 min post-stimulus reflects electrophysiological activity about an hour earlier, i.e., in our experiment very soon after LPS injection. Thus, our data suggest that much or all of the initial neuronal activation induced by ip LPS in these areas requires PGE_2 signaling.

We hypothesized that the midbrain raphe nuclei, i.e., the DR and MnR, are likely to be a site where the stimulation of brain capillary PGE_2 release by peripheral immune mediators gives rise to 5-HT neural signals that cause anorexia [23, 192]. The midbrain raphe is a principal source of ascending 5-HT projections, and there is extensive evidence that 5-HT neural signaling is necessary for ip LPS anorexia. This evidence includes reports that midbrain raphe 5-HT neurons express EP3 receptors [153] and are activated by PGE_2 [114, 154], that inhibition of 5-HT activity by direct MnR administration of the 5-HT1A receptor ($5\text{-}HT_{1A}R$) agonist 8-OH-DPAT, which is a functional 5-HT antagonist because most $5\text{-}HT_{1A}R$ are negative-feedback autoreceptors [148], attenuated ip and icv LPS anorexia in rats [147, 150], that DR neurons release 5-HT after LPS treatment [108], and, finally, that under the same conditions as tested here, antagonism of 5-HT2C receptors (2CR) reversed both LPS-induced anorexia and midbrain raphe c-Fos expression (see Chapter 5)

Our intracranial injection experiments add direct evidence implicating midbrain raphe PGE_2 signaling in LPS anorexia by showing, first, that PGE_2 can act in the midbrain raphe to inhibit eating and, second, that inhibition of PGE_2 signaling in the midbrain raphe reduces ip LPS anorexia. These experiments do not, however, indicate whether or not this midbrain raphe contribution to LPS anorexia involves 5-HT neurons.

The effects of LPS and NS-398 on c-Fos expression in the PVN were similar to those in the DR and MnR; i.e., LPS had a marked effect on c-Fos expression that was completely reversed by NS-398. The LPS-induced increase of c-Fos expression in the PVN extends numerous previous reports under conditions [101, 103, 105, 107, 216] in which c-Fos was measured later after LPS and in which LPS was administered in different amounts and via different routes. These effects across widely varying conditions indicate that the PVN is an important site for both the initial and subsequent phases of the APR. A role for PGE_2 in the PVN has also been previously reported. Zhang et al. [236], for example, reported that ip administration of SC-236, another specific COX-2 antagonist, reversed LPS induced c-Fos expression in the PVN 4 h following an intravenous injection of 4 µg/kg LPS. LPS-induced activation of the PVN has been implicated in fever and activation of the HPA-axis [109, 210, 211]. Although there is no direct evidence implicating the PVN in illness anorexia, this seems very likely given the PVN's important role in the control of eating. Also, several reports indicate that PGE_2 affects signaling in PVN corticotropin-releasing hormone (CRH) neurons [102, 110, 114], and CRH appears to play a role in the control of eating independent of its role in the HPA-axis.

Our data, together with the literature reviewed above, suggest the foundations of the neural network for LPS anorexia. The results of our PGE_2 and NS-398 infusions, as well as the c-Fos data, suggest the midbrain raphe as the focus of this network, i.e., we propose that peripheral immune signaling activates inducible COX-2 in endothelial cells in the midbrain raphe, leading to PGE_2 release, stimulation of neuronal EP3 receptors, and activation of the neural network mediating anorexia. Based on the data reviewed above, we hypothesize that many or most of these midbrain raphe neurons express 5-HT. Next, the fact that the PVN is an important target of ascending 5-HT fibers from the midbrain raphe together with the similarity of the c-Fos effects measured in the DR and MnR with those measured in the PVN, and the dissimilarity of midbrain raphe c-Fos and c-Fos in other forebrain sites, suggest that the PVN is the primary forebrain node in this network. We propose this network as a working model for future studies of LPS anorexia.

LPS-induced c-Fos expression was significantly reduced, but not eliminated, by NS-398 in the cRPa and A1, suggesting that PGE_2 contributes to, but is not necessary for, the full initial neuronal activation induced by ip LPS in these areas. Furthermore, these data suggest that the neural activity remaining after NS-398 treatment is not sufficient to mediate LPS anorexia. It has been reported that neurons of the RPa can be affected by LPS and other inflammatory mediators [97, 237]. These studies, however, used larger LPS doses (250 µg and 1 mg/kg) and measured c-Fos only at later time points (3 and 4 h) after LPS administration [103, 105] than we did in the present study. Our data, therefore, extend these findings by demonstrating that a dose of LPS that mimics the clinical features of the APR induced c-Fos in the RPa at a time when LPS anorexia also develops.

Because anorexia and fever develop with about the same latency, it is likely that some or all of the areas in which LPS and NS-398 affected c-Fos expression here are involved in mediating LPS's febrile effects. Our RPa data are especially interesting in this connection. The RPa is an important source of upper motor neurons projecting to sympathetic preganglionic neurons in the spinal cord and to the NTS and dorsal motor nucleus of the vagus, suggesting their potential involvement in control of gastrointestinal, pancreatic and brown adipose responses (reviewed in [97, 238, 239]). Nakamura et al. [97] reported that the number of cells expressing c-Fos in the rRPA, but not in the cRPA, was increased one hour after injection of PGE_2 into the lateral ventricle or preoptic area (POA) in urethane-anesthetized rats. In contrast, using the same rostral-caudal definition, we found that ip LPS increased c-Fos expression in the cRPa as well as in the rRPa and that ip NS-398 significantly reduced cRPa c-Fos, but not rRPa c-Fos. Taken together, these data suggest, first, that ip LPS activates the cRPa by a mechanism that involves in part PGE_2 signaling that does not arise in the POA or other sites near the lateral ventricles, and, second, that the PGE_2-dependent input from the POA to the RPa may not be activated by ip LPS. In so far as ip injection of the moderate LPS dose that we used is considered to be a valid model of illness, the latter conclusion suggests that the pathophysiological relevance of intra-POA PGE_2 injections deserves further research. It is also important to note

in this context that we did not determine c-Fos expression in all brain areas that have been implicated in fever or visceral elements of the APR, in particular the POA [97, 240] and the lateral hypothalamic area [238].

In contrast to the PVN results, in the CeA NS-398 did not affect LPS-induced c-Fos expression and in the Arc LPS alone had no significant effect. That LPS induces c-Fos expression in the CeA has been reported before under various conditions [101, 103-106, 209, 216, 217]. The failure of NS-398 to reduce this effect suggests that PGE_2 signaling is not a necessary part of the initial LPS-induced neuronal activation in this area. This is similar to the result of a study in which ip administration of the specific COX-2 antagonist SC-236 blocked c-Fos expression 4 h after intravenous infusion of 4 µg/kg dose LPS [236]. Administration of the non-specific COX antagonist indomethacin has also been reported to reduce LPS-induced c-Fos under various conditions and at various timepoints [112, 113, 120]. Our failure to detect an increase in c-Fos in the Arc appears inconsistent with several previous studies [101, 103-105], perhaps because of differences in LPS dose or time of sampling. For example, in one study [101], the high ip dose of 1 mg/kg LPS did not induced c-Fos mRNA expression 2 h after injection, but did so 4 h after injection [105].

Ip LPS also increased c-Fos in the brainstem areas NTS and A1 of the VLM, and NS-398 eliminated these effects in the NTS and partially in the A1. These data are also consistent with previous reports [101, 103-105, 216]. Furthermore, in ip LPS-challenged mPGES-1 knock out mice the induction of c-Fos expression was strongly reduced in the NTS and the VLM [217]. The activation of the A1 area suggests that noradrenergic (NA)/CA pathways may be part of the mechanism mediating the COX-2 effect on LPS anorexia. Indeed, Lacroix et al. [101] reported that indomethacin decreased the number of tyrosine hydroxylase-immunoreactive cells that express LPS-induced c-Fos mRNA. Therefore our findings suggest the involvement of the NTS and the A1 area in LPS anorexia and PGE_2 might be necessary for the full initial neuronal activation by ip LPS in the NTS but not in the A1.

7 Dose-related LPS-induced anorexia is associated with dose-related increases in brain c-Fos expression in rats

7.1 Introduction

The acute phase response (APR) of the innate immune system is the organism's initial response to bacterial infection, various toxins and other challenges. The APR can continue into a chronic response and also plays an important role in the activation of the adaptive immune system (reviewed in [191, 192]). The APR includes both peripheral responses and brain-mediated responses such as fever, anorexia and somnolence. The poor present understanding of the brain mechanisms of the APR hinders development of effective therapies for a number of clinical features of the APR, including illness anorexia [192]. To further investigate the mechanisms of illness anorexia, we used immunocytochemical methods to examine the brain mechanisms mediating the acute anorectic response to systemic injections of bacterial lipopolysaccharide (LPS).

LPS, a constituent of the cell wall of Gram-negative bacteria, is released during bacterial proliferation or lysis. It appears to be the major stimulus for the APR following Gram-negative bacterial infection and its administration is a widely used model. Circulating LPS, usually in the form of a complex with LPS-binding protein and often also with soluble CD14, binds to Toll-like receptor 4 (TLR-4) on macrophages and other immune cells [11]. TLR-4 activation causes the synthesis and release proinflammatory cytokines including interleukin-1 (IL-1), IL-6, and tumor necrosis factor-α (TNF-α), which then initiate a cascade of reactions involving other immune cell types and mediators and, ultimately, leading to the APR [15, 191-193].

How changes in circulating cytokines and other immune mediators affect the brain mechanisms controlling eating, fever and other brain-mediated elements of the APR remains unresolved. The neuronal mechanisms mediating LPS anorexia have been investigated by mapping changes in the expression of the *c-fos* gene, which occurs in some neurons in response to altered neurochemical or electrophysiological activity [184]. Ip injection of LPS elicits expression of c-Fos protein in several brain areas at various times after injection [101, 104, 111, 241, 242], including areas related to autonomic and endocrine control and to the control of normal eating, such as the paraventricular nucleus (PVN), arcuate nucleus (Arc), central nucleus of the amygdala (CeA) [101, 103-105], nucleus tractus solitarii (NTS) [101, 103, 105-107], area postrema (AP) [103, 105, 107], A1 noradrenergic neurons (A1) in the ventrolateral medulla (VLM) [101, 103, 105] and both midbrain and medullary raphe nuclei, for example the dorsal raphe nucleus (DR) [105, 108] and raphe pallidus nucleus (RPa) [103, 105]. In addition, our studies report brain site(s) that are initially activated by LPS-induced signals and further report the possible involvement of PGE_2, serotonin (5-HT) and catecholamines (CA) (see Chapters 5, 6 and 7).

In this study we attempted to identify brain sites that might mediate the initial central effects of peripherally administered LPS by searching for areas in which ip injections of two doses of LPS that produced differential degrees of anorexia also produced different degrees of c-Fos expression 90 min post-injection. Our logic is based on the assumption that brain areas that display dose-related degrees of c-Fos expression soon after LPS injections are more likely to be causally related to the dose-related anorexia than brain areas that do not display such graded c-Fos effects. To our knowledge, this strategy has not been used before.

We conducted four studies to identify likely brain sites of the initial processing of LPS-induced signals in male rats. In the first study, we identified significant, dose-related decreases in food intake within 60 min of ip injection of 12.5 or 100 µg/kg LPS. The second study characterized the effects of these two LPS doses on c-Fos expression 90 min later in several brain areas known to be

involved in the control of food intake. c-Fos expression 90 min post-stimulus reflects the electrophysiological activity, i.e., when LPS-anorexia developed under our conditions. In the third study, we investigated the role of CA neurons in the A1 area of the VLM, NTS and PVN by using double immunostaining for c-Fos and tyrosine hydroxylase (TH), a marker of CA neurons. CA neurons in the NTS and VLM play a critical role in the induction of the hypothalamo-pituitary-adrenal (HPA) response to immune related stimuli [120], in part via direct, predominantly CA projections [136].

Numerous studies indicate that 5-HT is a major neurochemical mediator of LPS anorexia [23, 191-193]. For example, ip administration of LPS or IL-1β increases the neurochemical responses of 5-HT neurons [145, 146], and midbrain 5-HT neurons project to several forebrain areas. Furthermore, a number of studies implicate the 5-HT2C receptor (2CR) in LPS anorexia. For example, antagonism of 2CR receptors partially [150, 151] or completely (see Chapter 5) attenuated peripheral and central LPS-induced anorexia. The midbrain raphe nuclei, i.e., the DR and median raphe nucleus (MnR), have been implicated in the initiation of LPS anorexia. That is, we have shown that PGE_2 injected directly into the midbrain raphe inhibits eating and that inhibition of PGE_2 signaling in the midbrain raphe reduces ip LPS anorexia (see Chapter 6). These experiments did not indicate whether or not this midbrain raphe contribution to LPS anorexia involves 5-HT neurons. Therefore, in our fourth study we investigated the role of 5-HT neurons in LPS anorexia by using double immunostaining for c-Fos and tryptophan-hydroxylase (TPH), a marker of 5-HT neurons, in the DR, MnR and RPa.

7.2 Materials and Methods:

7.2.1 Animals and housing

Male Long-Evans rats were bred in our colony and housed individually in a room maintained at 22 ± 2 ℃, with 12:12-h light-da rk cycles (lights on: 2300 h). Rats were offered ground chow (Provimi Kliba NAFAG #3433, Kaiseraugst, Switzerland) and water ad libitum except as noted below. All procedures were approved by the Veterinary Office of the Canton of Zurich.

7.2.2 Effects of LPS on Food Intake

Procedure. Twenty-two rats (body weight 417 ± 8 g, mean ± SEM) were housed individually in plexiglass cages (16 x 42 x 22 cm) with stainless steel grid floors. A plexiglass tunnel (30 cm long and 8 cm in diameter) protruded horizontally from one side of the cage, approximately 5 cm above the cage floors; at a 90° angle with the cage floor. The end of the tunnel opened over a food cup mounted on top of an electronic balance (PM 3000, Mettler-Toledo, Greifensee, Switzerland). The balances interfaced with a computer in an adjacent room, and custom-designed software (VZM, Software Entwicklung Krügel, Munich, Germany) recorded the weight of each balance (± 0.1 g) at 30-s intervals and later converted these data into spontaneous eating patterns, using a meal definition as any eating bout of at least 0.2 g that was separated from other bouts by at least 15 min.

Spontaneous eating was recorded for 1, 2, and 12 hours. Between 1000 and 1100 h each day the program was halted, access to the tunnel was blocked by a sliding plexiglass door, the food cups were filled with fresh ground chow and the rats were weighed. Just prior to dark onset, rats received ip injections of 1 ml/kg 0.9% saline (NaCl, B.Braun Medical, Emmenbrücke, Switzerland) and the

computer was restarted. After approximately two weeks of adaptation to these procedures, the effects of LPS (*Escherichia coli*, serotype 0111:B4; Sigma, Buchs, Switzerland) on spontaneous eating were tested using within-dose crossover designs. LPS solutions in saline were freshly prepared each day, with LPS concentration adjusted so that injection volume was 1 ml/kg, and the procedure described above was followed. Due to tolerance effects [243] each rat received only one LPS injection. The order in which individual rats received LPS and saline injections was randomized. Eleven rats received 12.5 µg/kg LPS, and 11 received 100 µg /kg LPS. These doses were selected on the basis of prior studies [188, 199]

7.2.3 Statistical Analyses

A robust statistical approach was adopted to increase statistical power [201, 202]. Logarithmic or inverse transformations were used as required to improve normality. In addition, to reduce the influence of extreme values, data were converted to standard scores using the median absolute deviate method, and standard scores with absolute values > 1.96 (i.e., $P < 0.05$) were excluded. Food intakes at the three measurement times were analyzed with separate one-way ANOVA (Sigma Stat version 3.5, Systat Software, San Jose, CA, USA) followed by Bonferroni-Holm tests. Spontaneous meal size and number during the 12-h nocturnal period were analyzed similarly. Three post-hoc comparisons were tested: (1) saline versus 12.5 µg/kg LPS, (2) saline versus 100 µg/kg LPS, and (3) difference (1) versus difference (2). The criterion for a dose-related effect was that all three tests be significant. Data are reported as means ± standard error of the mean (SEM), and the standard error of the difference (SED) is given to indicate experiment-wide residual variability.

7.2.4 Effects of LPS on c-Fos expression

Procedure. Two sets of rats (n=17 and n=16) were housed in plastic cages (16 x 55 x 22 cm) with wood-chip bedding (Rettenmaier & Söhne, Rosenberg, Germany) and adapted to the procedure described above with the exception that, in order to adapt the rats to the 90-min food deprivation before sacrificing for c-Fos expression, food was presented 4 h after the dark onset. Thus, on the experimental day, the same three treatments as above were administered and 90 minutes after the injection the rats were anesthetized with ip injections (1 ml/kg) of 50 mg/kg sodium pentobarbital (Nembutal, Abbott Laboratories, Abbott Park, USA), thoracotomized, and transcardially perfused (20 ml/min) with 100 ml 0.1 M phosphate buffer (PB, pH 7.4); followed by 300 ml of 4 % paraformaldehyde in 0.1 M PB. The brains were removed, postfixed in 4% paraformaldehyde solution for 2 h at room temperature and then in 20% sucrose in 0.1 M PB for 2 days at 4 °C. Forty-micron thick sections of hindbrain (14.3 -7.3 mm posterior to bregma [200]) and forebrain (1.8 - 2.7 mm posterior to bregma) were cut on a freezing sliding microtome, collected in six serial ordered sets, and stored in a 40% 0.1M PB, 30% ethylene glycol, and 30% glycerol solution at –20 °C.

7.2.5 Single-label Immunocytochemistry

One set of hindbrain and forebrain sections of each set of rats were processed for c-Fos expression using diaminobenzidine (DAB; Chemie Brunschwieg, Basel, Switzerland) standard immunocytochemistry technique. Sections were rinsed in 0.1 M PB buffer for 10 min followed by 30 min incubation in 1% sodium borohydride. After another PB rinse and a 10 min- incubation in 0.5 % H_2O_2 in 0.1 M PB, and another PB rinse, tissue sections were incubated for 1 h in 1 % normal goat serum (NGS, Vector Laboratories, Burlingame, CA, USA) solution in 0.1 M PB with 0.3 % Triton-X (0.3 % PBTx), and then overnight in primary antibody solution (1:10,000 rabbit polyclonal c-Fos antibody, Ab-5

Calbiochem, Cat#PC38, La Jolla, CA, USA, in 0.3 % PBTx and 1% NGS). The next day, sections were incubated for 1h with biotinylated anti-rabbit goat IgG (1:300; BA-1000, Vector Laboratories). Finally, the antibody complexes were visualized using the streptavidin-DAB-peroxidase reaction (ABC, 1:300, Vector Laboratories, Reactolab SA, Servion, Switzerland). Sections then were mounted on gelatinized microscope slides, dehydrated in an increasing series of alcohols, defatted in xylene, and cover slipped with Permount (Fisher Scientific, Wohlen, Switzerland).

7.2.6 Double-label immunocytochemistry

TH and c-Fos: Another set of sections from the first set of rats was processed with double-label immunofluorescence to identify c-Fos and TH. The procedure described above was followed, except the forebrains were incubated overnight at room temperature and the hindbrains were incubated 48 h at 4 °C, in primary antibody solution (1:10,000 rabbit polyclonal c-Fos antibody, Ab-5, Cat#PC38; Calbiochem) in 0.3 % PBTx and 1% NGS). The following steps were done in the dark. The second day, sections were incubated for 1 h with secondary antibody solution (1:400 Cy-3 AP Goat Anti-rabbit IgG (H+L), Jackson ImmunoResearch, Cambridgeshire, UK in 0.3 % PBTx and 1% NGS). Tissue sections were then rinsed in 0.1 % PBTx for 30 min followed by a preincubation in 0.3 % PBTx and 1% NGS and then overnight at room temperature in primary antibody solution (1: 1000 TH antibody, Sodiag SA, Losone, Switzerland in 0.3 % PBTx and 1% NGS). The third day sections were washed again for 30 min and incubated 1 h at room temperature with secondary antibody solution (1:400 Cy-2 AP Goat Anti-mouse IgG (H+L), Jackson ImmunoResearch, in 0.3 % PBTx and 1% NGS). Sections were then mounted on gelatinized microscope slides, dehydrated in an increasing series of alcohols, defatted in xylene, and cover slipped with Permount (Fisher Scientific, Wohlen, Switzerland).

TPH and cFos: Another set of sections from the second set of rats was processed for co-localization of c-Fos and tryptophan hydroxylase (TPH) expression using DAB immunocytochemistry technique. Sections were rinsed in 0.1 M PB buffer for 10 min followed by 5 min incubation in 3 % H_2O_2 in 0.1 M PB. After another PB rinse tissue sections were incubated for 15 min in blocking solution made in 0.1 M PB (Avidin/Biotin blocking kit, Cat#SP-2001, Vector Laboratories, Inc. Burlingame, CA, USA). Sections were rinsed in 0.1 M PB buffer for 10 min followed by 1 h incubation in 1 % normal donkey serum (NDS, Jackson ImmunoResearch, Cambridgeshire, UK) solution in 0.1 M PB with 0.3 % Triton-X (0.3 % PBTx), and then overnight in primary antibody solution (1:10,000 rabbit polyclonal c-Fos antibody, Ab-5 Calbiochem, Cat#PC38, La Jolla, CA, USA, in 0.3 % PBTx and 1% NDS). The next day, sections were incubated for 1 h with secondary antibody solution (1:400 anti rabbit made in donkey Biotin-SP, Jackson ImmunoResearch, in 0.3 % PBTx). The antibody complexes were visualized using the ABC (1:400, Vector Laboratories) and nickel chloride and DAB (NiDAB; DAB substrate kit for peroxidase, Cat#SK-4100, Vector Laboratories). After another PB rinse tissue sections were incubated with blocking solution as mentioned above followed by 1 h incubation in 1 % NDS solution in 0.1 M PB with 0.3 % PBTx, and then overnight in primary antibody solution (1:5000 sheep anti tryptophan hydroxylase polyclonal antibody, Cat#AB1541, Chemicon-LucernaChem, Switzerland). The next day, sections were incubated for 1 h with secondary antibody solution (1:400 anti sheep made in donkey Biotin-SP, Jackson ImmunoResearch, in 0.3 % PBTx). The antibody complexes were visualized using ABC (1:400, Vector Laboratories) and DAB (ImmPACT DAB Peroxidase Substrat, Cat#SK-4105, Vector Laboratories). Sections then were mounted on gelatinized microscope slides, dehydrated in an increasing series of alcohols, defatted in xylene, and cover slipped with Permount.

7.2.7 Image Analysis

LPS-induced c-Fos: In both sets of rats DAB-visualized c-Fos expression was quantified in the PVN, Arc, CeA, NTS, AP and A1. Because there were no brain sections available of the several raphe nuclei in the first set of rats the quantification of DAB-visualized c-Fos expression in the raphe magnus nucleus (RMg), RPa, DR and MnR was only done in the second set of rats. The number of cells expressing c-Fos was quantified bilaterally using Image Pro software (Version 6.0, MediaCybernetics, Silver Spring, MD, USA) in the following regions (locations are caudal to bregma as per [4]) : PVN (1.80mm), Arc (2.65mm), CeA (2.65mm), NTS (mean of 3-5 sections, 13.65 - 14.30mm), AP (13.65mm), A1 (13.65mm), RMg (mean of 3-5 sections, 11.60 - 9.08mm), RPa (mean of 10-14 sections, 10.52 - 14.08), DR (mean of 4 sections, 9.30 – 7.80mm) and MnR (mean of 3-4 sections, 8.3-7.3mm). Digital images of each section were made on Olympus AX70 microscope (Center Valley, PA, USA). Bilateral counts in each area were done using Image Pro software. Cells were considered c-Fos positive if their nuclei contained dark, punctate blue-black immunolabeling and were counted using constant minimum and maximum optical densities and object size criteria, which were based on the group means and validated with visual counts. Mean count/section were analyzed (bilateral counts are presented for midline structures and the mean of the left and right sections for others.

Co-localisation of c-Fos and TH: Labeled cells were counted by eye in images made with the Cy2 and Cy3 excitation wavelengths individually and together in the PVN, NTS and A1 area of VLM. The criteria for double labeling of individual cells was: 1) presence of red nuclear labeling in the Cy3 image (c-Fos), 2) green labeling in the Cy2 image (TH), and 3) double labeling of nuclear c-Fos and cytoplasmic TH immunoreactivities in the Cy3-Cy2 image.

Co-localisation of c-Fos and TPH: One set of tissue sections from the second set of rats was used to obtain counts of TPH-positive neurons and to determine the proportion that was activated to express c-Fos. Sections containing TPH positive neurons were imaged as mentioned above and analyzed in the RPa,

DR and MnR. In these sections, TPH immunoreactive profiles that were clearly cytoplasmic were counted as neurons whether or not a nucleus was visible. TPH neurons were considered c-Fos-positive when their nuclei contained visible blue-black immunolabeling, regardless of intensity. THP neurons were considered c-Fos-negative when they displayed a nucleus lacking c-Fos immunoreactivity. Single and double-labeled cells were counted bilaterally.

7.2.8 Statistical Analyses

Because there were differences in the intensity of the background staining in the single-label c-Fos runs done for the two sets of rats, the data in each set were expressed as percents of the control (saline) mean and then combined for analysis. Nevertheless, even after logarithmic or inverse transformations and removal of extreme values as described above, the data were not sufficiently normal for parametric analysis so were analyzed using ANOVA on ranks, with the differences between pairs of means examined with the Mann-Whitney Rank Sum Test. Most double-label data were sufficiently normal for one-way ANOVA and Bonferroni-Holm tests, which were done as above. In these data, separate analyses were done of the numbers of cells single labeled for c-Fos, single labeled for TH or TPH, double labeled for c-Fos and TH or TPH, and labeled for c-Fos but not TH or TPH (computed by subtraction). Data are reported as means ± standard error of the mean (SEM), and, for parametric analyses, the standard error of the difference (SED) is given to indicate experiment-wide residual variability.

7.3 Results

7.3.1 Effects of LPS on Food Intake

By 1 h after ip injection, 100 µg/kg LPS significantly reduced nocturnal food intake (Table 1, ($F\ (2,15)$ = 4.84, p <0.01; SED = 0.4). By 2 h and 12 h after ip injection, both 12.5 and 100 µg/kg LPS significantly reduced nocturnal food intake, and this effect was dose-related (Table 1, ($F(2,16)$ = 28.40, p <0.01, SED = 0.5 and ($F(2,16)$ = 273.67, p <0.01, SED = 0.8)). As previously reported [92], ip LPS inhibited eating mainly by decreasing spontaneous meal frequency, and this decrease was dose-related (Table 2; ($F(2,16)$ = 42.71, p < 0. 01, SED = 0.7)). Nocturnal meal size was significantly reduced only after 100 µg/kg LPS (Table 2; $(F(2,17)= 7.5, p<0.01; SED = 0.3))$.

Table 1. Cumulative food intake after LPS injections. The food intake within the first hour was significantly reduced by 100 µg/kg LPS but not by 12.5 µg/kg LPS. After 2 h and 12 h both doses of LPS reduced food intake significantly, and this effect was dose-related.

	Saline	LPS (12.5 µg/kg)	LPS (100 µg/kg)
1 hour	2.6 ± 1.2	2.6 ± 1.2	1.6 ± 0.9*
2 hours	5.0 ± 1.6	3.0 ± 1.0*	1.6 ± 0.9*+
12 hours	23.7 ± 2.4	15.7 ± 2.5*	2.8 ± 2.4*+

*Significantly different from saline, Bonferroni-Holm test after significant ANOVA, $P < 0.05$.

+Significantly different from LPS 12.5 µg/kg, Bonferroni-Holm test after significant ANOVA, $P < 0.05$.

Table 2. Spontaneous eating parameters after LPS injections. LPS dose-relatedly reduced nocturnal meal frequency. Nocturnal meal size was not significantly affected by 12.5 µg/kg LPS, but was reduced by 100 µg/kg LPS.

	Saline	LPS (12.5 µg/kg)	LPS (100 µg/kg)
Meal frequency	8.7 ± 2.2	6.2 ± 1.5*	1.3 ± 0.9*+
Meal size	3.0 ± 0.9	2.6 ± 0.4	1.7 ± 1.0*

*Significantly different from saline, Bonferroni-Holm test after significant ANOVA, $P < 0.05$.

+Significantly different from LPS 12.5 µg/kg, Bonferroni-Holm test after significant ANOVA, $P < 0.05$.

7.3.2 Effects of LPS on c-Fos Expression

Figures 1 - 3 show the effect of LPS on c-Fos expression. In the forebrain, both doses of LPS increased c-Fos expression in the PVN (Figure 1, $H(2) = 16.74$, $p < 0.01$), CeA (Figure 1, $H(2) = 12.56$, $p < 0.01$) and the Arc (Figure 1, $H(2) = 15.76$, $p < 0.01$). The effect of LPS on c-Fos expression was dose-dependent in the PVN and the Arc and, although the trend was there, it was not dose-dependent in the CeA. Saline alone did not affect c-Fos expression in any of the three forebrain regions.

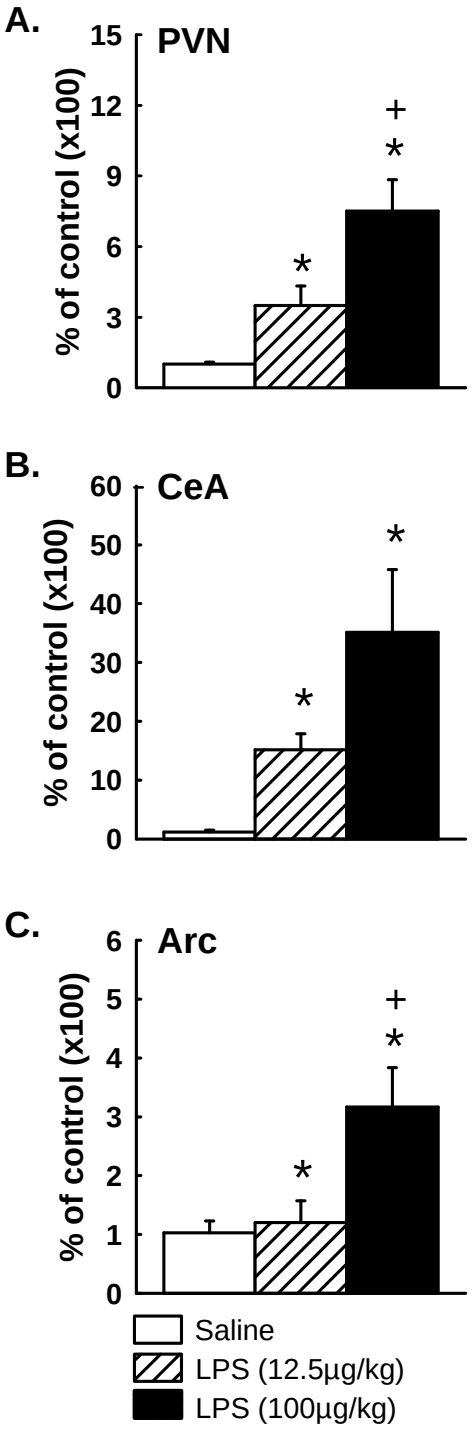

Figure 1a. Ip injection of 12.5 and 100 µg/kg LPS significantly increased the numbers of cells expressing c-Fos in the PVN (A), CeA (B) and Arc (C). The effect of LPS on c-Fos expression was dose-dependent in the PVN and Arc. The effects were significantly dose-related.

*Significantly different from saline, Mann-Whitney Rank Sum Test after significant ANOVA on ranks, $P < 0.05$.

+Significantly different from LPS 12.5 µg/kg, Mann-Whitney Rank Sum Test after significant ANOVA on ranks, $P < 0.05$.

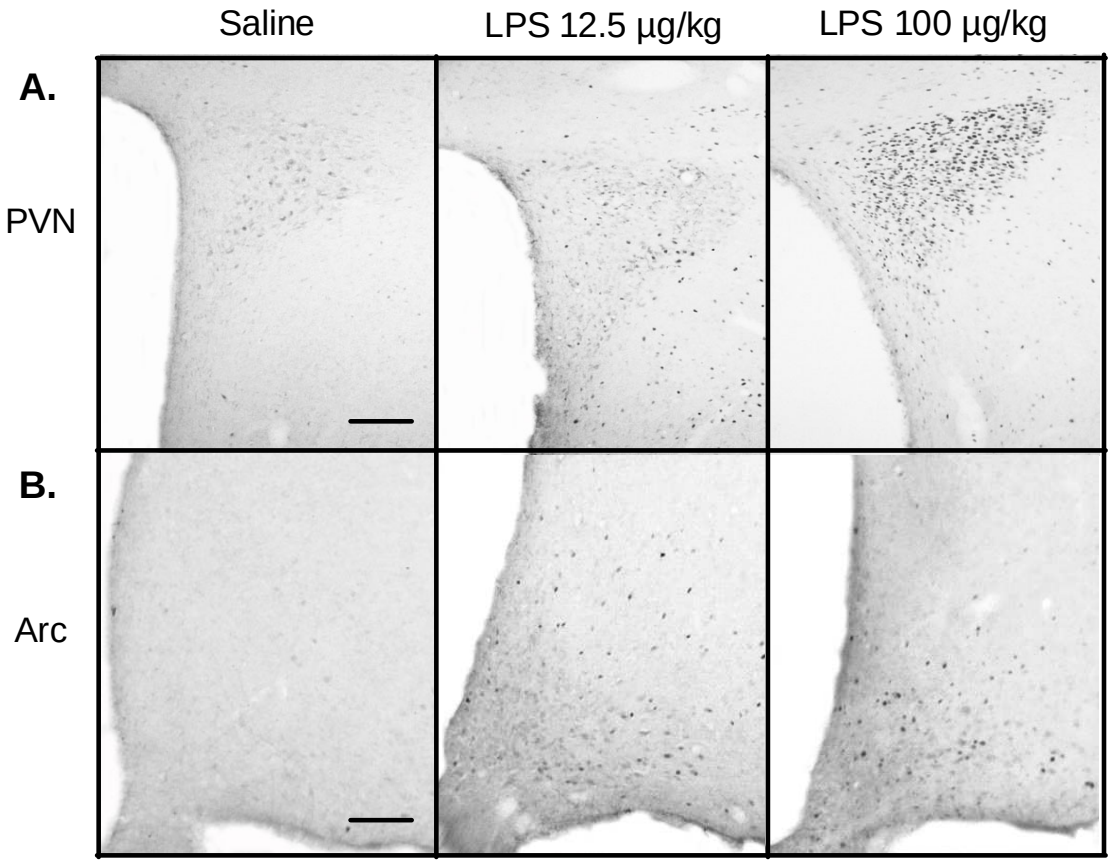

Figure 1b. A: Photomicrograph depicting c-Fos immunoreactivity in the PVN (scalebar 100 µm)

B: Photomicrograph depicting c-Fos immunoreactivity in the Arc (scalebar 100 µm)

In the hindbrain, LPS increased c-Fos expression in all the regions examined: NTS (Figure 2, ($H(2) = 9.245$, $p < 0.05$), A1 (Figure 2, ($H(2) = 9.15$, $p < 0.05$), AP (Figure 2, ($H(2) = 15.39$, $p < 0.01$) and the four raphe regions (Figure 3, ($H(2) = 6.80$, $p < 0.05$; ($H(2) = 8.38$, $p < 0.01$); ($H(2) = 9.73$, $p < 0.01$); ($H(2) = 9.64$, $p < 0.01$), for RMg, RPa, DR and MnR respectively). The effect of LPS on c-Fos expression was only dose-dependent in the DR. Saline alone did not affect c-Fos expression in any of the hindbrain regions.

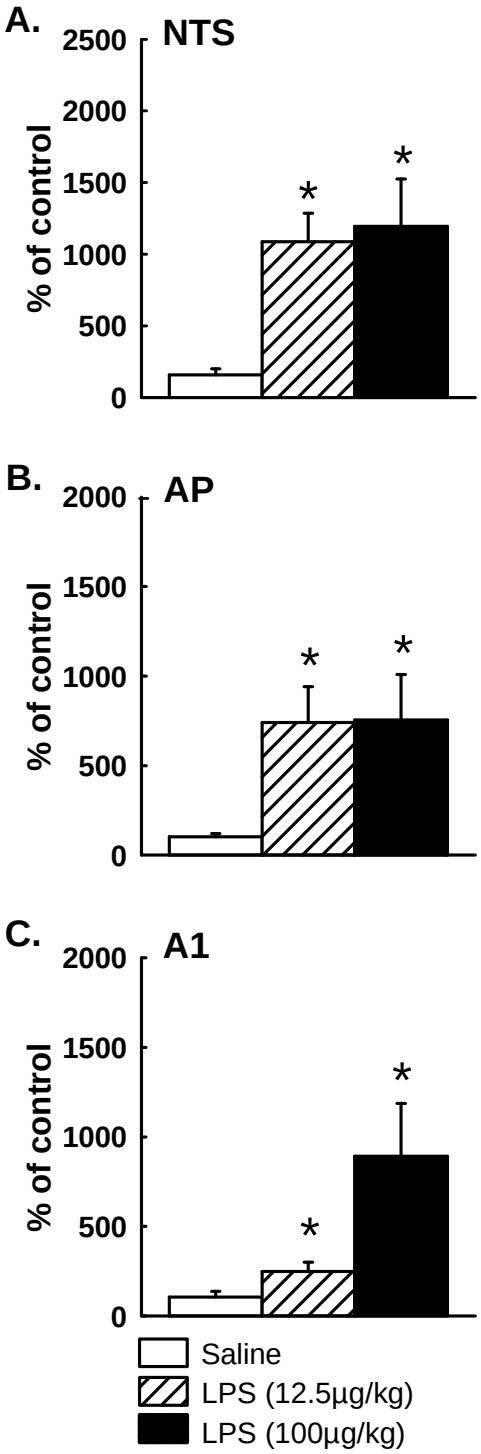

Figure 2. Ip injection of 12.5 and 100 µg/kg LPS significantly increased the numbers of cells expressing c-Fos in the NTS (A), AP (B) and A1 (C). None of the effects was significantly dose-related.

*Significantly different from saline, Mann-Whitney Rank Sum Test after significant ANOVA on ranks, $P < 0.05$.

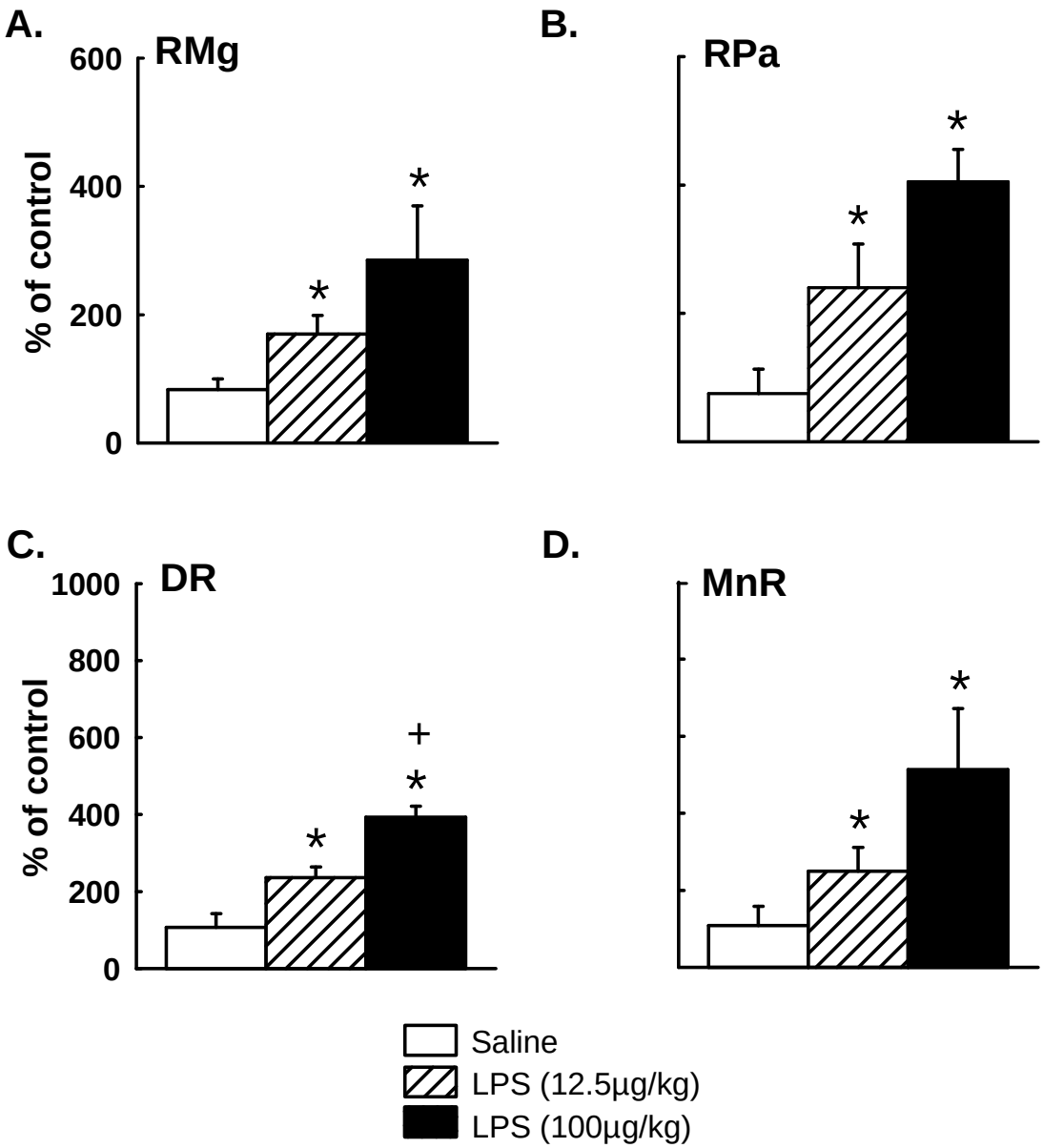

Figure 3a. Ip injection of 12.5 and 100 µg/kg LPS significantly increased the numbers of cells expressing c-Fos in the RMg (A), RPa (B), MnR (C) and DR (D). The effect of LPS on c-Fos expression was significantly dose-dependent only in the DR.

*Significantly different from saline, Mann-Whitney Rank Sum Test after significant ANOVA on ranks, $P < 0.05$.

+Significantly different from LPS 12.5 µg/kg, Mann-Whitney Rank Sum Test after significant ANOVA on ranks, $P < 0.05$.

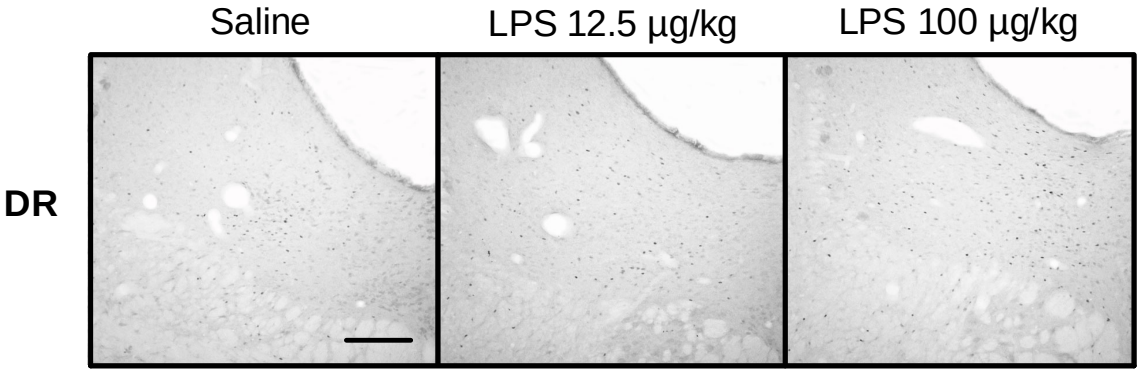

Figure 3b. Photomicrograph depicting c-Fos immunoreactivity in the DR (scalebar 100 µm)

7.3.3 Co-localization of c-Fos and TH

TH and c-Fos double labeling was examined in the A1, NTS and PVN. Only 100 μg/kg LPS significantly increased c-Fos expression in the NTS (Figure 4, ($F(2,12)$ = 6.812, $p < 0.05$; SED = 25.8 cells), but there was no effect in the A1 and PVN (Figure 4, (H (2) = 4.079, $p > 0.05$) and ($F(2,14)$ = 0.243, $p > 0.05$; SED = 24.4 cells)). Both doses of LPS increased the c-Fos expression in the A1 in TH-positive cells (Figure 4, (H (2) = 8.863, $p < 0.01$)) and there was no significant co-localization in the NTS and PVN (Figure 4, ($F(2,11)$ = 0.768, $p > 0.05$; SED = 25.8 cells) and ($F(2,12)$ = 0.387, $p > 0.05$; SED = 0.9 cells). Both doses did also not affect the number of TH-positive cells in all three areas examined (Figure 4, (H (2) = 15.39, $p > 0.05$) for A1, ($F(2,13)$ = 3.955, $p > 0.05$; SED = 9.3 cells) for NTS and ($F(2,13)$ = 0.255, $p > 0.05$; SED = 17.6 cells) for PVN.

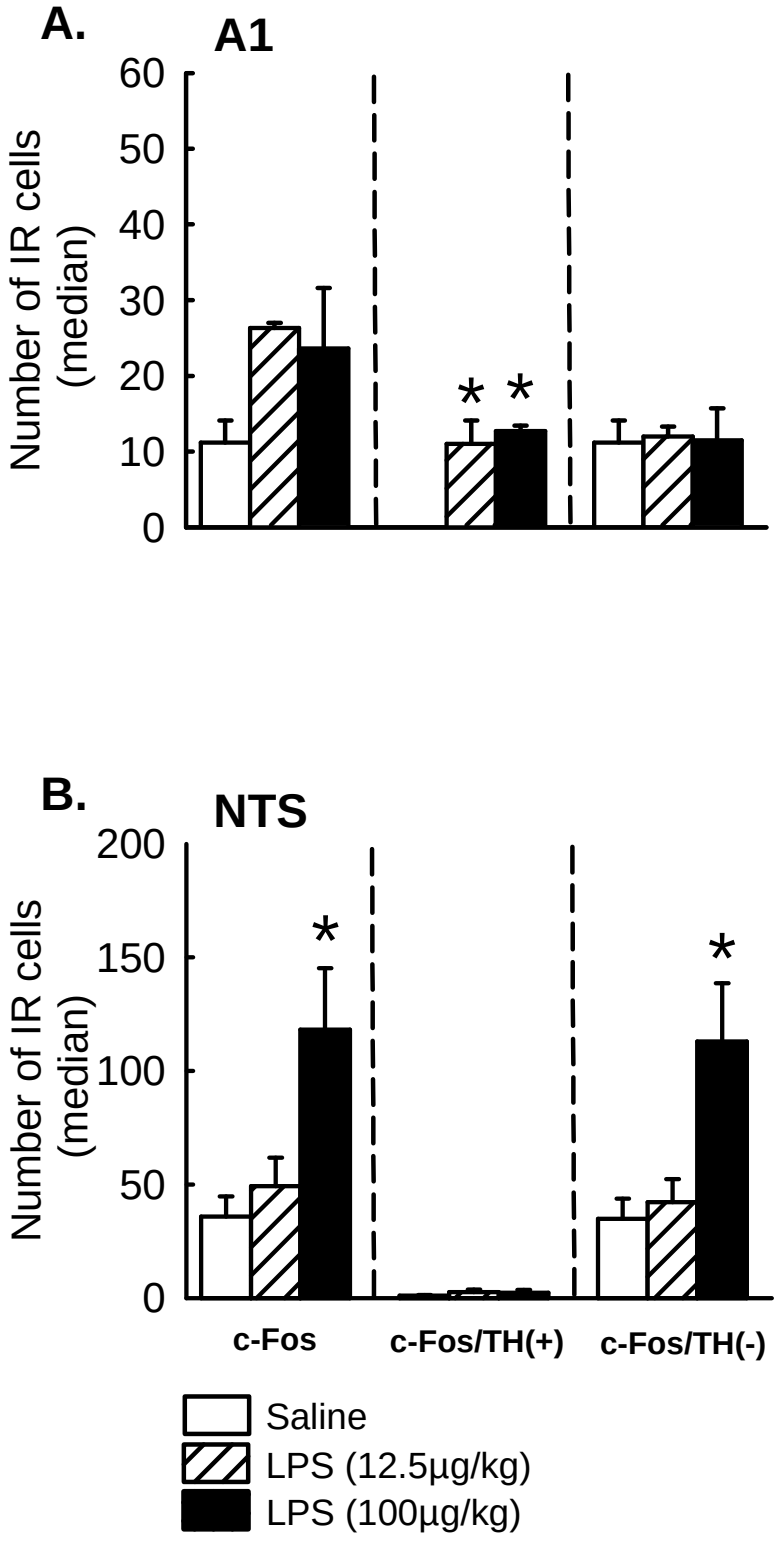

Figure 4a. Ip injection of 12.5 and 100 µg/kg LPS significantly increased the numbers of TH-positive cells expressing c-Fos in the A1 (A), but not the NTS (B).

*Significantly different from saline, Bonferroni-Holm test after significant ANOVA, $P < 0.05$.

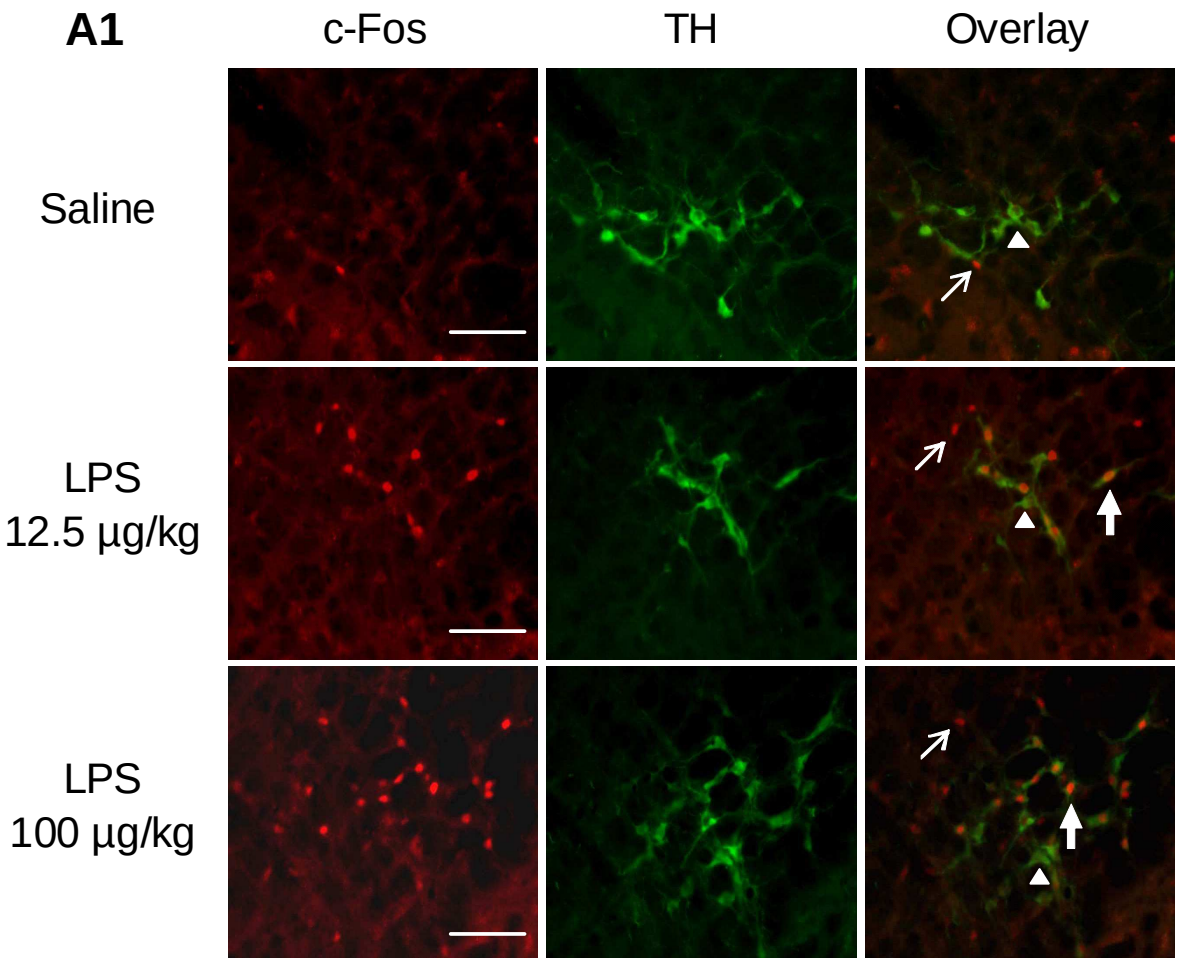

Figure 4b. Photomicrographs depicting c-Fos/TH co-localisation in the A1 area (scalebar 100 µm)

Arrow, depicting c-Fos staining only. *Arrow head*, depicting TH only and *thick arrow*, depicting co-localisation of c-Fos and TH

7.3.4 Co-localization of c-Fos and TPH

TPH and c-Fos double labeling was examined in the RPa, MnR and DR. Both doses of LPS increased c-Fos expression in the RPa and MnR, as occurred in the single-label assays; however, only 100 μg/kg LPS significantly increased c-Fos expression in the DR (Figure 5, (H (2) = 9.708, $p < 0.01$), ($F(2,13)$ = 13.76, $p < 0.01$; SED = 1.4 cells) and ($F(2,12)$ = 6.799, $p < 0.05$; SED = 11.6 cells) for RPa, MnR and DR). The larger dose of LPS increased c-Fos expression in all three areas in both TPH-positive cells (Figure 5, ($F(2,11)$ = 7.230, $p < 0.05$; SED = 0.5 cells), ($F(2,11)$ = 19.557, $p < 0.01$; SED = 0.3 cells) and ($F(2,13)$ = 6.168, $p < 0.05$; SED = 2.7 cells) for RPa, MnR and DR) and TPH-negative cells (Figure 5, ($F(2,10)$ = 17.74, $p < 0.01$; SED = 0.9 cells), ($F(2,11)$ = 8.444, $p < 0.01$; SED = 1.0 cells) and ($F(2,12)$ = 5.020, $p < 0.05$; SED = 11.6 cells) for RPa, MnR and DR). The smaller LPS dose increased it only in the RPa and MnR in TPH-positive cells (Figure 5, ($F(2,11)$ = 7.230, $p < 0.05$; SED = 0.5 cells) and ($F(2,11)$ = 19.557, $p < 0.01$; SED = 0.3 cells) but not in the DR (Figure 6, ($F(2,11)$ = 19.557, $p > 0.01$; SED = 0.3 cells)). The smaller LPS dose increased only in the RPa in TPH negative cells (Figure 5, ($F(2,10)$ = 17.74, $p < 0.01$; SED = 0.9 cells)) but not in the MnR and DR (Figure 5, ($F(2,11)$ = 8.44, $p > 0.01$; SED = 1.0 cells) and ($F(2,12)$ = 5.0, $p < 0.01$; SED = 11.6 cells)). The effect of LPS on c-Fos expression in TPH negative cells was only dose-dependent in the RPa.

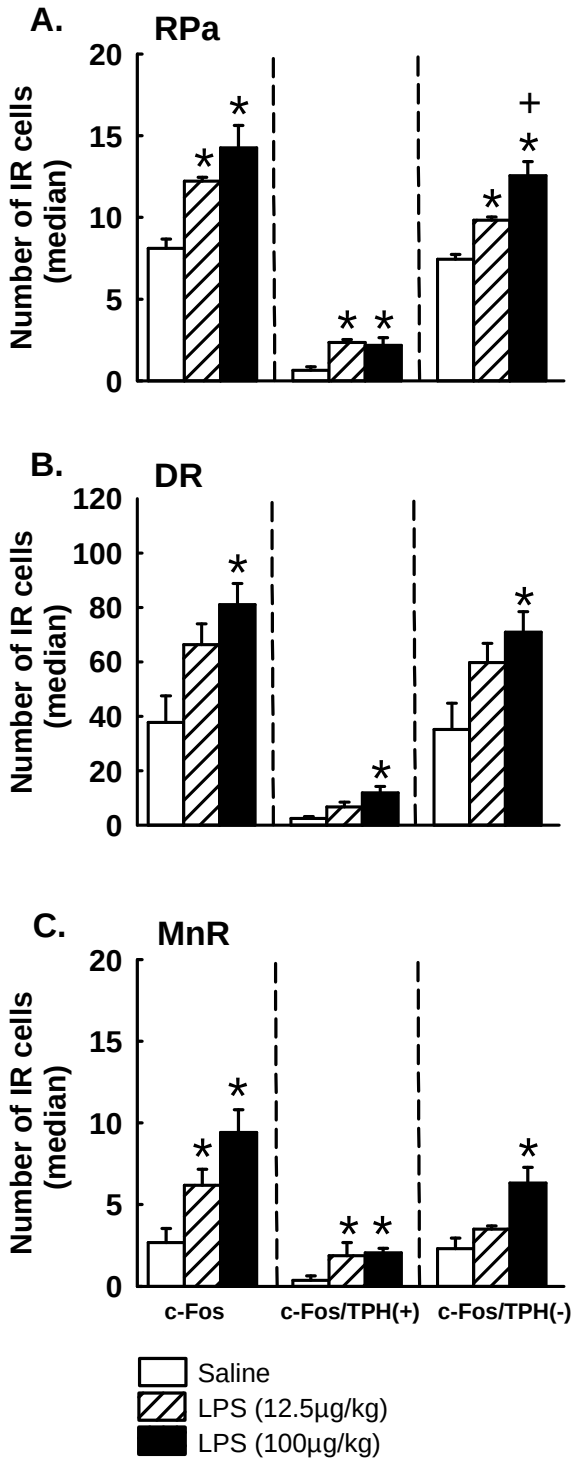

Figure 5a. Ip injection of 12.5 or 100 µg/kg LPS significantly increased the numbers of TPH-positive cells expressing c-Fos in the RPa (A), DR (B) and MnR (C). None of the effects was significantly dose-related. LPS also increased the numbers of non-TPH-positive cells expressing c-Fos in the RPa, and this effect was dose-reated (A).

*Significantly different from saline, Bonferroni-Holm test after significant ANOVA, $P < 0.05$.

+Significantly different from LPS 12.5 µg/kg, Bonferroni-Holm test after significant ANOVA, $P < 0.05$.

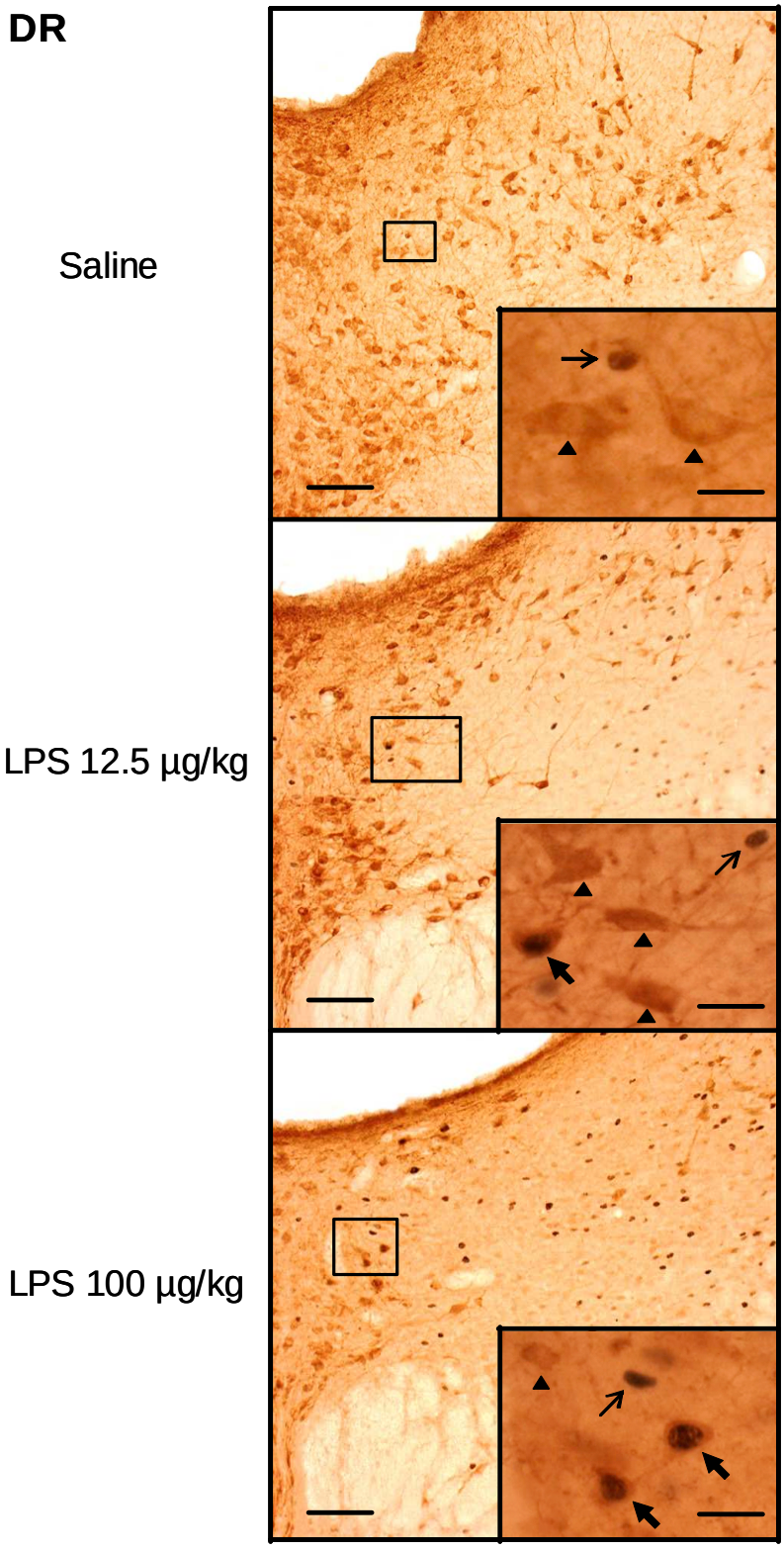

Figure 5b. Photomicrographs depicting c-Fos/TPH co-localisation in the DR (scalebar 100 µm; in the *inset* scalebar 25 µm)

Arrow, depicting c-Fos staining only. *Arrow head*, depicting TPH only and *thick arrow*, depicting co-localisation of c-Fos and TPH

7.4 Discussion

In the initial 1-2 h after injection, LPS dose-relatedly reduced food intake and increased c-Fos expression in 3 of the 10 brain areas studied: DR, PVN, and Arc. Both LPS doses significantly increased c-Fos in the other 7 areas, but in none was a significant dose-related effect detected. Nevertheless, there were clear trends for graded effects in the other raphe areas, i.e., the MnR, RMg and RPa, as well as in the CeA. Our failure to detect significantly dose-related effects in these areas might be related to the use of non-parametric tests in the analyses of these data, which was necessitated by their non-normal distributions. We conclude, therefore, that under our conditions, LPS's initial graded anorectic effect is associated with dose-related increases in c-Fos in the DR, PVN and Arc, and perhaps in the other raphe areas and CeA. These functional associations implicate these areas in induction of anorexia. There were also significant increases in LPS-induced c-Fos expression in DR, MnR and RPa neurons that express TPH and A1 neurons that express TH, although only the effect in the RPa TPH-expressing neurons was dose-related. These data provide further evidence that 5-HT neurons in the RPa, DR and the MnR and CA neurons in the A1 contribute to LPS-anorexia.

Measurements of c-Fos mRNA or protein have been reported previously in rats that received larger doses of LPS (250-1000 µg/kg) and were sampled at later times (3-6 h) following LPS injections, but none of these studies reported whether anorexia occurred or when it developed under the conditions used. Our data extend these reports by indicating that more modest doses of LPS elicit c-Fos expression in numerous brain areas, that this occurs soon after LPS-injection, and that the effect is dose-related in some areas. Better understanding of the dose- and temporal-characteristics of LPS-induced c-Fos expression would certainly help delineate the neural networks mediating anorexia and other elements of the APR.

7.4.1 Anorexia

As in a previous study [92], LPS-anorexia was expressed primarily as a decrease in spontaneous meal frequency. This suggests that LPS elicits neuronal responses that (1) may stimulate the normal physiological controls of postprandial satiety, i.e., processes that prolong inhibitory controls of eating during the intermeal interval, (2) may inhibit stimulatory controls of eating, i.e., hunger signals that develop during the intermeal interval, and (3) probably does not interact with the normal physiological controls of meal size. The larger dose of 100 μg/kg LPS, however, did decrease meal size as well as meal frequency. This contrasts with the previous study [92], in which the same dose of LPS decreased only meal frequency under similar conditions. This difference could be related to the much stronger anorexia elicited here than previously, which in turn might be explained by our use of Long-Evans rats rather than Sprague Dawley rats.

7.4.2 Neural Mechanism

The DR and PVN are strongly interconnected nuclei that are involved in the coordinate regulation of a number of endocrine and metabolic responses. The similar graded c-Fos responses in these two areas 90 min after LPS injections, together with the similar graded anorectic response beginning at this time, suggest that DR-PVN circuitry may form a functional dyad from which LPS-induced neural activation spreads to the other brain areas to produce anorexia and perhaps other elements of the APR. Although the activation of the MnR was not significantly dose-related, the c-Fos effects there were nearly identical to those in the DR, so it may well be that these two heavily interconnected areas also operate coordinately in this proposed circuit, i.e., that the functional dyad is DR/MnR-PVN.

This hypothesis is consistent with several previously reported phenomena. Several reports implicate the PVN in LPS anorexia [101, 103-105] and

indomethacin, a non-specific inhibitor of cyclooxygenase (COX) blocked the central activation in the PVN induced by LPS [101, 111] or IL-1β [112-114].

In regard to the midbrain raphe, perhaps most important are our recent discoveries that administration of the specific 5-HT_{1A} autoreceptor agonist 8-OH-DPAT into the MnR attenuated LPS anorexia [150] and that PGE_2 released by brain endothelial cells in the DR in response to peripheral LPS initiates a neural signal that is sufficient to inhibit eating (see Chapter 6). In addition, ip administration of the COX-2 antagonist NS-398, which reduces PGE_2 synthesis, blocked both the anorectic and c-Fos responses to 100 µg/kg LPS under similar conditions as tested here (see Chapter 6). Furthermore, the DR is a main source of 5-HT projections to the PVN [121-124] and other forebrain sites, and 5-HT is well known to play an important role in LPS anorexia [23, 191-193]. For example, ip injections of the specific 2CR antagonist SB 242084 attenuated LPS anorexia (see Chapter 5). Because 2CR mRNA is present on neurons in the PVN [158-161], it is possible that activation of DR and MnR 5-HT (i.e, TPH-expressing) neurons we observed here contributed to the dose-related activation of PVN cells. This hypothesis requires careful examination, however, because in both the DR and MnR, c-Fos was detected in more cells that did not express TPH than in cells that did and because in neither area were the effects in TPH-expressing cells significantly dose-related. Finally, we speculate that the activated PVN neurons may be corticotrophin-releasing hormone (CRH)-expressing neurons because transcriptional activation of CRH was reported in the PVN of LPS-treated rats [104].

5-HT projections from the DR and MnR may also be responsible for the observed activation of the Arc and CeA. Arc pro-opiomelanocortin (POMC) (i.e., alpha-melanocyte-stimulating hormone-expressing) neurons have 5-HT inputs [244]. These neurons, of course, project heavily to the PVN [116-118] thus suggesting another route through which the PVN may have been activated. The CeA also expresses 2CR [218, 219].

Both doses of LPS increased c-Fos in the two brainstem areas probed, i.e., the NTS and A1, which is consistent with previous reports [101, 103-105, 216]. Part of this increased activation might be due to CA neurons in the A1 area because c-Fos expression was significantly co-localised with TH positive neurons in this area, but was not in the NTS or PVN.

Activation of the RPa may be involved in LPS anorexia under our conditions, but activation of these areas may of course also be involved in the febrile or other effects of LPS because anorexia and fever develop with about the same latency. Upper motor neurons projecting to sympathetic preganglionic neurons in the spinal cord, the NTS and dorsal motor nucleus of the vagus arise in the RPa, suggesting their potential role in brown adipose tissue, pancreatic and gastrointestinal responses [238]. The RPa receives innervation from the medial preoptic area (POA), a major site of action in the induction of fever [88-90]. Nakamura et al. [97] reported that the rostral RPa mediates the pyrogenic neurotransmission from the POA to the peripheral sympathetic effectors contributing to fever development. They also reported that RPa neurons are activated following injection of PGE_2 into the POA, and these in turn activate sympathetic preganglionic neurons to produce fever [97]. Interestingly, however, the RPa neurons producing this effect were non 5-HT neurons. In our study, the numbers of cells expressing c-Fos in TPH neurons were similar after both doses of LPS, which suggests that the stimulation of these neurons does not depend on LPS dose. Nevertheless, because we observed a dose-related increase in the numbers of non-TPH expressing RPa neurons that expressed c-Fos after LPS, it is possible that these are the neurons that are involved in the production of fever or other visceral elements of the APR. Finally, the RMg may have been activated directly via binding of either PGE_2 on its EP3 receptors [153] or 5-HT on 2CR [158, 160, 161] or indirectly via fibers from the DR [245], MnR [124] or RPa [180].

8 General discussion

The peripheral and central mechanisms mediating anorexia during bacterial infections are not yet fully understood. Part of the reason is undoubtedly the complexity of the system, which seems to involve synergistic, reciprocal and antagonistic interactions among several cytokines and neurochemicals. The overall aim of this thesis was to further investigate the central neural mechanisms mediating LPS anorexia. More specifically, we wanted to identify whether several brain areas that are involved in autonomic and endocrine regulation and in the control of normal eating might also play a role in the initial anorectic effect of peripherally administered LPS. Furthermore, we wanted to identify the specific neurochemical phenotypes of neurons involved. The studies performed at least in part fulfilled these aims. First, the data indicate that several brain sites known to be involved in the control of normal eating are likely to play a role in the initiation of LPS anorexia. Second, the results provided further, and in part stronger, evidence for the involvement of 5HT2CR (2CR) and of prostaglandin E2 ($PGE_{2)}$ in LPS anorexia.

The 2CR antagonism study (Study 1) confirmed the necessity of the 2CR for LPS anorexia. Indeed under our conditions, no anorexia occurred after pharmacological blockade of 2CR signaling with SB 242084. This study did not reveal a single brain area where 2CR signaling seemed crucial, but suggested several candidates. Further work is required to identify which of these is most important, whether the inhibition of LPS-induced neuronal activation in several brain areas was due to local effects on 2CR or to indirect effects mediated by projections from other brain areas.

Similar to Study 1, the COX-2 antagonism study (Study 2) indicated that PGE_2 signaling can also be more crucial for LPS anorexia than previously reported and also revealed a number of candidate areas where PGE_2 signaling may contribute. Study 2 also revealed a particular brain site, the midbrain raphe nuclei, where PGE_2 signaling is necessary for LPS anorexia.

Our third study showed a dose-related anorectic effect of LPS on food intake which was correlated with a dose-related neuronal activation in the three brain areas PVN, Arc and DR, implicating these areas and their interconnections in the graded anorectic effect of LPS. Moreover, the third study also showed that this activation did in fact involve the 5-HT neurons of the midbrain raphe nuclei which revealed that the significant LPS-induced c-Fos expression occurred in TPH-positive neurons in the DR, MnR and RPa. In addition, we could also show that the CA neurons in the A1 area of the VLM were activated by ip LPS administration, but it remains unresolved whether this effect is involved in LPS anorexia.

The central strategy of these experiments was the use of immunohistochemical detection of c-Fos protein to localize brain areas where changes in neural activity were associated with changes in LPS anorexia following manipulation of PGE_2 or 5-HT signaling or of LPS dose. c-Fos protein is the product of the immediate-early gene c-*fos*, which is expressed beginning about one hour following electrophysiological activation of some populations of neurons [184]. Immunohistochemical measurement of c-Fos is routinely used to identify the neuroanatomical distribution and numbers of individual neurons activated during eating and numerous other functional states. Measurement of c-Fos and other early genes is a unique and very useful technique for functional brain imaging in laboratory animals. Nevertheless, it should be recalled that, for a number of reasons, c-Fos expression data are a relatively crude measure of neuronal activity. These limitations include that: 1) not all activated neurons express c-Fos protein [246], 2) immunocytochemical detection does not reveal the degree of activation of the neuron, 3) c-Fos expression does not reveal decreases in neural activity, 4) the time course of c-Fos expression does not necessarily match that of the behavioral phenomenon of interest, and 5) c-Fos expression may not be related to the expression or release of the neuropeptide or neurotransmitter of interest.

In all three of our studies, ip LPS administration resulted in a consistent increase in c-Fos immunoreactivity in all brain areas examined, except the Arc.

Arc neurons were activated in the first and third, but not in the second study, although the experimental setup, including the dose and route of LPS administration, were the same in all three. Such inter-experiment variability appears to be consistent with existing literature [101, 103-105], indicating that the effect of LPS on Arc neuronal activation is in fact situationally variable and depends on factors which we did not control. We can only speculate about the nature of these factors: perhaps LPS simultaneously activates anorexigenic and inhibits orexigenic neurons. For example, Heisler et al. [215] proposed that the POMC neurons in the Arc are activated through the 2CR, whereas AgRP neurons are inhibited at the same time through activation of the 5HT1B receptor (5-HT_{1B}R). In response to an activation of 5-HT input into the Arc, the overall c-Fos response would thus be determined by the combination of these two effects and might therefore be easily altered.

Based on the results of all three of our studies, summarized above, we propose the following model of a neural mechanism for the initiation of LPS anorexia. First, peripheral immune signaling activates inducible COX-2 in BBB-EC, leading to the release of PGE_2. This response appears to be widespread. Second, PGE_2 released by midbrain BBB-EC binds to neuronal EP3R in the DR and MnR, which, third, directly or synaptically activates 5-HT neurons in these areas. Fourth, the activated 5-HT neurons transmit an LPS-related signal to the PVN, and fifth, the activity of neurons in the PVN and interconnected areas, such as the Arc or CeA, produces anorexia. Thus, we propose that the midbrain raphe is a crucial starting point from which LPS-induced neural activation spreads through the neural network controlling eating. We focus on the role of the PVN as an initial relay in this network because of the strong anatomical connections of the midbrain raphe to the PVN and because, in Study 3, the DR and the PVN had similar, clearly dose-related degrees of activation.

While our results identify midbrain raphe 5-HT neurons as one anatomical substrate contributing to LPS anorexia, they do not allow us to conclude that these 5-HT neurons are directly activated by PGE_2 through EP3R. An additional co-localization study would be necessary to examine whether the midbrain raphe

neurons activated by LPS are specifically 5-HT neurons expressing EP3R. It is also possible that 2CR signaling was influenced indirectly via projections from non-5-HT neurons that expressed EP3R. Therefore, it would be interesting to examine LPS-induced neuronal activation after specific blockade of EP3R in the midbrain raphe. Unfortunately, so far no specific EP3R antagonist is available, and in EP3R knock-out mice [96] the EP3R blockade would not be restricted to the midbrain raphe nuclei. Another technique to silence the EP3R in the midbrain raphe nuclei would be an adenoviral vector-mediated RNA interference to reduce EP3R expression. This technique has been successfully used to silence estrogen receptor-α [247] and could also be used for the midbrain raphe nuclei EP3R. If the midbrain raphe were crucial for the initiation of LPS-induced anorexia, we would expect a decrease in the eating-inhibitory effect of peripheral LPS and a decrease in neuronal activation in the midbrain raphe and presumably in their hypothalamic projection sites as well.

To further investigate the role of specific DR 5-HT neuronal forebrain projections in LPS anorexia, especially the DR-PVN or DR-Arc projection, it would also be interesting to examine the effect of a specific blockade or ablation of midbrain raphe 5-HT neurons. This could be done by local injection of a 5-HT1A autoreceptor agonist or by specific lesion of the midbrain raphe 5-HT neurons using anti-SERT-SAP (Advanced Targeting System, San Diego, CA). The anti-SERT is bound to the saporin (SAP), which is a ribosome-inactivating protein. This complex utilizes a monoclonal antibody to the third extracellular domain of the 5-HT reuptake transporter (SERT). Because SAP is transported retrogradely, it is usually injected into downstream projection sites, e.g., into the PVN. Because the PVN receives 5-HT projections from both the DR and MnR, this strategy would potentially identify the necessity of midbrain 5-HT projections to the PVN in LPS anorexia, but would not disclose which midbrain raphe nucleus is crucial. There is also evidence for somatodendritic uptake of SAP [248], however, which would open the possibility of a direct injection into the DR.

Overall the neurochemical mechanisms mediating LPS anorexia are very complex. Our studies indicate that during the initiation of LPS anorexia the

midbrain raphe and its projections to the forebrain regions PVN, Arc and CeA might play the most important role. Further we could also show that 5-HT, PGE_2 and CA might be necessary mediators in specific brain areas during this initial phase of LPS anorexia. The exact signaling pathway could not be evaluated here and it is also not clear if these brain areas and neurotransmitters are necessary during the initiation or for the subsequent phases of LPS anorexia. Finally, we showed that brain sites known to be involved in the normal control of eating also change activity during LPS anorexia, which supports the concept based on previous data that illness anorexia arises from altered neural processing within the same networks that mediate normal eating.

9 References

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10 Abbreviations

2CR	Serotonin-2C-receptor
5-HT	Serotonin
$5\text{-HT}_{1A}\text{R}$	Serotonin-1A-receptor
$5\text{-HT}_{1B}\text{R}$	Serotonin-1B-receptor
AgRP	Agouti-related protein
Arc	Arcuate nucleus
AP	Area postrema
APR	Acute phase response
BAT	Brown adipose tissue
BBB	Blood-brain-barrier
B cells	B lymphocytes
CA	Catecholaminergic
CeA	Central nucleus of the amygdala
CGRP	Calcitonin gene-related peptide
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
CRH	Corticotrophin-releasing hormone
COX-2	Cyclooxygenase-2
CSF	Cerebrospinal fluid
CVO	Circumventricular organs

DR	Dorsal raphe nucleus
EC	Endothelial cells
EPR	Prostaglandin receptor
GLP-1	Glucagon-like peptide-1
HPA	Hypothalamic-pituitary-adrenal
Icv	Intracerebroventricular
IFNγ	Interferon-gamma
IL-1β	Interleukin-1 beta
Ip	Intraperitoneal
IR	Immunoreactivity
Iv	Intravenous
LBP	LPS-binding protein
LPS	Lipopolysaccharide
α-MSH	Alpha- melanocyte stimulating hormone
MC	Melanocortin
mCD14	Membrane bound CD14
MC3-R	Melanocortin receptor 3
MHC	Major histocompatibility complex
MnR	Median raphe nucleus
mPGEs	Microsomal prostaglandin synthase
MTII	MC3-R/MC4-R agonist
NA	Noradrenergic

NFkB	Nuclear factor kappa beta
NS-398	COX-2 antagonist
NTS	Nucleus tractus solitarii
NO	Nitric oxid
PBN	Parabrachial nucleus
POA	Preoptic area
POMC	Pro-opiomelanocortin
PVN	Paraventricular nucleus
RMg	Raphe magnus nucleus
RPa	Raphe pallidus nucleus
SB 202484	2CR antagonist
SDA	Subdiaphragmatic vagal deafferentation
SHU-9119	MC3-R/MC4-R antagonist
T_C	T cytotoxic cell
T cells	T lymphocytes
T_H	T helper cell
TH	Tyrosine-hydroxylase
TLR-4	Toll-like receptor-4
TNF-α	Tumor necrosis factor alpha
TPH	Tryptophan-hydroxylase
VLM	Ventrolateral medulla

11 Curriculum Vitae

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Publications:

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Presentations (* denotes presenting author):

B. S. Kopf *, N. Geary, W. Langhans, L. Asarian. "Serotonin 2C receptor (5HT2CR) signaling is involved in lipopolysaccharide (LPS)-induced neuronal activation and anorexia". Society for the Study of Ingestive Behavior (SSIB) 2008 (Paris, France).

B. S. Kopf *, N. Geary, W. Langhans, L. Asarian. "Intraperitoneal (ip) bacterial lipopolysaccharide (LPS) elicits rapid, graded increases in c-Fos expression in the raphe pallidus nucleus (RPa) and central nucleus of the amygdale (CeA) in male rats". Society for the Study of Ingestive Behavior (SSIB) 2007 (Steamboat Springs, CO, USA).

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B. S. Kopf *, N. Geary, W. Langhans, L. Asarian. "Sex differences in bacterial lipopolysaccharide (LPS)-induced c-Fos expression in the rat brain". Society for the Study of Ingestive Behavior (SSIB) 2006 (Naples, FL, USA).

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