The Effects of Sepsis on Osmoregulatory Neurons Mediating Thirst and Vasopressin Release

Jerneja Stare

Department of Physiology, Faculty of Medicine
McGill University, Montréal, QC
April 2017

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Doctor of Philosophy.

To Babi, Bazi and Očko.

TABLE OF CONTENTS

Abstract

The ability of osmosensory neurons within the hypothalamus to sense changes in blood osmolality is essential for maintaining hydromineral homeostasis. Typically, an increase in blood osmolality excites the osmosensitive neurons of the organum vasculosum laminae terminalis (OVLT), which then stimulate downstream neurons to induce a parallel increase in thirst sensation and arginine vasopressin (AVP) secretion to promote fluid expansion and maintain blood pressure. Previous studies show that thirst and AVP secretion are altered in sepsis, a deadly disease defined by the systemic inflammatory response to a severe infection, and that this impairment may be due to a deficit in the osmoregulatory pathway. Using the cecal ligation and puncture (CLP) rat model of sepsis, we show that septic rats drink significantly less under systemic hypertonic conditions. Furthermore, sepsis impairs the osmoresponsiveness of neurons in the OVLT and attenuates that of AVP-secreting magnocellular neurosecretory neurons (MNCs). Notably, we found that OVLT neurons are hyperpolarized and electrically silenced. In contrast, CLP increased the proportion of MNCs displaying spontaneous electrical activity with a parallel increase in circulating AVP. Therefore, sepsis affects the properties of osmoregulatory neurons in a manner that can affect systemic osmoregulation.

Résumé

La capacité des neurones osmosensoriels de l'hypothalamus de détecter des changements dans l'osmolalité du sang est essentielle pour maintenir l'homéostasie hydrominérale. Typiquement, une augmentation de l'osmolalité du sang excite les neurones osmosensibles de l'organe vasculaire de la lame terminale (OVLT), qui stimulent ensuite les neurones en aval pour induire une augmentation parallèle de la sensation de soif et de la sécrétion d'arginine vasopressine (AVP) pour favoriser l'expansion de fluide et maintenir la pression sanguine. Des études antérieures ont démontré que la soif et la sécrétion d'AVP sont altérées dans le sepsis, une maladie mortelle définie par une réponse inflammatoire systémique à une infection sévère, et que cette déficience peut être causée par un déficit de la voie osmorégulatrice. En utilisant la ligature et ponction caecale (LPC) dans le rat comme modèle de septicémie, nous montrons que les rats septiques boivent beaucoup moins dans les conditions hypertoniques systémiques. De plus, le sepsis altère l'osmoresponsivité des neurones dans l'OVLT et atténue celle des neurones magnocellulaires (NMCs) sécrétant l'AVP. Notamment, nous avons constaté que les neurones OVLT sont hyperpolarisés et électriquement silencieux. En revanche, la LPC augmente la proportion de NMC présentant une activité électrique spontanée avec une augmentation parallèle de l'AVP en circulation. Par conséquent, le sepsis influence les propriétés des neurones osmorégulateurs d'une manière qui peut affecter l'osmorégulation systémique.

Povzetek

Sposobnost hipotalamičnih osmotsko občutljivih nevronov, da zaznavajo spremembe osmolalnosti krvi, je ključna za vzdrževanje vodnega in elektrolitskega ravnovesja. Zvišanje krvne osmolalnosti vzburi osmotsko občutljive nevrone v organum vasculosum laminae terminalis (OVLT), kateri nato preko vzburjenja nižje ležečih nevronov hkrati povzročijo povečan občutek žeje in izločanje antidiuretskega hormona (ADH), ter posledično zadrževanje vode in dvig krvnega tlaka. Predhodne študije so pokazale, da sta zaznavanje žeje in izločanje ADH spremenjena pri sepsi, ki je življenje ogrožajoče stanje, za katerega je značilen sistemski vnetni odziv na hudo okužbo, in da bi to lahko bila posledica okvare delovanja v osmoregulatorni poti. Z uporabo tehnike ligacije in perforacije cekuma kot eksperimentalnega modela sepse (angl. *cecal ligation and puncture; CLP*) na podganah smo dokazali, da septične podgane v sistemskem hipertoničnem stanju popijejo bistveno manj tekočine. Poleg tega sepsa ovira delovanje osmotsko občutljivih nevronov v OVLT in zmanjšuje odzivnost nevrosekretornih nevronov, ki sproščajo ADH (angl. *magnocellular neurosecretory neurons; MNCs*). Ugotovili smo namreč, da so OVLT nevroni hiperpolarizirani in električno neaktivni. Nasprotno pa CLP povečuje delež MNCs, ki kažejo spontano električno aktivnost s posledičnim zvečanjem cirkulirajočega ADH. Sepsa torej vpliva na značilnosti osmoregulatornih nevronov na način, ki ima lahko vpliv na sistemsko uravnavanje osmotskega ravnovesja.

Acknowledgements

This thesis would not have come to fruition if it were not for the collaborators and co-authors who have directly contributed to the works described herein, or for the family and friends who have supported me in my pursuit of the Doctoral degree, or for the financial support from the Canadian Institutes of Health Research.

First and foremost, I would like to thank my supervisor, Charles W. Bourque, for his support. The writing in this thesis was inspired by yours. May I grow to be as knowledgeable as you are.

I would like to also like to thank our collaborators, Shidasp Siami, Tarek Sharshar, and Willis Samson, who contributed data and expertise to this body of work. Thank you to my advisory committee, Ellis Cooper, Reza Sharif-Naeini and David Stellwagen, for helping to steer this ship with your feedback. I would also like to thank Christine Pamplin, Rosetta Vasile, and Yvonne Gardner for their support and help with the bureaucracy throughout the years; and Guylaine Gadoury, Johanne Deslauriers, and Nancy Laughren for their training and "on the ground" support at General Hospital Animal Care Facility.

Thank you to Masha Prager-Khoutorsky and Eric Trudel for your time and effort in teaching me, and your support and encouragement, and to all of the past and present lab members: Anna Bumagin; Katrina Choi; Ariane Gagnon; Claire Gizowski; Pierre Yves Laroche; Jessica Sudbury; Daniel Voisin; and Cristian Zaelzer. Thank you to Kelly Bullock for your feedback on parts of this thesis, Mario Calderon for your help on the résumé, and *hvala* to Ana Mihor for help with the povzetek.

A sincere thank you to my family Renata Kobe, Marko Stare, Sara Verderber, Aurelio Herrera, Ava Herrera Kobe, Tai Herrera Kobe, Gašper Stare, Babi, and Bazi; I am forever indebted to you all for your relentless support. To my friends Chinye Azuh, Theda Backen, Giselle Boukhaled, Emily Coffey, Kate Fathers, Joe Makkerh, Martha Shiell, Lucy Williams, and Beth Yaworsky: thank you for walking parts of this journey with me. Last, but not least, thank you to Mark Verway for giving me nothing else to say.

Preface

The works presented in this thesis are the first to investigate the effects of sepsis on the firing behaviour and membrane properties of osmoregulatory neurons that participate in activating osmotic thirst and vasopressin release. It is the first work to investigate both osmotic control of vasopressin release and the role of vasopressin in body-fluid homeostasis in a rat sepsis model. The contribution to originality is demonstrated by the successful publication in peer-reviewed journals of the following manuscripts and abstracts.

Manuscripts:

Stare, J., C.W. Bourque (2016) "Measuring rat serum osmolality by freezing point osmometry," *Bio-protocol* 6 (19): e1950.

Stare, J., Siami, S., Trudel, E., Prager-Khoutorsky, M., Tarek S. and Bourque, C.W. (2015) "Effects of peritoneal sepsis on rat central osmoregulatory neurons mediating thirst and vasopressin release," *Journal of Neuroscience* 35(35): 12188-12197.

Abstracts:

Siami, S., Stare, J., Prager-Khoutorsky, M., Trudel, E., Sharshar, T., & Bourque, C. (2015). Altération de la soif osmotique par l'hyperpolarisation des osmorécepteurs centraux au cours du sepsis. *Anesthésie & Réanimation*, *1*, A352-A353.

Stare, J., Siami, S., Trudel, E., Prager-Khoutorsky, M., Sharshar, T., & Bourque, C. (2015). The effects of sepsis on osmosensory neurons mediating thirst. *The FASEB Journal*, *29* (1 Supplement), 968-17.

Contributions of Authors

The data presented herein have been previously published unless otherwise noted and have been produced in collaboration with other authors.

Section 2.4.1 Methods (Serum osmolality, Representative Data)

I designed, executed, and analyzed all of the experiments presented in this section. I prepared the figures and manuscript in partnership with my supervisor for publication in the open-access journal Bio-protocol.

Section 3.1 Effects of sepsis on OVLT osmosensory neurons mediating thirst

This section was published in collaboration with other authors in the Journal of Neuroscience. My supervisor and I wrote the manuscript and addressed reviewers' comments.

Figure 3.1.3.1 I executed and analyzed the experiment presented in this figure. I prepared the figure for publication.

Figure 3.1.3.2, Figure 3.1.3.3 A-C These experiment were performed and analyzed by Shidasp Siami. I re-analyzed the datasets as an exercise in quality-control and prepared the figure with my supervisor for publication.

Figure 3.1.3.3 D-E Immunohistochemistry and microscopy was performed by Masha Prager-Khoutorsky. I developed a counting method, and performed the counts (3.1.3.3 E) for this figure as well as for others (data not shown).

Figure 3.1.3.4 Myself and Eric Trudel performed the experiments presented in A, B, and C (left graph). The dataset in C (right graph) is a combination of my experiments, and those of Shidasp Siami. I analyzed all of the data and prepared the figure for publication.

Figure 3.1.3.5 Myself and Eric Trudel performed the experiments presented in C (right three graphs). Shidasp Siami performed and analyzed the experiments in A, B, and C (left graph). I

analyzed or re-analyzed for C (left graph) all of the datasets and prepared the figure for publication.

Section 3.2 Effects of sepsis on magnocellular neurons (MNCs) of the supraoptic nucleus (SON)

This section was published in collaboration with other authors and is the second half of the publication appearing in the Journal of Neuroscience.

Figure 3.2.3.1 I performed, and analyzed the experiment presented in this figure. I prepared the figure for publication.

Figure 3.2.3.2A & B I performed these experiments and analyzed the dataset.

Figure 3.2.3.2C Myself and Shidasp Siami performed these experiments. I analyzed the combined datasets and prepared the figure for publication.

Figure 3.2.3.3 Shidasp Siami performed these experiments. I analyzed the dataset and prepared the figure for publication.

Figure 3.2.3.4 I performed and analyzed the experiment presented in this figure. I prepared the figure for publication.

Section 3.3 Vasopressin and water balance in septic rats

With the exception of the radioimmunoassay (*Figure 3.3.3.1C*), which was kindly done by Willis Samson, I performed and analyzed all of the experiments in this section. This section is part of a manuscript currently being prepared for submission.

Symbols and Abbreviations

, **, **	statistically significant
ACSF	artificial cerebrospinal fluid
ANG II	angiotensin II
ANOVA	analysis of variance
AP	action potential
ATPases	adenosine triphosphatases
AV3V	anteroventral third ventricle
AVP	arginine vasopressin
BCE	before the common era
Ca^{2+}	calcium ions
$CaCl_2$	calcium chloride
CD14	cluster of differentiation 14
CDI	Central diabetes insipidus
CLP	cecal-ligation and puncture
CNS	central nervous system
CVO	circumventricular organ
D-glucose	D-isomer of glucose a.k.a dextrose
ddH_2O	double distilled water
ECF	extracellular fluid
eGFP	enhanced green fluorescent protein
ESP	early sleep phase
F-I	frequency – current
F_{max}	maximum firing frequency
G	guanine nucleotide-binding protein
GABA	γ-aminobutyric acid
h	hour
HEPES	4-*(2-hydroxyethyl)-1-piperazineethanesulfonic acid* (buffering agent)
Hz	hertz
I	current
I-V	current – voltage

i.v.	intravenous
ICU	intensive care unit
IL-1	interleukin-1
IL-1α	interleukin 1 alpha
IL-1β	interleukin 1 beta
IL-6	interleukin 6
INFγ	interferon gamma
iNOS	inducible nitric oxide synthase
K^+	potassium ions
K_{ATP}	ATP-sensitive potassium channel
KCl	potassium chloride
kg	kilogram
KOH	potassium hydroxide
LPBN	lateral parabrachial nucleus
LPS	lipopolysaccharide
$MgCl_2$	magnesium chloride
mL	milliliter
MNC	magnocellular neurosecretory cells
MnPO	median preoptic nucleus
MOD	multiple organ dysfunction
mOsm	milliosmoles
ms	milliseconds
MSP	mid-sleep phase
mV	millivolts
Na^+	sodium ions
NaCl	sodium chloride
NaH_2PO_4	monosodium phosphate
$NaHCO_3$	sodium bicarbonate
NeuN	NeuN
NF-κB	nuclear factor kappa beta
NO	nitric oxide

NOS	nitric oxide synthase
nS	conductance
ns	not significant
NTS	nucleus tractus solitarius
OT	oxytocin
OVLT	organum vasculosum laminae terminalis
p	*p*-value
pA	current
PAC	pulmonary artery catheter
PBS	phoshphate-buffered saline
pg	pictogram
PVN	paraventricular nucleus
RAAS	renin-angiotensin-aldosterone system
RMP	resting membrane potential
RT	room temperature
SCN	suprachiasmatic nucleus
SEM	standard error of the mean
SFO	subfornical organ
SIADH	syndrome of inappropriate antidiuretic hormone secretion
SIRS	systemic inflammatory response syndrome
SON	supraoptic nucleus
SVR	systemic vascular resistance
TLR-4	toll like receptor 4
TNF	tumour necrosis factor
TNFα	tumour necrosis factor alpha
TRPV1	transient receptor potential cation channel subfamily V member 1
TRPV4	transient receptor potential cation channel subfamily V member 4
V	volt
V1	arginine vasopressin receptor type 1
V1a	arginine vasopressin receptor type 1 subtype a
V2	arginine vasopressin receptor type 2

VLM	ventrolateral medulla
V_{max}	maximal action potential upstroke velocity
z-stack	focus stacking
Δ	difference
Ω	ohm

1.0 INTRODUCTION

1.1 Physiology of Water-Electrolyte Homeostasis

1.1.1 Osmoregulation

1.1.1.1 A brief history of life

"The principle of nature is water."

– Thales of Miletus (624–547 BC)[1]

Water is the source of all life. About 3.6 billion years ago[2], liquid water provided the requisite environment for nature's prebiotic chemistry experiments that led to the formation of organic compounds (e.g. amino acids, nuclei acids), the bare necessities of life[3,4]. How the biochemistry occurred and which prebiotic elements first formed are debatable hypotheses, but the critical step in our ancient collective history as living organisms was the formation of membranes[4,5]. The first membranes, presumably formed by the spontaneous aggregation of amphiphiles such as lipids, provided the necessary separation of the external environment from the *internal milieu*. They gave our ancestral protocell a preliminary means to stabilize its internal environment and individualize from the chaotic world it lived in. However, the protocell's primitive membrane was permeable to water, a feature inherited by all living organisms.

1.1.1.2 Osmosis

Water spontaneously flows across semi-permeable membranes (meaning, permeable to water, but not necessarily to solutes) along its electrochemical gradient from the less concentrated side, to the more concentrated side, until the solute concentrations on both sides of the membrane equalize[6]. Solutes, therefore, affect the movement of water. This process, termed *osmosis* in 1826 by René Henri Dutrochet, is a double-edged sword: water is a biological necessity, but too much or too little is lethal[3]. If a cell is placed into a diluted aqueous environment (i.e. hypo-osmotic or hypotonic) relative to its internal solute concentration, water will flow into the cell in an effort to achieve solute equilibrium, causing the cell to swell and lyse. Conversely, if the cell is placed into a concentrated solution (i.e. hyperosmotic or hypertonic), then the net efflux of water out of the cell will cause the cell to shrink and shrivel.

The force that drives the movement of water across the membrane is referred to as osmotic pressure; it is directly related to the difference in solute concentrations on either side of a cell membrane[7,8]. The early challenges thus faced by ancient protocells revolved around volume regulation by maintaining a balance between electrolytes and water a.k.a. hydromineral homeostasis to avoid osmotic stress.

1.1.1.3 Unicellular osmoregulation

Osmoregulation is the sum of all adaptive biological processes that regulate the internal osmotic pressure of an organism to achieve hydromineral homeostasis[3]. Primitive cells evolved a number of effective structural and biochemical mechanisms that allowed them to influence their internal osmolality (a measure of solute concentration expressed in osmoles per kg of solvent; see section 2.4)[8]. One of the earliest mechanisms adopted by protocells was the use of adenosine triphosphatases (ATPases) to actively drive water and salts against their osmotic gradients (e.g. proton pumps, potassium (K^+) ATPases)[4]. Simple cells also developed contractile vacuoles to expel water in order to maintain a hyperosmotic cytoplasm, or created osmolytes (compounds that affect osmosis[9]) such as glycerol, mannitol, or free amino acids to compensate for changes in osmotic gradients of extracellular salts[10]. Prokaryotes evolved ion channels that respond to membrane stretching or shrinkage: most notable is the mechanosensitive channel large conductance protein of *Escherichia coli,* which senses membrane stretching during hypotonic shock and responds by mediating osmolyte efflux[11]. All of these mechanisms were preserved in multicellular organisms to varying degrees, but the complexity of higher organisms demanded more expansive and coordinated response systems to preserve hydromineral homeostasis.

1.1.1.4 Multi-cellular osmoregulation

"The true medium in which we live is neither air nor water but the plasma or the liquid part of the blood that bathes all the tissue elements." – Homer W. Smith (1959)[12]

As the complexity of the organisms evolved from single cell to multi-cellular entities, systemic osmoregulation became necessary. The external milieu of the cells was no longer the Earth's oceans but rather the extracellular fluid (ECF) produced by the organism itself. The

concentration and composition of electrolytes, and water volume in the ECF, needed to be maintained within a specific range to provide the conditions necessary for fundamental biochemical reactions to occur[3,9]. When the hydromineral balance is tipped in favour of salts, as can be caused by increased salt intake or water loss, the ECF becomes hyperosmotic. When the balance is tipped in favour of water, as occurs with increased water intake or salt loss, the ECF is said to be hypo-osmotic.

Multicellular organisms adapted regulatory mechanisms that maintained not only cytoplasmic osmolality relative to ECF, but also ECF osmolality relative to their external environment. Some put in more effort than others. Osmoconformers, comprising mostly marine invertebrates (e.g. mussels, scallops, starfish) and craniates (e.g. sharks, skates, hagfish), are species that maintain an internal environment in osmotic equilibrium (i.e. isosmotic) with the waters they inhabit[9,13]. Osmoregulators (predominantly vertebrates), on the other hand, maintain their ECF osmolality at a species-specific "set point". For example, mammals maintain an ECF osmolality roughly around 300 mOsm/kg[9], with humans at ~288 mOsm/kg[14], and rats at ~294 mOsm/kg[15]. Both osmoconformers and osmoregulators evolved neuroendocrine systems to coordinate the function of specialized organs, such as skin, gills, bladders and kidneys, for solute and water regulation[13].

1.1.1.5 Systemic osmoregulation

Mammals face innocuous osmotic challenges daily, as caused by eating and drinking, dehydration, exercising or heat-induced sweating[3,9]. Changes of as little as 1 – 3% in ECF osmolality are enough to induce an osmoregulatory response[9]. Deviations greater than 3% have pathological implications: for example, in one study conducted in healthy humans, increases of over 10 mOsm/kg (~3.5%) in serum osmolality caused neurological symptoms including headache, reduced concentration and alertness, and lethargy[16]. Hydromineral homeostasis is achieved by balancing behaviourally motivated and neuroendocrine-mediated input and output of water and salts[3,9] (Fig 1.4.1). Water and salt gain are actively modulated by changes in behaviours driven by thirst and salt appetite[17,18]. Conversely, water and salt loss occur normally via benign mechanisms such as sweat and exhalation of humidified air, but the majority is excreted as urine. The primary effector organs of systemic osmoregulation are thus the brain, which senses global changes in ECF osmolality and mediates the appropriate behavioural and

hormonal responses, and the kidneys[3,9]. The kidneys reabsorb or excrete water, electrolytes, and metabolites, and can partially regulate their own function by producing renin to activate the renin-angiotensin-aldosterone system (RAAS), whose products, the hormones angiotensin II (ANG II) and aldosterone, promote reabsorption of sodium ions (Na^+). However, the hypothalamus is the primary osmoregulatory: it houses nuclei that regulate natriuresis (salt unloading) and diuresis (water excretion) in the kidney via sympathetic innervation, the RAAS pathway, and through the release of hypothalamo-neurohypophysial hormones such as oxytocin (OT, a hormone primarily known for its role in parturition and lactation[19]) and the anti-diuretic hormone vasopressin (AVP) from the posterior pituitary[3,9] (discussed in section 1.1.4). Integral to systemic osmoregulation is the maintenance of water balance. When fluid loss occurs, compensatory mechanisms are engaged to minimize the loss of solutes and water. However, these mechanisms alone do not restore body fluid volume and composition, therefore the organism must be motivated to drink to survive.

1.1.2 Thirst

Aquatic animals have it relatively easy from an osmoregulatory perspective by virtue of being bathed in water[13]. On the contrary, terrestrial mammals face the largest water gradients across their skin because of the relatively dry environments they live in. These animals need to seek water in order to preserve their internal fluids within a very limited range of osmolalities with the goal of maintaining their interstitial and intracellular fluids isotonic relative to each other[3]. Thirst is an undeniable sensation that urges the body to consume fluids and is crucial for survival. Because the sensory consciousness of thirst is a subjective experience dictated not just by physiological need, but also by culture, habit, and psychogenic need, it is not yet clear where this "feeling" is generated in the brain[17,20] (see section 1.1.2.1).

Regulatory thirst as a result of physiological need is generated by cerebral mechanisms that respond to extracellular or intracellular fluid depletion from humoral and visceral signals, or stimulated with dipsogenic (i.e. thirst-inducing) hormones, such as ANG II and relaxin[17,20]. ECF depletion involves an absolute depletion of both water and NaCl, thus the effective osmolality of the blood remains unchanged. This type of thirst, referred to as hypovolemic thirst, contributes primarily to supporting volume status and is only briefly discussed (section 1.1.2.2). Intracellular fluid depletion prompts osmotic thirst, on the other hand, and is caused by loss of water or

increase in solute, increasing serum osmolality (section 1.1.2.3). Serum hyperosmolality shifts the osmotic gradient across cellular membranes to draw water into the interstitial fluid, dehydrating the cells. As the name implies, osmotically stimulated thirst is a key component of osmoregulation and is central to the works discussed in this thesis.

1.1.2.1 Neural circuitry of thirst

The generation and inhibition of the sensory perception of thirst involves several neural circuits in the brain (Fig 1.4.2), with variations depending on the sensory neural circuitry and type of stimuli involved. However much remains to be discovered about thirst-generating systems. The earliest studies on water consumption somewhat incompletely identified the hypothalamus as the "thirst center" of the brain[20]. Direct electrical stimulation[21] or hypertonic NaCl application[22] to hypothalami provoked polydipsia (excessive water intake) in goats, whereas lesioning regions within the hypothalamus produced hypodipsia (reduced drinking) in rats[23,24]. Within the hypothalamus, along the lamina terminalis, lie two highly vascularized nuclei called circumventricular organs (CVOs) that are located outside of the blood-brain-barrier: the subfornical organ (SFO) and the organum vasculosum laminae terminalis (OVLT: discussed in section 1.1.3)[9]. These two sensory CVOs, along with a third CVO in the medulla oblongata called the area postrema, receive status updates on blood osmolality and volume by direct biochemical sensing, hormone detection, or innervation from other nuclei[25]. The OVLT, in conjunction with the median preoptic nucleus (MnPO), form the anteroventral third ventricle (AV3V) region of the hypothalamus[26]. The AV3V and SFO are extensively reciprocally connected[27-32]. The MnPO integrates sensory input from the SFO and OVLT, and afferent innervation from - but not limited to - the nucleus tractus solitarius (NTS)[28], the lateral parabrachial nucleus (LPBN)[28,33-35], and the ventrolateral medulla (VLM)[28] which relay information from, for example, baroreceptors and visceral afferents.

Evidence suggests that the MnPO, OVLT, SFO, LPBN and another nucleus implicated in thirst generation, the median raphé[36,37], project to the lateral hypothalamic area[38-43] which in turns sends projections to the cerebral cortex[44,45]. Neuroimaging studies in humans[46,47] corroborate findings from decerebrated rats[48] that implicate the cerebral cortex as the effector region in response to osmotic stimuli. Direct electrical stimulation of the anterior cingulate cortex elicited drinking behaviour in monkeys[49]. In hyperosmolar humans, increased cerebral

blood flow correlative with augmented perception of thirst was detected predominantly in the anterior and posterior cingulate cortex, insula, lateral terminalis, parahippocampal gyrus, and in regions of the cerebellum[46,47]. Human magnetic resonance imaging studies have demonstrated increased cerebral blood flow to, and therefore implicit activation of, the OVLT region during dehydration and hyperosmolality[47,50]. The OVLT relays osmoregulatory information to the cingulate and insula cortices via medial hypothalamic nuclei[38]. Though our understanding of the neural mappings for thirst is far from complete, the combined interoception and motivation of water consummatory behaviour is likely seated in the anterior cingulate cortex, insula, and parahippocampal areas[20,51]. These brain areas are affiliated with complex cognitive processes, including social and emotional behaviours, supporting the notion of non-physiological modulation of thirst[52].

1.1.2.2 Hypovolemic thirst

Hypovolemic thirst is caused by blood loss, vomiting, diarrhea, or edema (sequestration of interstitial fluid)[17]. Decreased circulating blood volume and concomitant decreased blood pressure unload arterial and atrial baroreceptors, respectively[53]. These afferent receptors project to the area postrema and NTS to induce autonomic and neuroendocrine changes that affect renal and cardiovascular function to promote salt and water retention, and restore blood pressure. At the kidney level, reduced blood flow activates the RAAS pathway to produce renin[3,17]. Renin catalyzes the conversion of circulating angiotensinogen into angiotensin I, which is further catalyzed to form ANG II. Circulating ANG II is highly dipsogenic and effectuates primarily through the SFO and area postrema. Systemic injection of the effector peptide stimulates drinking behaviour even in satiated, euvolemic rats[54]. Baroreceptor input and ANG II humoral signaling are integrated in the AV3V to stimulate thirst and salt appetite through higher brain areas[26,55]. In rodents, lesioning of the area postrema and portions of the NTS[56,57] or the LPBN (which receives inputs from the NTS) produces an exaggerated response to extracellular dehydration[17,58]. Furthermore, activation of a subset of SFO neurons inhibits drinking[59], suggesting that the aforementioned nuclei also play a role in thirst inhibition.

1.1.2.3 Osmotic thirst

Osmotic thirst, along with AVP-mediated renal water retention, is particularly essential to water homeostasis. This form of thirst occurs as a result of cellular dehydration and ECF hyperosmolality which stimulates osmoreceptors and activates corresponding neural circuits to motivate water intake and concomitant secretion of AVP from the posterior pituitary[17]. For the sake of simplicity, this section will summarize the joint responses to osmotic challenge in thirst and circulating AVP, followed by a discussion on the OVLT and its relationship with the magnocellular neurons (MNCs) that secrete AVP into the periphery (section 1.1.4.1). Osmoregulation of AVP and its effects on renal filtration is discussed in greater detail in section 1.1.4.2.

Systemic homeostatic response mechanisms vary depending on the direction of change in osmolality[9] (Fig 1.4.1). Experiments in humans and animals have shown that when the ECF is made hyperosmotic by injections of NaCl, or other osmolytes such as mannitol, the sensation of thirst in humans (or water intake in animals) and circulating AVP increase[14,47,60-63]. Hyperosmotic ECF further increases the excretion of Na^+ (natriuresis)[64-68], and inhibits salt appetite[69], which in rats is partially mediated by OT[70-73]. A rise in plasma osmolality over ~ 295 mOsm/kg achieves maximal AVP-mediated anti-diuresis in humans[74]. Further fluid replenishment is mediated by graded increases in thirst as osmolality rises, with severe thirst starting at ~ 300 mOsm/kg[60,75]. Conversely, if the ECF is made hypo-osmotic, salt stores must be conserved and replenished and water must be discharged. In hypo-osmotic conditions, basal AVP secretion is suppressed in rats[15] and humans[75], effecting renal diuresis[9]. Although no study has investigated the "inhibition" of thirst as a result of ECF hypo-osmolality, the current conjecture is that the desire to drink is also suppressed because the osmotic thresholds for thirst and AVP release are similar in humans and animals[9,60,74,76-79].

1.1.2.3.1 Peripheral osmoreceptors

Drinking quenches thirst and inhibits AVP release, even before serum osmolality is corrected, by mechanisms that include peripheral osmoreceptors[47,76-82]. The identity of these receptors is unknown, though one recent study implicated the transient receptor potential action channel subfamily V member 4 (TRPV4), an ion channel known to be activated by hypo-osmotic stimuli[82-84]. It is known, however, that peripheral osmoreceptors located in the alimentary tracts

and associated blood vessels detect ingested water and solutes and provide feedback to the central nervous system (CNS) via afferent neurons supplying the NTS[9,85-89] (Fig 1.4.2). The parallel nature of the behavioural and neuroendocrine response to changes in ECF osmolality indicates that osmotic regulation of thirst and AVP-secretion are initiated by a common neural circuit that is dependent on sensory input from cerebral osmoreceptors[9,17,60,90].

1.1.2.3.2 Central osmoreceptors

The original concept of osmoreceptors was proposed by Verney in 1947 in the context of hyperosmotic stimulation of AVP release[91]. Subsequent work by many researchers narrowed down the location of intracranial osmoreceptors to the anterior preoptic area of the hypothalamus[92-94], specifically to the AV3V region of the hypothalamus[95,96]. Hypotheses proposing that osmoreceptors were located in CVOs[40,62,63] were supported by AV3V lesion studies in animals. Experiments in which lesions encompassed or targeted the OVLT were shown to produce adipsia and inhibit AVP release during osmotic stimulation, despite dehydration (a condition that results in hyperosmotic hypovolemia)[26,93,96-99]. Osmoreceptors are also located in the SFO[83,100,101], which has been shown to participate in hyperosmotically-induced thirst and AVP secretion[102-104]. However, the currently accepted view is that the SFO is the primary sensor for circulating ANG II, whereas the OVLT is the primary osmosensor, although both nuclei are capable of responding to both stimuli[3,9,17,98,105].

1.1.3 Organum vasculosum laminae terminalis (OVLT)

The OVLT is a small globoid nucleus within the hypothalamus[106] that projects to multiple sites within the CNS. It is located along the anterior wall of the third ventricle, dorsal to the optic chiasm and immediately ventral to the MnPO. The ventricular surface of the OVLT is covered by ependymal cells (a type of glial cell that contributes to cerebrospinal fluid production), and tanycytes (a subtype of ependymal cells), whose long projections extend into the OVLT to encircle neurons and wrap around local capillaries[40,106,107]. The capillaries of the anterior preoptic arteries that feed into the OVLT are lined by fenestrated endothelia and pass through a layer of tightly packed astrocytes[108,109], but are not surrounded by the astrocytic processes that would normally be associated with the traditional blood-brain-barrier[106,110]. The OVLT has been documented to express: cytokine receptors[111-115]; receptors for hormones such as

estrogen[116], ANG II[90,117-119], and prolactin[120]; nitric oxide synthase (NOS)[121]; and Na^+ [117] and mechanosensitive ion channels[122-124]. Thus, blood-borne signals can diffuse into the OVLT and act on local neurons, providing a mechanism by which humoral signals communicate with the CNS.

The OVLT is in a privileged position to coordinate a diverse array of centrally-mediated physiological processes, including fever and sickness behaviour[125-127] (see section 1.3.3), cardiovascular regulation[128-131], and osmoregulation[9]. Electrophysiological experiments performed in rodent hypothalamic slices demonstrate that acute increases in perfusate osmolality effected a proportional increase in firing rate in a portion of OVLT neurons[123,132-134]. Studies measuring *c-fos* activity (an indirect marker of neuronal activation) to identify OVLT neurons that are responsive to hypertonic NaCl stimuli show that these neurons[135] line the highly vascular midline of the OVLT, and cluster at higher density in the dorsal cap region of the nucleus[38,106,136-139]. The dorsal cap borders the MnPO, and is an area that is also responsive to relaxin[140]. Furthermore, OVLT neurons in the dorsal cap project to the supraoptic nucleus (SON; see section 1.1.4.1), whereby they stimulate AVP-release from MNCs in hyperosmotic conditions[90,141] (Fig. 1.4.3). Destruction of the OVLT increases the threshold plasma osmolality necessary to induce drinking behaviour and AVP secretion by 7 – 10% in dogs[63].

Acute systemic hyperosmolality also increases sympathetic nerve activity[128,130], likely mediated in part by synaptic inputs into the paraventricular nucleus (PVN)[142]. The PVN is a heterogenous mix of MNCs and parvocellular neurons which mediates neuroendocrine secretion, and sympathetic nerve activity through synaptic projections to other sympathoregulatory areas of the CNS[128]. Several studies have shown that stimulation of the OVLT neurons causes a sympathoexcitation in response to hyperosmotic stimuli. Furthermore, excitation of the OVLT effects a sympathetically-mediated increase in vascular resistance and arterial blood pressure in the renal and visceral microcirculatory systems, providing evidence of the OVLT's pressor role in the cardiovasculature[130]. Lesioning the anterior lamina terminalis, particularly the OVLT, attenuates the drinking response, AVP secretion, and sympathetic nerve activity in response to hyperosmotic stimuli[95,143-147], indicating that these regulatory functions depend on the osmolality-sensing capabilities of the OVLT[9,128].

1.1.3.1 Osmosensation in OVLT

Osmosensation is a mechanical process[9]. As discussed in section 1.1.1.2, hyperosmolar ECF dehydrates cells causing cell-shrinkage, whereas hypo-osmolar ECF causes cell volume expansion. Osmosensory neurons translate such intracellular volume changes into electrical signals (i.e. altered firing frequency or pattern) using mechanosensitive ion channels. Because synaptic transmission from other neurons or osmolyte secretion from osmotically-responsive glial cells can alter the action potential discharge of neurons, osmosensory neurons by definition must have cell-autonomous osmosensory transduction mechanisms in order to be considered intrinsic osmoreceptors. The cellular mechanisms of osmosensation have been predominantly studied in the MNCs of the SON and PVN in rodents (see section 1.1.4.1.2), however this discussion will focus on what is known specifically for the OVLT.

Electrophysiological recordings from the OVLT *in vivo*[148] and *in vitro*[123,132-134,149] indicate that the OVLT is responsive to changes in fluid osmolality, and that this response is independent of synaptic input[149]. Intracellular recordings of OVLT neurons demonstrated that perfusion with hypertonic and hypotonic solutions respectively induced and reduced firing frequency[141]. The strongest evidence of OVLT neurons as intrinsic osmosensors came from whole-cell recordings of acutely isolated mouse OVLT neurons[123,133]. Ciura and colleagues demonstrated that perfusion with mannitol-based hypertonic solution elicited a membrane depolarization and associated increase in firing rate in responsive OVLT neurons. Furthermore, hyperosmotic stimulation or applying manual suction to isolated neurons in iso-osmotic conditions to mimic cell shrinkage elicited an increase in conductance and neuronal excitability. Neither the excitatory response nor the change in conductance was present in neurons isolated from mice lacking the transient receptor potential cation channel subfamily V member 1 (TRPV1) even in the presence of cell volume change. Zaelzer and colleagues went on to show that a truncated variant of TRPV1 was responsible for both the osmotic and thermo-responsive elements of OVLT neurons[150]. Osmoregulatory defects have also been reported in mice lacking TRPV4[122,151], though it remains to be determined if it serves a hypo-osmolality transducer function in the OVLT[133].

Although TRPV1 knockout mice show attenuated AVP secretion in response to hyperosmotic stimuli[152], the role of TRPV1 in osmotically-driven thirst remains controversial. Since TRPV1 channels are essential for increasing the electrical discharge of osmosensitive

OVLT neurons, functional loss of these channels would be expected to translate into suppressed osmotic thirst[123]. Surprisingly, TRPV1, TRPV4, and double TRPV1/TRPV4 knockout mice were shown to drink equal amounts of water in response to an array of systemic osmotic stimuli, including hypernatremia, dehydration, and intraperitoneal or subcutaneous NaCl or mannitol injections[153,154]. Furthermore, the number of *c-fos* positive cells in the OVLT remained similar in normal and knockout mice despite hyperosmotic challenge[153,154]. These data do not preclude the involvement of other osmoresponsive elements, including the role of glia and peripheral osmoreceptors, that function independent of TRPV1 and TRPV4 (see section 1.1.4.1.2).

1.1.4 Vasopressin (AVP)

"Of the extracts of the three organs in question that of pituitary body is by far the most marked. The rise of blood-pressure produced is rapid, its amount varying with the initial pressure." – Oliver and Schäfer, 1885[155]

Oliver and Schäfer were the first to describe the vasopressor activity of what was later identified as AVP in extracts from the posterior pituitary[155,156]. In 1913, reports of successful treatment of diabetes insipidus, a condition marked by polydipsia and polyuria (excessive urination), with subcutaneous injections of posterior pituitary extract highlighted the antidiuretic properties of the concentrate[157-159]. Experimentally, extracts were shown to reduce thirst and urine volume while increasing urine tonicity[160,161]. It was not until the 1950s that the pressor and anti-diuretic properties of posterior pituitary extract were attributed to a single hormone: AVP[162].

Congruent with the 'form follows function' biological principle, AVP was structurally defined[163,164] decades after the vasopressor and anti-diuretic function of the hormone were identified[156,162]. AVP is a nonapeptide, structurally distinguished from other forms of mammalian vasopressin (i.e. lysine-vasopressin in pigs[165]) by the presence of arginine at the eighth position and differs from OT by only two amino acids at positions three and eight[166]. It is synthesized as a pre-prohormone, which includes an N-terminal signal peptide, vasopressin, neurophysin, and a C-terminal glycoprotein called copeptin[167]. Post-synthesis and processing in the Golgi apparatus, AVP is transported along the axons of MNCs and stored in neurosecretory vesicles in Herring bodies of the posterior pituitary (a process lasting from 1 to 2 hours)[167,168]

until chemical or electrical stimuli induce action potential propagation in the nerve terminals located in the posterior pituitary[169]. Depolarization of MNC axon terminals generates an influx of calcium ions (Ca^{2+}) through voltage-gated Ca^{2+} selective channels to trigger fusion of vesicles with the terminal membrane[170-173], releasing AVP into the extracellular space where it diffuses into the global circulation via fenestrated neurohypophyseal capillaries[166,174]. Once in the periphery, AVP acts on guanine nucleotide-binding (G) protein-coupled receptors $V1_a$ and V2 to exert its effects[162], but is quickly metabolized within 10 to 35 minutes by liver and kidney vasopressinases[94].

1.1.4.1 AVP secretion

AVP is functionally multifarious. It acts as a neuromodulator within the CNS to influence emotional and social behaviours, cognition, circadian rhythm, thermoregulation, nociception, and autonomic function[162,166,167]. In the periphery, it performs in a classical neuroendocrine fashion, maintaining hydromineral and hemodynamic homeostasis by altering renal filtration and counteracting hemodynamic changes during cardiovascular distress. In mammals, AVP, along with OT, is synthesized in large MNCs concentrated in the SON, PVN, and in accessory nuclei dispersed throughout the brain[174-176]. Few neurons synthesize both neuropeptides[174]. Much of what is known about AVP secretion comes from studies performed in the SON of the anterior hypothalamus, located superior to the optic chiasm. The SON contains a homogenous composition of MNCs, shielded by a classical blood-brain-barrier[177]. In the rat SON, AVP-MNCs are clustered in the caudal portion of the nuclei and make up the majority (~60 – 70%) of cells[178,179].

The function of AVP-MNCs is regulated by many intrinsic and extrinsic mechanisms thus these neurons are said to be integrative sites for coordinating the organism's response to physiological and environmental challenges[174]. The activity of MNCs is regulated by synaptic inputs from many nuclei that are responsive to peripheral cardiovascular and osmolality changes. Glial cells can also modulate the electrical activity of these cells by regulating gliotransmission and the number of available synapses[180]. MNCs may also auto regulate by somato-dendritic release of AVP[181,182], via production of nitric oxide (NO) which can inhibit intrinsic electrical activity[183,184] and afferent pre-synaptic transmissions[185,186] (see section 4.4), or by intrinsic

osmosensory mechanisms (section 1.1.4.1.2)[9]. The sum of these processes alters the electrical activity of MNCs, which in turn dictates AVP secretion into the circulation.

1.1.4.1.1 Activity-dependent release

AVP release is electrical-activity dependent: the propagation, form, frequency and pattern of action potentials facilitate the quantity of hormone released[173]. In *in vivo* and *in vitro* rat experiments, basal MNC firing rate (i.e. action potential frequency, expressed in Hz), was recorded at ~ 3 Hz in isotonic conditions (~ 294 mOsm/kg)[187-190] which maintains basal serum levels of AVP at 2 – 3 pg/mL[15], consistent with reported human serum AVP concentrations in healthy, iso-osmotic conditions[94]. The basal firing frequency of MNCs thus effectively determines the osmotic set point[9,191]. Increases in MNC firing rate above basal frequencies and up to ~ 15 Hz – such as can occur with experimental dehydration, hemorrhage, and hyperosmotic stimuli – linearly correlate with increased AVP secretion[173,187-190,192,193]. AVP is maximally secreted at firing frequencies at ~ 10 to 15 Hz, plateauing the linear relationship[193-195].

Stimulated AVP-releasing MNCs exhibit a distinct phasic firing pattern that are electrophysiologically defined as trains of action potentials, or 'bursts', with intra-burst frequencies of 3 to 15 Hz followed by an inter-burst quiescent period lasting from 4 to 100 seconds[191]. Bursting increases the duration of action potentials, which is positively correlated with firing rate, according to intracellular recordings obtained from rat hypothalamic explants[196]. K^+ channels play an important role in this form of facilitation: pharmacologically blocking K^+ channels prolongs the duration of action potentials and enhances AVP release, whilst simultaneously reducing frequency-dependence[195,197]. Electrical stimulation of rat neurohypophysis to attain continuous or phasic activity with mean firing frequencies of 6 Hz and similar distributions of interspace intervals (i.e. the time between action potentials), demonstrated that phasic firing elicits more AVP release than continuous firing[198]. Continuous firing at moderate to high frequencies (10 – 15 Hz) has been shown to induce fatigue and drastically reduce AVP secretion, a phenomenon that can be reversed by increasing the duration of inter-burst quiescence[173,199]. In conditions in which AVP increase is necessary to preserve physiological homeostasis, facilitating AVP release by altering firing behaviour may provide the rapid response necessary to protect the system without exhausting the neurons.

1.1.4.1.2 Osmotic control of AVP secretion

Experiments *in vivo* and *in vitro* established that the firing rate of AVP-secreting MNCs is linearly proportional to physiological ranges of ECF osmolality. Systemic hypo-osmolality caused by intragastric water loading reduces MNC firing rate[188], whereas induced systemic hypertonicity increases MNC firing rate[187-189,191,200-205]. Various synaptic and cellular elements influence the response of MNCs to shifts in osmolality. Peripheral osmoreceptors have been demonstrated to affect MNC activity[206], likely through afferents from the VLM and NTS[207-209]. MNCs also receive synaptic inputs from central osmoresponsive sites including the SFO, MnPO, and OVLT[207,210]. Specific to the OVLT, electrophysiological evidence indicates that osmoresponsive glutamatergic OVLT neurons influence MNCs via excitatory inputs[141,211-213] and that electrolytic destruction of the area encompassing the OVLT diminishes osmotically-induced AVP secretion[97,98,105,214]. Additionally, glia within the SON release taurine[215,216], a primary osmolyte used by the brain to protect against hyponatremia-induced swelling[217,218], in response to cellular swelling[11,219,220] via volume-regulated anion channels[221]. Taurine inhibits neuronal firing during hypotonic challenges by imposing a glycine-mediated inhibitory tone on MNCs[215,222,223]. Glial cells also reversibly alter their morphological features to tune MNC activity in response to physiological stimuli, such as dehydration, hypernatremia, parturition and lactation[180,224,225].

The first evidence of cell autonomous osmosensory transduction came from recordings attained from SON MNCs: neurons fired in response to increasing perfusate osmolality independent of synaptic transmission[200]. MNCs depolarize and fire in the presence of hyperosmotic fluid in association with an increased cation conductance[201,202,226], which appears to be active at resting membrane potential (RMP) [227,228]. Inversely, hypo-osmotic perfusate hyperpolarize acutely isolated MNCs[202] and inhibit cation conductance[227,228]. Whole-cell patch clamp experiments from isolated MNCs evidenced that hypotonic swelling and concurrent decrease in cation conductance could be reversed by applying negative pressure to the recording pipette[229]. Mechanical recovery of cell volume also reversed hyperosmotic cellular swelling and incidental increase in membrane conductance. The associated changes in cell volume and conductance led to the proposition that cell autonomous osmoresponsiveness is conferred on MNCs by mechanosensitive non-selective cation channels[227]. Work by Sharif-Naeini and colleagues identified the channel in MNCs as the truncated form of TRPV1[152], which confers

intrinsic osmoresponsiveness when injected into otherwise non-osmoresponsive cells[150]. Hyperosmotically-stimulated MNCs isolated from TRPV1 knockout mice failed to display changes in firing frequency or membrane conductance even in the presence of cell shrinkage. This result could be duplicated in wildtype mice by pharmacologically blocking TRPV1 channel function. Disrupting the organization of cytoskeletal elements, including actin and microtubules[230], abates the influx of positive charges into MNCs in response to hyperosmotic cell shrinkage, whilst stabilizing these elements enhances osmotically-induced nonselective cation channel activity[229,231]. One study showed that microtubules in SON MNCs physically interact with TRPV1 channels to provide the "push" force necessary to promote channel activation during cellular shrinkage[231]. Other ion channels may also be involved in cell autonomous osmosensory transduction. Electrophysiological recordings from isolated MNCs have identified stretch-activated K^+ channels[232,233] as putative contributors, though other, as of yet unidentified, Na^+ and Ca^{2+} conducting channels could also play a role[9].

1.1.4.2 Role in osmoregulation

Although the pressor effect of AVP was the first functional characteristic recognized, AVP and its ancestral gene products are predominantly involved in osmoregulation[165]. In this context, changes in circulating AVP follow a linear function in response proportional to plasma osmolality[15,75]. The sensitivity (i.e. slope) of the osmolality-AVP relationship is referred to as osmoregulatory gain[234] (section 1.1.5). An increase in 1% blood osmolality (~3 mOsm/kg) results in an increase of at least 2.9 pg/mL AVP in normovolemic rats[15]. Because AVP is secreted in iso-osmotic conditions, some basal renal water reabsorption occurs at rest[75]. Experimental hyperosmolality linearly increases serum AVP[14,15,47,61-63,74,94], whereas basal AVP is inhibited by serum hypo-osmolality[15,75,235,236]. Injection of exogenous AVP in dogs[237-239] and humans[235,240,241] actuates a dilution of serum osmolality with concomitant anti-diuresis and increased natriuresis. Diuresis, and therefore serum osmolality, is altered by the function of renal V2 receptors, which are tightly controlled by physiological ranges of AVP[166].

Anti-diuresis ensues when AVP binds to AVP type 2 (V2) receptors located along the basolateral membranes (i.e. facing the blood stream) of endothelial cells lining the renal distal tubules and collecting ducts[166]. Activated receptors initiate the mobilization of protein kinase A, which effects the translocation of vesicles to the luminal membrane (i.e. the urine side),

directing the water-conducting aquaporin-2 channels lining the vesicle membranes to the luminal surface. Expression of aquaporin-2 increases intracellular water content, osmotically equilibrating the ECF, and achieving a net effect of water reabsorption from the renal filtrate and concentrated urine. Urine concentration and excreted volume are linearly proportional to serum AVP in humans and animals[61,75,235,242-244], with maximal diuresis ensuing at lower AVP concentrations[60].

1.1.4.3 Role in hemodynamic function

AVP in the cardiovasculature is principally effective during times of hemodynamic challenge[174]. Afferents from baroreceptors and chemoreceptors lining the chambers of the heart, aortic arch, and carotid that monitor the volume and pressure status of the cardiovascular system, project to the area postrema and the NTS to tonically inhibit AVP-release. As blood volume or pressure drops to pathological levels, baroreceptor-mediated afferent discharge rate drops, stimulating AVP-secretion[245]. Stimulation of the caudal region of the NTS selectively excites SON AVP cells[246,247] likely indirectly via projections from the NTS to the VLM, parabrachial nucleus, and locus coeruleus[248-250]. AVP contributes to hemodynamic homeostasis by regulating vascular tone, baroreceptor reflex (i.e. blood pressure), cardiac function, and sympathetic nerve function, predominantly through the $V1_a$ receptor pathways[166]. $V1_a$ receptors are found on the smooth muscles of various blood vessels throughout the vasculature[251,252]. Binding of AVP rapidly phosphorylates the receptor via G protein-coupled receptor kinases and messenger-dependent kinases such as protein kinase C and phospholipase C, releasing intracellular Ca^{2+} stores[166]. Ca^{2+} interacts with the myosin machinery to contract the smooth muscle. AVP further contributes to vasoconstriction by blocking ATP-sensitive potassium (K_{ATP}) channels[253]. In normal conditions, K_{ATP} channels inhibit voltage-sensitive Ca^{2+} channels in smooth muscle cells by altering the membrane potential, thereby regulating vascular tone[254].

The systemic effects of $V1_a$ activation are complex and balance the prevalent vasoconstrictive consequences at different degrees in various organs. That is, mesenteric, cerebral, pulmonary, and coronary circulations are less sensitive to AVP-mediated vasoconstriction relative to other vasculatures, in part due to the presence of NO in these organs that promotes vasodilation at basal and low doses of AVP[162,255-259]. Overall, AVP is a weak vasoconstrictor at basal levels in normal conditions[260-263]. Appreciable changes in mean arterial

blood pressure are attained in healthy humans and dogs only at supra-physiological levels (~50+ pg/mL)[264,265]. This is due to the concomitant effect of AVP on heart rate as any increase in blood pressure is masked by a larger reduction in heart rate[162]. However, AVP contributes to maintaining arterial blood pressure during hypotension in a dose-dependent fashion[162,166,266,267]. AVP levels rise exponentially relative to severity of hypotensive hemorrhage but only after a greater than 7 - 10% blood volume loss[15,268,269]. Pharmacologically blocking V1 receptors during experimental hypovolemia in animals induces hypotension[161,270]. Hypervolemia, on the other hand, suppresses AVP release and causes diuresis[15,75,271].

1.1.5 Osmoregulatory gain

As discussed in section 1.1.4.2, serum concentrations of AVP are closely correlated with serum osmolality[60]. Non-osmotic stimuli of AVP release can alter the osmotic control of AVP by adjusting the gain of the osmoregulatory system. For example, elevated AVP during hemodynamic stress alters the osmotic modulation of AVP-secreting MNCs so that the osmotic threshold is shifted leftward (i.e. the set point or "osmostat" is reduced) without affecting the sensitivity of the relationship. This shift thereby necessitates a greater amount of circulating AVP to maintain normal serum osmolality[15,269,271-273]. Isotonic hypovolemia enhances AVP release and engages other systemic responses to cooperatively augment salt and water intake in an effort to recuperate the ECF volume. Conversely, hypervolemia inhibits osmotic control of AVP and promotes diuresis to discharge excess water[15,235]. Osmoregulatory gain also fluctuates within normal physiological parameters following the periodicity set out by the circadian rhythm[274].

1.1.5.1 Diurnal fluctuations of osmoregulatory gain

The relationship between AVP and serum osmolality is dynamic, following a diurnal rhythm. A shift in osmotic threshold or slope of the relation, as seen in the hemodynamic distress scenario, may facilitate AVP secretion at the end of the sleep period[274]. AVP progressively rises during the sleep phase, independent of serum osmolality, to prevent dehydration and enuresis (involuntary urination), and protect sleep, peaking just before wakefulness[236,275-280]. In human males, peripheral AVP increases overnight by 2 pg/mL on average[275], similar to levels induced by dehydration[243]. The circadian cycle is imposed on MNCs in part by GABAergic inputs from the "master clock"[281] - the suprachiasmatic nucleus (SCN)[282]. Increased SCN activity,

demonstrated using *in vivo* and *in vitro* recordings from rats, peaks in the middle of the sleep phase[282,283], silencing presynaptic osmosensory projections from the OVLT to the SON[274,284]. SCN firing progressively declines, with the nadir occurring by wake time[282,283], just as osmotic modulation of MNCs is enhanced[236,276,277]. Thus declining SCN activity towards the late sleep phase facilitates AVP secretion by disinhibiting MNCs. Decreased osmoregulatory input into the SON is therefore one potential mechanism contributing to the normal dissociation seen between plasma osmolality and circulating AVP. Outside of these normal fluctuations, and in the absence of cardiovascular distress, inappropriate AVP serum concentrations relative to blood osmolality lead to detrimental osmoregulatory disruption and serious medical complications.

1.1.6 Exampled disorders of hydromineral homeostasis

Osmotic homeostasis is particularly important for mammals whose brains are encased within a skull that does not provide room for swelling[9]. Therefore symptoms associated with dysregulated ECF osmolality are neurological in nature, as exhibited in the following examples.

1.1.6.1 Central diabetes insipidus (CDI)

Central diabetes insipidus (CDI) is characterized by polydipsia and polyuria caused by AVP deficiency[285], as mentioned in section 1.1.4. It is recognized by the onset of high volume hypotonic urine, often occurring in conjunction with urine hypernatremia, extreme cravings for fluid, and iso- or hyperosmotic serum. Diagnosis of CDI is made based on a water deprivation test as serum osmolality will increase in the presence of inappropriately dilute urine. CDI can be caused by damage to the brain through infections, autoimmune disorders, cancers, or it can be idiopathic. However, it is most commonly acquired via structural damage that disrupts the synthesis of AVP at the site of MNCs within the hypothalamus or the ability of the posterior pituitary to secrete the hormone[286,287]. It is a frequent complication of pituitary surgeries that occurs in ~30% of post-operative patients[288], and can either be transient or permanent[289]. Pathophysiologically, the closer the insult or lesion to the MNC bodies, the more likely the disease will be permanent due to the loss of AVP-producing neurons[244,286]. Furthermore, CDI can be caused by damage to areas encompassing the OVLT[290]. If fluid intake is inadequate to keep pace with water loss, patients will suffer from hyperosmolality and associated neurogenic symptoms that can include lethargy, seizures, and coma[291]. CDI is manageable with good

drinking habits to ensure hydration, and treatment with synthetic vasopressins such as desmopressin[285].

1.1.6.2 Syndrome of inappropriate antidiuretic hormone secretion (SIADH)

A prime example of a pathological dissociation between plasma osmolality and AVP is the syndrome of inappropriate antidiuretic hormone secretion (SIADH). SIADH is an inappropriate secretion of AVP with concomitant increase in extracellular water, without an increase in volume[292]. SIADH is considered to be the most frequent subtype of hyponatremia[293,294]. Hyponatremia, usually defined as blood sodium levels of <135 mmol/L, is a serious and highly prevalent electrolyte disorder, affecting nearly 30% of hospitalized patients[295-297]. Hyponatremia manifests clinically with a slew of neurological symptoms that range in severity relative to the concentration of sodium. These symptoms include altered gait and reduced attention span at the mild end of the spectrum[298,299], to headaches, confusion, seizures, coma, and in severe cases, death [291,296]. Furthermore, hospitalized hyponatremic patients have an increased morality rate relative to nonhyponatremic patients[293]. SIADH is euvolemic hyponatremia with associated plasma hypo-osmolality, and hyperosmotic and hypernatremic urine[292]. It presents in the absence of volume depletion in the context of edema, with normal renal and adrenal function and has been found to occur in infectious states, including pneumonia, HIV, and meningitis[300,301]. Despite plasma hypo-osmolality, AVP secretion is not suppressed[302]. AVP secretion is considered to be caused by non-osmotic stimuli such as, for example, hypotension, ANG II, and stress, or can be caused by ectopic secretion by tumours[293]. Although the pathophysiological mechanisms that breaks or reduces the osmoregulatory gain of AVP regulation in SIADH are unknown, a recent hypothesis proposes that there is a link between the inflammatory response and AVP secretion in some electrolyte-water disorders[300,301].

Is sepsis, a disease known for its hyperinflammatory response and dysregulated AVP secretion, also a disorder of osmoregulation?

1.2 Sepsis

The term sepsis is often used clinically to describe a spectrum of conditions resulting from a systemic inflammatory response to an infection that results in damage to the host's tissues

and organs[303,304]. Any pathogen or infectious agent can trigger sepsis, though gram-negative and gram-positive bacteria are the most common isolates. Nearly a third of blood cultures from septic patients are negative for any detectable microorganism; in such cases sepsis is presumed to have developed in response to toxic bacterial agents (e.g. endotoxin)[305-307]. While sepsis can strike any person of any race and age, incidence is higher in the elderly and infants and in blacks over whites[308-310]. Those with traumatic injuries, particularly immuno-compromised individuals, are especially vulnerable to developing sepsis[307,308].

The hallmarks of sepsis are multiple organ dysfunction (MOD) or failure as a result of hypotension and tissue hypoperfusion. It can be complicated by distributive shock in the presence of treatment-resistant hypotension, which is associated with intensive care unit (ICU) mortality rates of up to 50%[311-315]. Survivors of the syndrome commonly suffer from life-long ailments related to organ damage, including brain dysfunction[316-318]. There are currently no sepsis-specific medications available: treatment strategies depend on antibiotic treatment, fluid resuscitation, and organ support[304,319].

1.2.1 On the origins of sepsis

The conceptualization of sepsis – including its re-classification from disease to syndrome - has evolved throughout history. In 700 BCE, the Greeks defined sepsis as rot or decomposition of organic matter[320]. Hippocrates further described this pungent diseased state as originating from the colon, which released "dangerous principles" into the body that caused toxicity. Alternatively, early Romans believed that invisible creatures from swamp environments caused sepsis, giving rise to sophisticated water delivery systems and public baths as part of their public health programs. Despite these initiatives, sepsis continued to be highly lethal.

It was not until Louis Pasteur proposed his "Germ Theory" in the late 19th century that advances in the understanding of sepsis were made[321]. One of Pasteur's contemporaries, the surgeon Joseph Lister, correctly deduced that the fermentation that Pasteur demonstrated in his now famous experiments also appeared in the wounds of patients[320]. Lister took inspiration from engineers that used carbolic acid to eradicate odours from sewage, and doused wound dressings in the acid. This drastically reduced the rate of sepsis in his hospital. Lister went on to further advance aseptic techniques as part of general surgery practice. At the same time, another father of modern microbiology, Robert Koch, furthered germ theory by outlining the principles

necessary to define an agent as infectious. Koch's work initiated the rapid expansion of microbe identification, shifting the focus of treatment towards eliminating pathogenic organisms. This produced the groundwork for the development of antibiotics, and confirmed what the putrefaction definitions of earlier civilizations were hinting at: that sepsis started with an infection caused by microorganisms[321].

The pathogenic mechanisms of infectious diseases were unclear and contradictory even after the identification of microorganisms[320]. How could such a small quantity of tiny organisms result in a seemingly disproportionate lethal illness? Was it the bacteria that was lethal, or some disease-causing agent that the microbes produced? In 1886, Ludwig Briefer isolated heat-sensitive "exotoxins" from the supernatant of cultured diphtheria[322]. This work inspired Koch's colleague, Richard Pfeiffer, to search for exotoxins in cholera, a common infection of the small intestine[323]. Pfeiffer inoculated vaccinated pigs with *Vibrio cholera*, and then performed dissections on the pigs that had died of the disease[322]. Surprisingly, he found no living *V. cholera* at the site of infection. He discovered that the bacteria had lysed and released poisonous substances that were normally contained within the bacterial cell. Pfeiffer observed that the toxicity of *V. cholera* was independent of the bacteria's viability. He furthered this finding by showing that heat-killed cholera still maintained a toxic potential, and postulated that this insoluble and heat-resistant factor was not secreted, but was rather an intrinsic part of the bacteria. Pfeiffer named this substance "endotoxin". Pfeiffer had in fact discovered lipopolysaccharide (LPS) endotoxin of gram-negative bacteria that went on to become the predominant lab model for studies of fever, host immune response, and the pathogenesis of sepsis[324,325] (see section 1.2.5.1.1). This discovery furthered the burgeoning idea that sepsis was not caused by infection alone, but rather the host's response to the infection. Sir William Osler famously articulated this shift in dogma in 1904[326] with the following:

"*Except on few occasions, the patient appears to die from the body's response to infection rather than from it."*

Once the mystery of bacteria had been unraveled by germ theory, it was generally believed that with the advent of antibiotics and the advancement of vaccines, infectious diseases could be eradicated[320,321]. Sepsis was reinterpreted to refer to a systemic infection caused by

pathogenic bacteria that spread into the bloodstream, commonly referred to as "blood poisoning"[304]. With modern antibiotics such as penicillin and streptomycin in hand, it was presupposed in the early 20th century that sepsis had finally met its match. This was not the case. Despite the elimination of the causative bacteria with antibiotics, many patients still died of sepsis. By the mid- to late- 20th century – alongside rapid advances in immunology, microbiology, and medication and hospitalization paradigms – treatment of sepsis improved to the point that patients were surviving beyond the stages of the syndrome that would have historically killed them[319,327]. New symptoms became apparent and sepsis could no longer be strictly defined as a systemic infection or an inordinate inflammatory response. Bone and colleagues said it well in 1992: "Roughly 30 years ago, sepsis, septic shock, and multiple organ failure were rarely seen. Simply put, we could not keep severely ill or injured patients alive long enough for these disorders to develop" (p.1481)[327]. These "disorders" referred to the hemodynamic manifestations of sepsis, and had been documented before in the context of shock.

Studies conducted during World War I were the first to recognize the traumatic and lethal shock that occurred in soldiers as a result of excessive loss of blood volume, referred to as hypovolemic or hemorrhagic shock[320]. However, shock was still seen in soldiers that did not have an obvious hemorrhage. This "wound shock" was thought to be actuated by release of "wound toxins" (likely referring to the mediators of the local inflammatory response) that led to blood pooling in injured tissue as a result of neurogenic vasodilation[320]. The surgeon Alfred Blalock and others confirmed that movement of blood plasma from the circulation into tissue caused non-hemorrhagic shock[328,329]. By this time, it was recognized that the symptoms of shock could be seen in other conditions: in fact, the idea that sepsis could result in shock had already been postulated by the late 1800s[320]. Blalock developed classifications for shock: cardiogenic (as seen in heart attacks), hematogenic (hypovolemia), neurogenic (brain or spinal cord injury), and vasogenic (septic shock). Further categories were developed in the 1960s and 1970s, which ultimately led to the re-classification of sepsis as a distributive shock (an abnormal distribution of blood, resulting in oxygen deprivation to tissues and organs) caused by excessive blood vessel dilation[320,330].

The 1960s also marked the beginning of an evolving understanding of the hemodynamic alterations in septic shock[331,332]. Septic shock was mistakenly believed to manifest in two forms: warm and cold[320,333]. Warm shock was thus called because patients presented with warm skin,

and a strong, forceful pulse (high cardiac output) despite hypotension; these patients were predicted to survive. Cold shock was characterized by cold skin, and a weak pulse (low cardiac output) with hypotension, and these patients were thought to be marked for death. In 1965, Wilson and colleagues challenged this doctrine by demonstrating that most patients with septic shock exhibited a higher cardiac output and lower systemic vascular resistance (SVR; the resistance to blood flow as a sum of all of the vasculature, excluding the pulmonary vasculature) compared to patients with hypovolemic or cardiogenic shock[334]. They also noted that another distinct feature of shock originating from sepsis was the "element of vasodilation", which contributes to the reduction in SVR. Despite the findings of Wilson and colleagues, clinical and experimental studies of the time persisted in suggesting that survival correlated with high cardiac output[320,335,336]. A significant breakthrough came with the invention of the flow directed pulmonary catheter (PAC) by Harold Swan and William Ganz, which allowed for real-time monitoring of pressure changes in the pulmonary vasculature and heart[320,337]. Up until then, invasive monitoring of hemodynamics and cardiac function was not common in sepsis patients because of the difficulty in using traditional diagnostic techniques[320,337]. Use of the PAC indicated that sepsis patients presented with hypovolemia, and confirmed the modern understanding of the hyperdynamic (high cardiac output/low SVR) nature of early septic shock following fluid resuscitation; "cold" shock was most likely a result of insufficient fluid support[320]. Further studies in the 1980s showed that myocardial dysfunction (i.e. decreased volume of blood ejected during systole) is reversible in many septic shock patients[338-340]. It is now well established that sepsis is biphasic: the early phase is hyperdynamic, while the late phase is hypodynamic. Today, the concepts of warm and cold shock are used for diagnostic purposes only and are not indicators of potential mortality[341]. As the field of septic shock research evolved, so did research in other fields.

The 1970s and 1980s were a golden time for the fields of cytokine and coagulation research. Interleukin-1 (IL-1) and tumour necrosis factor (TNF) had been identified and implicated as critical to the pathogenesis of sepsis[342-344]. Significant advances in the understanding of coagulopathies present in sepsis were made as links between inflammation and coagulation were established[320,345]. The complexity of sepsis became increasingly apparent as studies furthered the role of cytokine cascades and inflammation, the coagulation system, and hemodynamic alterations in sepsis pathogenesis[304,320] (see section 1.2.6). This conceptual shift

from infectious disease to multi-system progressive syndrome cemented the idea that sepsis was more than a systemic infection and inflammatory response: in fact, it was known that not all septic patients were bacteremic[327]. The increased diversity of recognized symptoms and pathologies confounded sepsis dogma and inhibited any advancement in clinical and basic research. A standardized definition of sepsis was needed.

1.2.2 Defining sepsis

In 1991, an international panel of experts defined sepsis as a systemic inflammatory response syndrome (SIRS) with an active infection[311,327]. The panel acknowledged that the severity of sepsis ran on a continuum, and defined severe sepsis as sepsis plus MOD, hypoperfusion, or hypotension. Septic shock, a subset of severe sepsis, was defined as severe sepsis plus persistent and resuscitation-resistant hypotension. A decade later, another international panel extended the list of signs for SIRS, acknowledging that these were not specific to sepsis, while upholding the 1991 definitions. This panel blurred the line between sepsis and severe sepsis, allowing them to be used interchangeably[304,346]. In 2016, the definitions were once more updated. Sepsis is now defined as a "life-threatening organ dysfunction caused by a dysregulated host response to infection"[312]. Emphasis was placed on multiple organ failure and resuscitation-resistant hypotension, and SIRS was removed from sepsis vocabulary given its ubiquitous nature in hospitalized patients[312,347]. These new criteria also formally eliminated the distinction between severe sepsis and sepsis. Finally, septic shock is now defined as "a subset of sepsis in which underlying circulatory and cellular/metabolic abnormalities are profound enough to substantially increase mortality", with emphasis placed on refractory (treatment-resistant) hypotension [312,313,348].

1.2.3 Symptomatology

The symptoms of sepsis are highly variable and depend on the type of causative infection, any pre-existing conditions in the patient, and the length of time between diagnosis and treatment[304]. There are currently no specific diagnostic tests to conclusively identify sepsis therefore diagnosis is made based on the type, combination, and order of symptoms. The 2003 international panel that updated the definition of SIRS and sepsis also outlined the associated diagnostic criteria still used today[346]. The early signs of sepsis are non-specific symptoms that

fall under the category of SIRS that also occur in other systemic responses (e.g. trauma) and include: tachycardia (elevated heart rate), tachypnea (rapid breathing), hypothermia or fever, and leukocytosis or leukopenia (increased or decreased white-cell count, respectively). On their own, the symptoms are unreliable unless there is a strong suspicion or confirmation of an infection. Sepsis is diagnosed when at least two SIRS-associated symptoms manifest, along with confirmed/suspected infection, plus evidence of early organ dysfunction and/or hypotension. The syndrome usually compromises the cardiovascular and respiratory systems first, with the kidneys and brain also often affected[304]. Symptoms vary depending on the type of organ or system that is malfunctioning: hypouresis (renal), arterial hypoxemia (respiratory), metabolic acidosis (general or renal), decrease in platelet count (hematologic), and delirium (neurological), amongst others[346,349].

1.2.4 Treatment

The goal of effective treatment of sepsis is to diagnose the syndrome as soon as possible because delays in appropriate antibiotic administration are associated with higher mortality rates in sepsis patients that will go on to develop septic shock [350-353]. A retrospective analysis by Kumar and colleagues determined that a delay in antibiotic treatment is the strongest predictor of mortality, with survival rates decreasing by 7.6% for each hour without treatment after documented hypotension[354]. Rapid treatment includes broad-spectrum antibiotics (and pathogen-specific antibiotics once a causative microorganism is identified) for at least one week, draining or removing the infection source, and management of hypotension to restore oxygenation by immediate perfusion of intravenous fluids[313,341,349]. If fluid resuscitation is inadequate to maintain a target arterial pressure (~60 – 65 mmHg)[355,356], the catecholamines norepinephrine, epinephrine, dopamine, dobutamine, or a combination of norepinephrine and AVP are administered[341,357]. Mechanical ventilation with sedation is provided to patients that show respiratory distress or neurological deterioration, and additional drugs can be provided for organ-specific support[313,341]. Patients who survive acute sepsis (first 2 - 3 days) are given nutritional support and continued treatment as appropriate. Of the patients that die, 20 - 35% will do so within first 3 - 7 days of developing sepsis, 40 - 60% within 30 days, and the remainder within 6 months[349,358]. Sepsis survivors, regardless of severity of the syndrome, have increased long-term

morbidity and mortality rates, and a decrease in quality of life, likely related to organ-specific damage sustained during the septic episode (see section 1.2.6).

1.2.5 Experimental sepsis

Much of our current understanding of sepsis pathophysiology comes from animal and patient studies. The primary challenge of basic sepsis research is capturing the complexity of the syndrome in a lab setting: the syndrome is difficult to reproduce and cannot be fully replicated by any existing preclinical model. In order for the model to be considered clinically relevant, it should mimic the human septic response to include progression from early to late sepsis as marked by initial hyperdynamic response, hypermetabolic and hyperdynamic cardiovascular states (e.g. increased glucogenesis, high cardiac output) and later hypometabolic and hypodynamic cardiovascular response (e.g. hypoglycemia, hypotension)[359-361]. Some models are used to specifically study the immune responses and apoptosis in certain cell types, as is commonly observed in human sepsis, however these are considered less clinically relevant as they capture only a minority of sepsis-associated symptoms[359-364].

1.2.5.1 Sepsis models

Sepsis models fall into two broad categories: exogenous models, and endogenous models[359-361]. Exogenous models are developed by injections of a pathogen or a toxin that can trigger an immune response. These models are dose-dependent and can include injections of viable or non-viable bacteria, fecal slurries, or components of microorganisms such as endotoxins. Exogenous models are predominantly used to study the immune response and have implications beyond sepsis. Endogenous models involve disruption of hosts' barriers that allow for bacteria to permeate into otherwise sterile tissues, causing infection. These usually involve surgery on the animal and use the hosts' microbiome to cause infection. As a result, they are referred to as "two-hit" or "multiple-hit" models because they involve multiple insults (e.g. the surgical procedure, followed by infection) that can result in an inappropriate immune response that leads to multiple organ failure, as seen in human sepsis[365,366]. Although there are many models of sepsis, the remainder of this section will focus on the two most popular: endotoxemia (exogenous) and cecal ligation and puncture (CLP; endogenous).

1.2.5.1.1 Endotoxemia / Lipopolysaccharide (LPS) model

This model uses single or multiple injections of various doses of LPS to induce a systemic inflammatory response[360,361,367], and is the predominant model used in sepsis research literature. LPS is an endotoxin expressed on the outer membrane of gram-negative bacteria, and can interact with the co-receptors CD14 and toll-like receptor-4 (TLR-4) to activate the innate immune system via NF-κB-mediated upregulation of transcription of pro-inflammatory mediators[368]. This model arose because it was originally thought that the major sepsis-causing organisms were gram-negative bacteria[369]. It is the simplest, most homogenous model currently available making it highly attractive as this experimental system can be controlled and easily reproduced. Using this model, advances have been made in understanding the inflammatory response that may apply to many disease states[370,371].

Headway in the context of therapeutic potential for the treatment of sepsis was made in the late 1980s when it was discovered that healthy humans receiving endotoxin injections showed an acute increase in tumour necrosis factor (TNFα), an early proinflammatory cytokine, that "likely mediate(s) septic shock"[372,373]. This led to a rapid push for clinical trials for anti-TNFα therapy in sepsis that were largely unsuccessful[367,368]. The trouble with the groundwork used to design these trials is that endotoxemia is not sepsis. LPS models do not replicate the magnitude, kinetics, and profile of cytokines seen in human sepsis and in the CLP model[362,367,374,375]. Single injections of LPS result in immediate and significant spikes in serum cytokine levels, whereas sepsis is considered to have low but prolonged and progressively increasing levels of cytokines. Furthermore, dosage of LPS can vary between labs: this is important as a different dose can result in different mechanisms coming into play. Particularly relevant to this thesis are example studies that showed that two different intravenous (i.v.) doses of LPS mediate different central pathways to induce a fall in arterial blood pressure: a low dose (1 mg/kg i.v.) initiates hypotension via the vagus nerve[376], whereas a high dose (15 mg/kg i.v.) initiates hypotension via the OVLT[377]. Use of endotoxins such as LPS for the study of sepsis has yielded many benefits, and has advanced our understanding of the inflammatory response, however the sepsis research community has shifted towards more complex models that better represent the human sepsis syndrome.

1.2.5.1.2 Cecal ligation and puncture (CLP) model

As stated earlier (section 1.2.1), the pathophysiology seemed deceptively straightforward until the last couple of decades when it became clear that sepsis is more than just a vigorous systemic inflammatory response. This shift in understanding resulted in a move to more complex animal models that have an infectious focus that develops into a systemic response as seen in the CLP model, instead of starting with a systemic response as seen in endotoxemia[378].

The CLP model begins with a surgical procedure to expose the cecum (a pouch between the ileum and the ascending colon; Fig. 2.14.1), ligating it causing local necrosis and ischemia to the tissue, and puncturing it to bring fecal matter, and therefore a sample of the resident gut microbiota, into the peritoneal cavity[360,379]. It is generally reproducible, most often performed in mice and rats, and the surgery is relatively simple in trained hands[359,380]. The number of punctures, the gauge of the needle used, and the amount of cecum ligated can be tailored to adjust for severity of the syndrome[360,379]. Adjusted severity usually translates into how quickly the infection progresses into sepsis, and from there to multiple organ failure and death: the syndrome can develop in rodents in as little as 2 hours[381], to up to 28 days[382]. Disadvantages specific to this model are that some hosts form abscesses to isolate the infection and don't develop sepsis, and consistency. Labs vary in the number of punctures, the gauge of the needle used, and the amount of cecum ligated, and therefore the severity of symptoms. The severity of CLP-induced sepsis affects mortality and can potentially represent a difference in underlying pathophysiology[379].

CLP in rodents follows a similar pattern of physiological hyperactivity at the initial stage of sepsis followed by hypoactivity in the late stage, as seen in human peritonitis-induced sepsis (see section 1.2.6)[359,360,383]. Furthermore, the CLP model begins with a polymicrobial infection, which represents ~ 36% of infection types in patients[384]. The group that developed the CLP model in rats compared several physiological features of "early sepsis", defined as 10 hours post CLP surgery, and "late sepsis", defined as 16 to 24 hours post CLP[360,383]. They found that early sepsis in rats was marked by a hyperinflammatory phase, hyperglycemia, hyperinsulinemia, and increased blood flow to organs, while rats in late sepsis showed hypoinflammation, hypoglycemia, hypoinsulinemia, and decreased blood flow. This list of outcomes has since been corroborated and expanded by numerous other studies[380]. In summary, the CLP model is

considered the "gold standard" for sepsis research and is used for the experiments described in this thesis (see section 2.2).

1.2.6 Pathophysiology

The pathophysiology of sepsis and septic shock involves numerous mechanisms, our understanding of which is constantly evolving[304,312]. The goal of the immune response to an infection is to isolate, restrict, and eliminate infective material, while repairing and protecting surrounding host tissues. The host immune response is dependent on the type of infecting microorganism and corresponding host receptor activation[385,386]. The response is complex and involves a coordinated cascade of events that are initiated by the advance guard cells of the innate immune system upon contact with invading microbes. Historically, gram-negative bacteria were the predominant causative microorganisms of sepsis, but their prevalence is declining and gram-positive and fungal infections are on the rise[305-307]. A critical distinction between a normal response to infection and sepsis is that the former occurs in peripheral tissue and is usually localized, whereas the latter occurs in the bloodstream at a systemic level. In the case of an acute and contained infection, the body coordinates a response that encourages appropriate inflammation at the site of the infection, while promoting anti-inflammatory effects systemically. Failure to eradicate an infection or mitigate excessive inflammation tips the balance in favour of inciting sepsis[387].

Inflammation in the blood stream results in broad tissue hypoperfusion and hypotension because of the damage dealt to the vascular endothelium[304]. Briefly, when leukocytes encounter a pathogen or toxin in the circulation, they release chemokines and pro-inflammatory factors to recruit additional immune cells and amplify the inflammatory cascade[388]. Normally, neutrophils and other leukocytes would need to migrate into the peripheral tissue to reach the site of inflammation. The interaction between leukocytes and endothelial cells within the vasculature stimulates the release of vasoactive mediators such as NO to change the permeability of the vascular endothelium to allow for migration[389,390]. In sepsis, the leukocytes remain in the vasculature (some may migrate to the causative site of infection or tissue injury) and augment the effects of mediators. The alteration of vascular permeability increases the "leakiness" of capillaries: this causes plasma leakage out of the bloodstream, general edema, a decrease in vascular resistance, and ultimately, vasodilation[391]. The combination of vascular changes results

in tissue hypoperfusion and a decline in blood pressure (note that blood pressure = cardiac output x SVR), and gives rise to the early hypovolemic nature of untreated sepsis[369,392]. Cardiac output increases to compensate for the decline in SVR in an effort to restore blood pressure. Although tissue perfusion initially increases, the quality of the perfusate decreases due to a decline in oxygenation of the blood.

As the inflammatory response in the vasculature progresses, the reactive oxygen species and lytic enzymes released by leukocytes in an effort to clear pathogenic material will cause excessive host damage[304,393]. Microruptures of the vascular endothelium will recruit coagulation factors, causing widespread fibrin deposits and result in microvascular thrombi. Disseminated intravascular coagulation occurs as continuous systemic activation of the coagulation response depletes platelets and proteins, potentially provoking internal bleeding. Hypoperfusion, hypotension, and extensive thrombosis in the microcirculation of organs lead to hypoxia, metabolic alterations, and nutrient deprivation resulting in MOD. Cardiac output progressively declines as the heart is also damaged. Furthermore, myocardial dysfunction, impaired hepatic function, cortisol deficiency, adrenal insufficiency, and adrenergic receptor hyposensitivity contribute to the development of lethal levels of hypotension seen in sepsis and septic shock[394]. If recovery does not occur during the hyperinflammatory state, the subsequent inappropriate systemic immunosuppression seen in late and chronic phases of sepsis renders patients vulnerable to nosocomial infections, perpetuating the syndrome[387].

1.2.6.1 Pathophysiology of vasodilatory hypotension

Vasodilation caused by decreased SVR is considered the primary cause of severe hypotension in sepsis[162,392,394-399]. Pathological vasodilation is the hallmark of vasodilatory shock, and sepsis is the most common cause of this form of shock[400]. The mechanisms of hemodynamic abnormalities in vasodilatory shocks are multifactorial and incompletely understood, but center around inappropriate vasodilation and loss of vasoconstriction mechanisms.

Overproduction of the potent vasodilator NO is an important etiology for vasodilation. Proinflammatory cytokines such as TNFα, IL-1, and interferon gamma (INFγ) stimulate inducible NO synthase (iNOS), via NF-κB signaling pathways, producing excessive NO in sepsis[401]. The literature consistently points to hyperproduction of NO as the culprit for most of

the cardiovascular and hemodynamic pathologies presenting in sepsis and septic shock [402,403]. Studies show that NO levels are high in sepsis patients[404,405]: blocking iNOS activity reverses endotoxin or cytokine-induced hypotension[406-409] and increases SVR, thus improving blood pressure, and reverses refractory hypotension in septic shock patients[410-412]. However, therapeutic use of NOS inhibitors in sepsis patients is controversial: a phase II double-blind, randomized and placebo-controlled multicenter study found infusion of a NOS blocker for 72h resolved septic shock[413], however the follow-up phase III trial reported increased mortality associated with the inhibitor and the study was terminated early[414]. Deaths were associated with increased circulatory failure and myocardial dysfunction, which may not be that surprising given that NO is an important contributor to vascular tone in physiological states[186,402].

In sepsis, vascular tone is progressively lost due to the inability of smooth muscles in the blood vessels to contract. Studies have shown that vascular smooth muscle cells are hyperpolarized due to NO-mediated activation of K_{ATP} channels and resulting inhibition of voltage-sensitive Ca^{2+} channels, forcing muscle relaxation[415-418]. Blocking K_{ATP} channels with glibenclamide restored arterial blood pressure in endotoxemic dogs and pigs by increasing SVR with minimal changes in cardiac output [416,419,420]. Septic shock patients did not see the same benefits in similar experiments in which treatment with glibenclamide had minimal effects on blood pressure, and did not reduce dependence on norepinephrine[421,422]. These divergent outcomes most likely represented a discrepancy in dosage: the high doses used in *in vivo* and *in vitro* studies to block K_{ATP} are beyond the doses given to patients[420]. Additionally, vascular smooth muscle cells lose sensitivity to the vasoconstrictive actions of catecholamines in septic shock[423-426]. Vasoplegia (catecholamine resistance) is a common element of vasodilatory shock, and a quintessential feature of all forms of shock in the final stages[400]. The term refractory septic shock refers specifically to the development of vasoplegia in late septic shock. Interestingly, septic shock patients at this stage are sensitive to the pressor action of exogenously administered AVP, strongly suggesting a role for AVP in the mechanism of vasodilatory hypotension in sepsis[427,428].

1.2.7 Role of AVP in shock

AVP plays an important role in vasodilatory shock, and all forms of protracted shock, including cardiogenic and hypovolemic[400]. The critical contribution of endogenous AVP to

survival in shock[429-431] was demonstrated by studies using natural AVP "knock out" Brattleboro rats - the first knockout animals used in research[432]. In an endotoxic shock model, infusion of endotoxin in normal and Brattleboro rats caused a greater reduction in blood pressure (70 mmHg vs. 40 mmHg, respectively) and substantially increased mortality in the latter group: the control rats survived until the experimental endpoint of 4 hours, whereas all of the Brattleboro rats died within 3.5 hours[430]. Rehabilitating AVP to serum levels equal to that of control rats restored blood pressure in the Brattleboro rats with hypovolemic shock[431].

Although AVP is important for cardiovascular regulation as discussed in section 1.1.4.3, it normally has minimal effects on vasoconstriction and therefore arterial blood pressure at basal levels[94,267,270,433-435]. It is not until very high levels of endogenous AVP are reached (10 to 200 pg/mL) in response to a challenge by, for example, hypovolemia or hypotension, that AVP's vasoconstrictive effects manifest[267,270,400,435]. Initial levels of AVP have been demonstrated to be appropriately elevated (10+ pg/mL) to combat hypotension in the early stages of hypovolemic shock[436,437], cardiogenic shock[428], and in vasodilatory shock caused by cardiopulmonary bypass[438,439], left ventricular assist placement[440], organ donation[441], and of course sepsis[428,442]. As shock progressively worsens and enters the vasodilatory phase, serum AVP levels decrease inappropriately relative to the degree of hypotension in the subjects[436,437,443-445]. Like other forms of shock, sepsis is associated with biphasic concentrations of serum AVP[400].

1.2.7.1 AVP in sepsis

Studies in early septic shock patients[428,442,446-448], and in endotoxemia [449-453] and CLP animal models[381,454-457], show that AVP levels during the early phase of septic shock are appropriately high (up to 21 pg/mL in patients[442,447]) relative to blood pressure. As the syndrome progresses to late septic shock, serum AVP levels fall dramatically in patients[427,428,442,447,458,459] and sepsis animal models[381,454-457,460,461]. In a pioneering study by Landry and colleagues, serum AVP concentration in late septic shock (1 - 2 days post onset) was evidenced to be deficiently low (3.1 pg/mL) compared to cardiogenic shock patients (22.7 pg/mL) with similar levels of hypotension[428]. Additional studies furthered the finding by demonstrating that ~23% of early septic shock patients[442,446] with hypotension developed relative AVP deficiency, with AVP inappropriately within the normal range (1.4 to 3.6 pg/mL) 36 hours after onset of shock[442]. Interestingly, Lin and colleagues found that AVP concentrations progressively increased as

sepsis developed in patients (sepsis: 10.6 ± 6.5 pg/mL, severe sepsis: 21.8 ± 4.1 pg/mL), and plummeted at the stage of septic shock (3.6 ± 2.5 pg/mL)[447]. Presumably, the vasodilatory and vasoconstrictive mechanisms discussed earlier (section 1.2.6.1) play a dominant role in sepsis and early septic shock and increased AVP during this phase is compensatory, whereas AVP deficiency contributes to the hypotension in late and irreversible vasodilatory septic shock.

1.2.7.2 Clinical relevance of AVP

AVP is a treatment option that has been extensively considered given that AVP deficiency is a hallmark of septic shock; exogenous AVP administration reduces catecholamine dependence thereby circumventing the development of pressor resistance in septic shock patients[341,384,428]. Landry and colleagues were the first to report the beneficial pressor effects of AVP infusion in AVP deficient septic shock patients[428]. Based on the rational that correction of AVP deficiency would improve blood pressure in septic shock patients, Landry and colleagues infused a small number of patients with low-dose AVP (0.1 units/min - which results in ≈30 pg/mL systemically - up to 0.4 units/min) for an hour[427,428]. In the absence of any other pressor drug, mean arterial pressure due to AVP-induced vasoconstriction increased to normal levels (~126 mmHg) and re-declined within minutes to hypotensive levels at the cessation of AVP infusion. This body of work inspired numerous observational studies and clinical trials[384,427,459,462-466]. Most of the results were promising: AVP increased mean arterial pressure without increasing cardiac output and improved renal function, decreased plasma cytokine levels, and, importantly, allowed for the reduction in associated high-dosage norepinephrine treatment[427,458,459,462,463,467,468].

The largest randomized and controlled trial for AVP versus norepinephrine (*Vasopressin and Septic Shock Trial*, aka VASST) compared mortality rates and safety of treatment with norepinephrine versus norepinephrine with low-dose AVP at different degrees of septic shock severity[384]. The authors found no difference between the treatments at 28 and 90-day mortality, organ dysfunction, or adverse effects. Retrospective analysis did, however, indicate a significant reduction in mortality in the AVP patient population with less severe septic shock, and an associated decreased risk for acute kidney injury[465,466]. Currently, AVP is used as a second-line treatment option to alleviate vasoplegia[341,355]. Further studies are necessary to determine the

efficacy of AVP as a monotherapy, and the mechanisms by which AVP asserts a beneficial effect.

1.3 Central Osmoregulation in Sepsis

Central neural pathways integrate interoceptive signals to harmonize dynamic visceral functions appropriately to achieve homeostasis even in diseased states (see sections 1.3.1. and 1.3.2) to regulate physiological processes, including inflammation, autonomic function, and of course, body fluid balance[469,470]. Accumulating evidence suggests that osmotic thirst is disrupted in sepsis and that this functional change might involve the OVLT[471-483] (section 1.3.3.1). Because the OVLT is an important contributor to AVP secretion from MNCs[207] (Fig. 1.4.3), altered osmosensory function in this CVO may contribute to osmoregulatory dysfunction of AVP secretion that has been reported by others[480,484-488] (sections 1.3.4).

1.3.1 Rethinking sepsis as a neuroendocrine disorder

Sepsis is a complex syndrome[304] that features many centrally mediated symptoms, including the establishment of sickness behaviour[489,490] (see section 1.3.3), the generation of appropriate and inappropriate neuroendocrine responses[491-493], and alterations in autonomic output that contribute to hypotension and hypovolemia[461,494]. Although much information is available concerning the peripheral immune responses associated with sepsis[303], little is known of the changes in neuronal excitability that mediate the central manifestations of the disorder. In fact, sepsis is often accompanied by CNS dysfunction, which can be masked by the tendency of patients to be placed in an induced coma[492]. The manifestation of delirium, diminished mentation, and coma, are collectively called sepsis-associated encelopathy and are a prominent and underappreciated instance of MOD. The CNS coordinates physiological systems and the inflammatory response during pathological states predominantly through the hypothalamic-pituitary-adrenal axis[495]. Evidence of this is available in literature discussing not only the role of the CNS in mediating inflammatory and anti-inflammatory response[470,495,496], but also in the altering levels of circulating hormones. Like AVP, the dynamics of several hormones released from the pituitary or regulated by neuroendocrine peptides are drastically altered in sepsis[491]. Several authors have thus suggested that sepsis is in fact a neuroendocrine disease[495,497], yet again shifting our understanding of what sepsis *is*.

1.3.2 Integrative central neural pathways in inflammatory states

The systemic response to an infection necessitates collaboration between the immune system and the CNS. The relationship involves a coordinated behavioural, endocrine, and autonomic response triggered by circulating pro-inflammatory cytokines, including IL-1α, IL-1β, IL-6, and TNFα[470]. Peripheral cytokines can communicate with the relevant central nuclei by either activating primary afferent nerves, such as the vagal nerves[498,499], or the humoral pathway by diffusion into CVOs. Additionally, cytokines can gain access to the brain by active transport across the blood-brain-barrier[500], or relay their message by stimulating cytokine receptors such as IL-1 receptors on the endothelia of cerebral blood vessel thereby triggering the production of local cytokines and prostaglandins in the brain[501,502].

In response to humoral and afferent feedback on immune status in septic and non-septic inflammation, microglia produce local pro-inflammatory mediators, most notably IL-1β and TNFα[470]. In sepsis, primed microglia react with an exaggerated response to systemic infection by producing high amounts of IL-1β[503]. It is likely that the locations of the corresponding IL-1 receptors–are the primary sites of the central response[504]. Most of the interaction between the microvasculature and immune system during an inflammatory response takes place in postcapillary venules[505], and though they are present throughout the brain, these venules are densest in the NTS, VLM, base of the hypothalamus, and the preoptic area (including the OVLT)[506]. In one exampled study, IL-1 receptor type 1 was found at high density in the endothelial cells lining cerebral venules throughout the OVLT, the SFO, the SON, and to a lesser extent in the PVN, cortex, NTS, and VLM of rats[501]. As mentioned in section 1.1.3, receptors for cytokines IL-6, TNF-a, and the LPS receptor TLR-4 have been documented in the OVLT[111-115]. Applications of the aforementioned cytokines or LPS to OVLT microcultures isolated from rat pups evoked calcium responses predominantly in astrocytes and microglia, but also in a fraction of neurons[507]. Thus these nuclei are implicated in the central response to inflammation. The resulting efferent CNS response supports visceral function and homeostasis via activation of sickness behaviours and neuroendocrine pathways [495].

1.3.3 Sickness behavior

Sickness behaviour is an exquisite example of the symbiotic relationship of the brain and the immune system. Anyone who has ever suffered through an infection is familiar with “feeling

sick". Being sick involves universal subjective behavioural alterations including general malaise, adipsia, anorexia, inability to concentrate, memory impairment, listlessness, lethargy, fatigue, depression, altered sleep patterns, weakness, social isolation, and loss of interest in gratifying experiences[470,496,508]. Rodents will additionally adopt a hunched posture, cease to groom, and show signs of dehydration[490]. These adaptive sickness behaviours accompany the classical signs of illness (nausea and fever)[470,496], and in the case of injury or infection, the physical manifestations of inflammation (redness, warmth, pain and swelling)[509]. Sickness behaviour is a change in motivational state that allows the organism to reorganize priorities to concede resources to the energetically expensive febrile response[490,508,510] and to better cope with infection[470,496]. It is one of the earliest (non-specific) symptoms of sepsis[492]. Which neural circuit mediates which behaviour is unknown, though it is feasible that the brain areas involved in a given behavior in the healthy state also mediate the corresponding sickness behavior. For example, the OVLT (section 1.1.3) likely plays a role in mediating loss of thirst sensation during infection.

1.3.3.1 Adipsia in sepsis animal models

Adipsia is part of the bevy of sickness behaviours caused by endotoxin[482]. The first published study to investigate the link between inflammation and thirst used endotoxin (which cannot cross the blood-brain barrier) from a variety of pathogens to test drinking behaviour in mice[471]. Both mice that had *ad lib* access to drinking water and mice that were dehydrated demonstrated dose-dependent adipsia when challenged with endotoxins (via intraperitoneal injections) with subsequent recovery 1 to 4 days later[471,472]. Antecedent vaccination with heat-killed bacteria conferred resistance to endotoxin-induced adipsia. In a CLP mouse model, sepsis also induced a short-term inhibition of drinking behaviour that resolved within a few days in surviving animals[489]. Using larger doses of LPS (640 – 1280 μm/kg, intravenously), Focà and coworkers demonstrated vigorous and long-lasting anti-dipsonegic effects in 48 hour dehydrated – and therefore hypovolemically and osmotically stimulated – mice that was partially reduced by blocking prostaglandin synthesis systemically[483]. The preoptic area, but not the SFO[473], mediates the inhibited drinking behaviour of endotoxic rodents through pathways that involve prostaglandins and NO[473,477,478]. Cytokines may also play a role in sepsis-mediated adipsia. Plata-Salaman and colleagues demonstrated that intracerebroventricular infusion of TNF and IL-1β

suppressed water intake in rats, though which neural circuit was responsible for this sickness behaviour was not clear[481]. However, direct injection of LPS into the POA showed dose-dependent decreases in water intake, unlike in non-thirst brain areas such as the nucleus caudatus or superior colliculus[473,477]. Since the POA includes the OVLT, it is therefore reasonable to posit that the OVLT is involved in the altered osmotically induced drinking behaviour in sepsis.

1.3.3.2 First hypothesis

As noted above, sepsis is associated with sickness behavior, a syndrome that features an inhibition of thirst[490]. Notably, systemic infusion of bacterial endotoxin has been shown to inhibit water intake provoked by dehydration[471-476], a stimulus that promotes both hypovolemia and hyperosmolality and therefore potently stimulates thirst[18]. Although hypovolemia and hyperosmolality stimulate thirst via distinct visceral sensory systems[3], the inhibitory effect of endotoxin on dehydration-induced thirst has been shown to involve the preoptic nucleus[473,477-479], an area encompassing the primary osmoreceptor of the brain: the OVLT[96]. Furthermore, 60% of recovering patients given an osmotic challenge 10 to 20 days after onset of septic shock did not respond with an appropriate increase in thirst perception[480]. Therefore, reduced water intake during and after sepsis may specifically involve osmotic thirst and be attributable to an inhibition of OVLT neurons. Using the rat CLP model, we tested the hypothesis that sepsis impairs osmotically induced thirst and that this is due to changes in OVLT neuron properties during the early phase of sepsis (section 3.1).

1.3.4 Osmoregulation of AVP in sepsis

Understanding the mechanisms behind changes in endogenous AVP dynamics is an essential avenue of sepsis research (section 1.2.7). Non-osmotic contributions to AVP regulation in sepsis are a substantive and important field of research, however despite the fact that systemic hyperosmolality is a more potent stimulator of AVP release than baroreceptor-mediated input[15,267,511], relatively few studies have investigated the central osmoregulatory mechanisms of AVP secretion in animals[484-486,488] and septic shock patients[480,487]. One previous study found that lesioning the AV3V area in endotoxemic rats[486] reverses the increase in MNC *c-fos* expression seen in rat brains in response to systemic LPS treatment[512], and substantially blunted LPS-stimulated increase in circulating AVP[449-453]. In human studies, the osmoregulatory pathway

mediating AVP release from the neurohypophysis was shown to be disrupted in a subset of late (>72h post-onset) septic shock patients. Using an intravenous infusion of hypertonic saline, Siami and colleagues demonstrated that half of patients with AVP deficiency responded with an appropriate increase in circulating AVP ("responders") while half did not ("non-responders")[487]. This alteration persisted in recovering patients, which was associated with a dramatic decline in thirst sensation and circulating AVP concentrations during an osmotic challenge[480].

1.3.4.1 Second hypothesis

Under normal conditions, excitation of OVLT neurons during systemic hypertonicity causes an increase in thirst via projections to the prefrontal cortex[20,38] (section 1.1.3) and a parallel increase in AVP release via excitatory projections to hypothalamic MNCs[9,141,284] (section 1.1.4.1; Fig 1.4.3). Therefore, a defect in the osmotic activation of OVLT neurons, as is demonstrated in the findings addressed in section 3.1, would be expected to impair AVP release as well. However, previous work has shown that circulating levels of AVP are elevated during the early stages of sepsis in both humans[442] and rats[454,461,513]. The mechanisms by which water intake and AVP responses become dissociated during sepsis remain unknown. We hypothesized that the electrophysiological activity of SON MNCs would be decreased in our CLP rat model. Furthermore, because MNCs are osmoreceptors[9] and AVP-MNCs make up the majority of the rat SON[179], we hypothesized that sepsis impairs osmotically induced action potential discharge in the SON (section 3.2).

1.3.4.2 Third hypothesis

We show in sections 3.1 and 3.2 that the osmosensory capacities of the OVLT and SON are depressed in septic rats, but that SON neurons are more spontaneously active. An increase in SON activity could explain the high circulating AVP levels seen in the early stages of sepsis in both humans and rats, and lead to the eventual depletion of AVP stores and thus lethal AVP deficiency (section 1.2.7.1). However, we did not specifically identify the SON neurons in section 3.2 as AVP-secreting MNCs. We therefore used transgenic rats expressing an enhanced green fluorescent protein (eGFP) tag driven by the AVP promoter[514], thereby allowing us to visually identify AVP-MNCs. We hypothesized that identified AVP-secreting MNCs would be more spontaneously active in CLP rats, and sought to further characterize the physiological

parameters (i.e. serum AVP, serum osmolality) that is associated with the early phase of experimental sepsis (section 3.3).

1.4 Figures

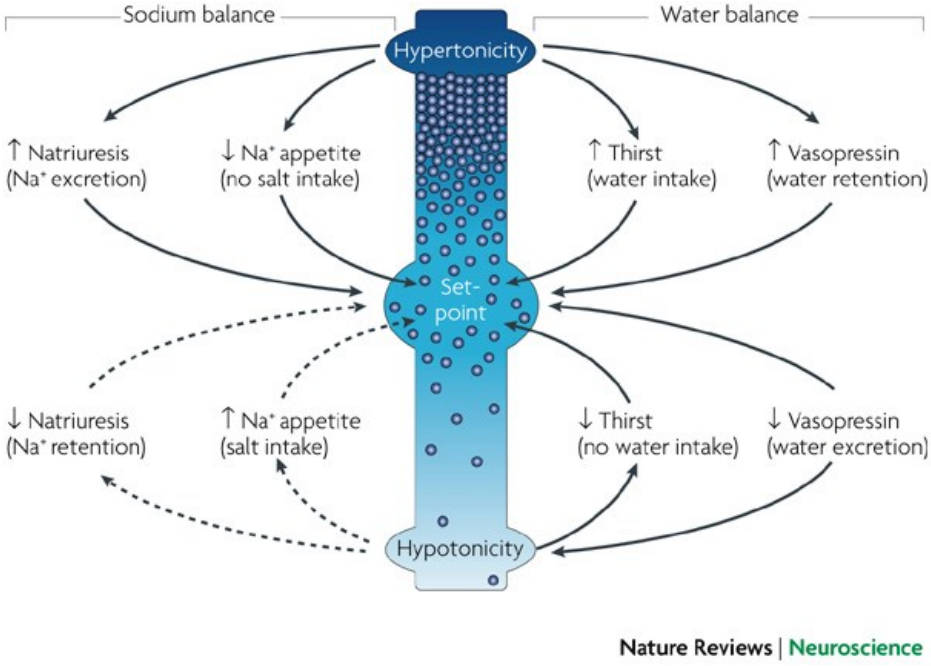

Figure 1.4.1 Systemic osmoregulatory mechanism vary depending on the polarity of ECF osmolality/tonicity. With permission from Ref. 9 © (2008) *Nature Publishing Group*.

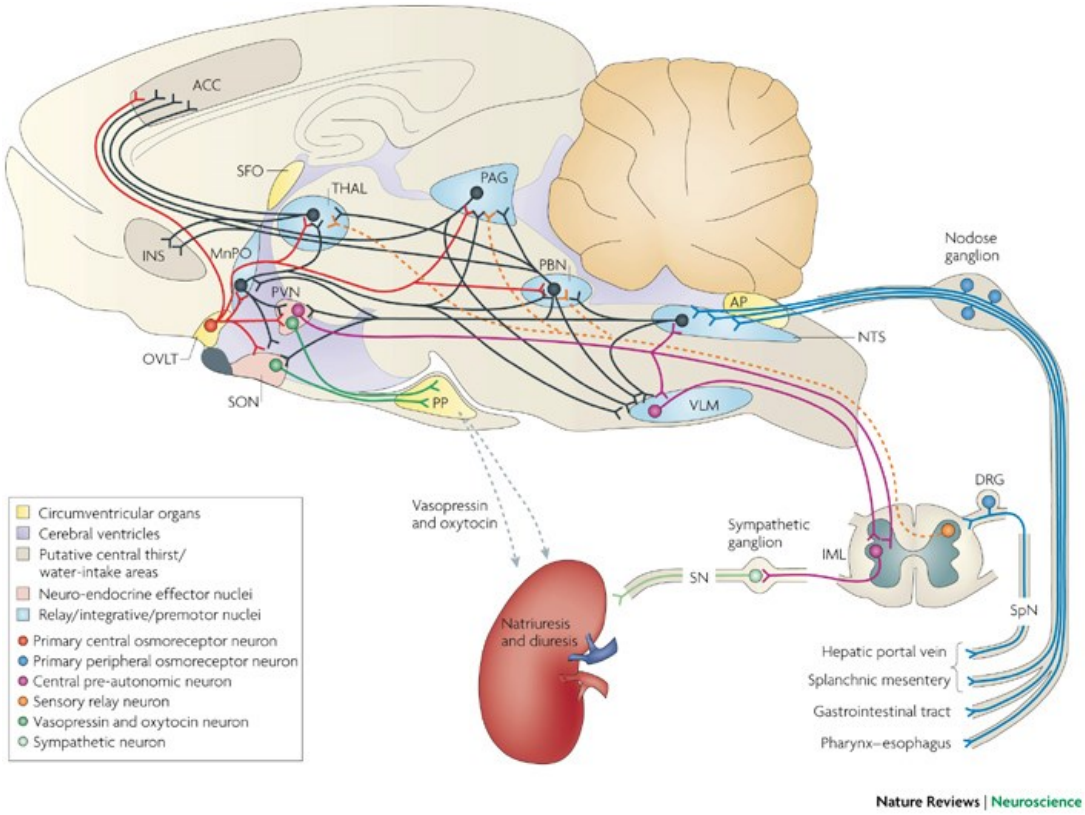

Figure 1.4.2 Sagittal view of the brain and the neural circuits implicated in systemic osmoregulation. ACC, anterior cingulate cortex; AP, area postrema; DRG, dorsal root ganglion; IML, intermediolateral nucleus; INS, insula; MnPO, median preoptic nucleus; NTS, nucleus tractus solitarius; OVLT, organum vasculosum laminae terminalis; PAG, periaqueductal grey; PBN, parabrachial nucleus; PP, posterior pituitary; PVN, paraventricular nucleus; SFO, subfornical organ; SN, sympathetic nerve; SON, supraoptic nucleus; SpN, splanchnic nerve; THAL, thalamus; VLM, ventrolateral medulla. With permission from Ref. 9 © (2008) *Nature Publishing Group*.

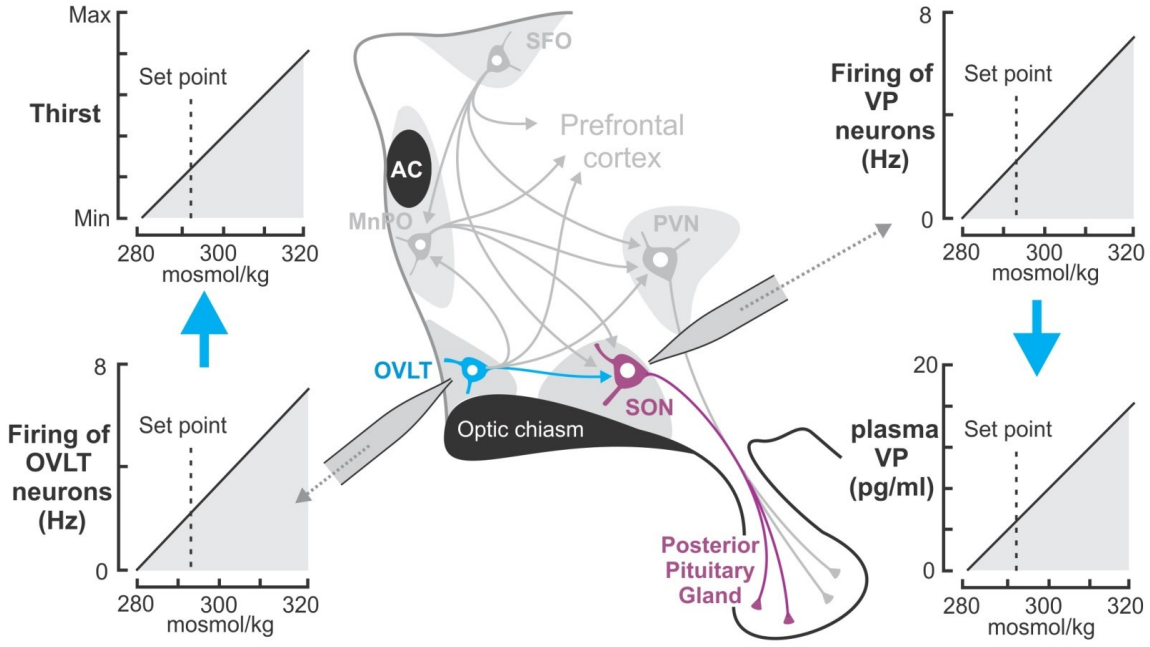

Figure 1.4.3. The common neural circuitry regulating thirst and AVP secretion. OVLT, *organum vasculosum laminae* terminalis; SON, Supraoptic nucleus; VP, vasopressin.

2.0 METHODS

All procedures described in this section were approved by the Facility Animal Care Committee of McGill University.

2.1 Animals (sections 3.1 – 3.3)

Experiments were performed on male Long–Evans rats (80 -120 g) obtained from Charles River Laboratories. Additionally, in-house bred transgenic Wistar rats (180 – 440 g) that express enhanced green fluorescent protein (eGFP) under the control of the AVP promoter[514] were used in section 3.3. Rats were housed under 12 h light/dark conditions, and food and water were provided *ad libitum*, except in experiments in which the drinking solution was replaced by 2% NaCl (section 3.1).

2.2 Cecal ligation and puncture (CLP) surgery (sections 3.1 – 3.3)

Sepsis was induced in rats using the CLP method as described by Rittirsch et al.[379] (Fig. 2.14.1A). Surgeries were performed on animals anesthetized with vaporized isoflurane (Pharmaceutical Partners of Canada) at 2 – 5% in a stream of O_2 (0.4 - 1.5 L/min). After a 3 - 4 cm midline laparotomy, the cecum was exposed and ligated below the ileocecal junction with a 3.0 silk suture (Ethicon), isolating ~65% of the cecum (Fig. 2.14.1B). It was then punctured twice with an 18 gauge needle - once at the proximal end and once at the distal end—and a small amount of fecal matter was extruded inside the peritoneal cavity. After these punctures, the cecum was repositioned into the abdominal cavity, and the overlaying muscles and skin were treated with a topical analgesia mixture (0.4% lidocaine from AlvedaPharma; 0.2% bupivacaine from Hospira) and sutured closed (with 3.0 monofilament nylon suture and 3.0 silk suture, respectively; Ethicon). The animals were then injected subcutaneously with sterile saline (0.5 ml/10 g body weight), placed in fresh cages individually, and monitored for recovery before being returned to the housing room.

Sham control rats underwent the same procedure, without ligation or puncture of the cecum. All rats were killed 18 - 24 h after the operation and necropsied to ensure the quality of the CLP or sham surgery. CLP rats that did not develop stereotypical septic symptoms according to an in-house monitoring scale based on the study by Rittirsch et al.[379], or developed secondary infections or an obstructed bowel, were not used for experiments.

2.3 Fluid intake measurements (sections 3.1, 3.3)

Drinking behavior was quantified by using drip-proof sipper sacs (Edstrom Industries). Sacs were filled with either water or 2% NaCl solution, weighed in grams, and placed into the rats' cages at the time of surgery (time point 0). After surgery, the animals were returned to the housing room, and the sipper sacs were weighed 12, 18, 20, 22, and 24 hours after surgery (section 3.1), or 6, 12, 18 and 24 hours post-surgery (section 3.3). Data from septic animals that died before 24 hours after surgery were included whereas data were excluded in cases of sipper sac leakage. Values at time points after death were capped at the last recorded measurement in section 3.1. Fluid intake was reported in milliliters at the conversion rate of 1.0151 g/ml for water and 1.0258 g/ml for 2% NaCl.

2.4 Serum osmolality measurements (sections 3.1, 3.3)

Because measurement of serum osmolality played a crucial role in the results described later on, this section is expanded to include a set of experiments conducted to verify this technique[515]. Osmolality, defined as the number of moles of solute per kilogram of solvent (mOsm/kg)[8], is a functional measurement of the osmotic pressure exerted by a solution. The formal definition of osmotic pressure is the force required to counteract the movement of solvent across a membrane that is permeable to the solvent but not to solutes[3,8]. Osmotic pressure is conceptually equivalent to the force required to move water across a membrane, as mentioned in section 1.1.1.2, but is difficult to measure. It is, however, directly proportional to the concentration of solute (but independent of the type of solute) and linearly related to the freezing point of a given solution. Therefore osmolality in lab settings is measured using freezing point osmometry.

Serum osmolality was measured from blood samples that were collected by cardiac puncture or by trunk post-decapitation from rats under 2 – 5% isoflurane anesthesia. Approximately 1 - 2 ml of blood was collected per animal in a microcentrifuge tube and placed on ice in a closed foam container prior to processing. Storage time and temperature recommendations prior to centrifugation of the whole blood sample vary and is to this day debated[516-518], however we routinely place the sample on ice in an insulated foam container with the lid closed for up to 9 hours without any adverse effects (see section 2.4.1 below).

Blood samples were then spun at 6000 x g for 5 min in a Spectrafuge 24D centrifuge

(Labnet International). The serum samples were collected into fresh microcentrifuge tubes and stored at 4°C for up to 3 weeks (*Representative Data*)[516,517]. Serum osmolality values were averaged over three to five measurements using a freezing point osmometer (model 3320; Advanced Instruments).

2.4.1 Representative Data

We investigated the effects of different coagulation times and temperatures on serum osmolality measurements determined immediately and after one day of cold storage. Seven adult male Westar rats were subjected to the cardiac puncture procedure outlined above. Collected blood was divided into six 1.5 mL microcentrifuge tubes, and left to coagulate at RT or on ice in an insulated foam container with the lid closed for various lengths of time. Each serum sample was separated as described above, and aliquoted into 2 microcentrifuge tubes: one tube was used for immediate osmolality measurement (time point "0h"), and the other was kept at 4°C for one day (time point "24h"). Reported values are means of 3 – 7 measures per sample with ± SEM. We first demonstrate that the osmometer used is calibrated and in good working order by plotting the standard curve (measured osmolality versus a control solution with a known osmolality; Fig. 2.14.2) We found that there was no appreciable impact of various coagulation times for blood samples left at room temperature (RT, ~22°C; 30 min and 60 min) or ice (~0°C; 30 min, 60 min, 90 min, and 9 hours; Fig. 2.14.3A, B). We also found that serum samples were stable for a day at 4°C (Fig. 2.14.3C).

2.5 Histological analysis and image processing (section 3.1)

For localization of the OVLT region, which lacks a blood–brain barrier, animals were anesthetized with isoflurane and injected intravenously with 0.5 ml of a 1% solution of Evans Blue (Sigma) dissolved in phosphate-buffered saline (PBS). After 30 min, the animals were killed by decapitation, and the brain was removed and fixed by immersion for 48 h in 4% paraformaldehyde dissolved in PBS. Explants were then prepared as described below and photographed in whole mount to identify the OVLT region.

For cell counts, animals were anesthetized with isoflurane and perfused (transcardiac route) with 10 ml of PBS, followed by 300 ml of 4% paraformaldehyde in PBS. Coronal sections were cut on a vibratome (50 μm thickness), and the eight consecutive sections lying rostral to

(and one including) the preoptic recess of the third ventricle were retained for staining and analysis. The sections were blocked with 10% normal goat serum (in PBS containing 0.3% Triton X-100) and incubated overnight with a chicken polyclonal anti-NeuN antibody (1:500 in PBS; EMD Millipore). Sections were then washed and incubated in goat anti-chicken secondary antibody (Alexa Fluor 568 conjugated; 1:200 in PBS; Life Technologies) and phalloidin (Alexa Fluor 647 conjugated; 1:300; Life Technologies) and mounted on coverslips using SlowFade Gold Antifade reagent (Life Technologies). z-Stack images were collected at 11 × 5 μm steps using an Olympus FV1000 confocal microscope equipped with a 20×, 0.85 numerical aperture oil-immersion lens.

Acquired z-stack images were thresholded for background and counted blind using Imaris 6.4 (Bitplane). Overlapping image stacks were stitched using XuvTools v2 (Free Software Foundation) in cases in which multiple frames were required to cover the entirety of the OVLT area. Briefly, two rectangular boxes covering a total area of 400 μm × 300 μm × 9 voxels were drawn over the OVLT region using actin-stained blood vessels and cells as location references. Spheres representing the NeuN-stained nuclei were rendered and automatically quantified by the program within each cube after thresholding for diameter (7.030 μm) and spot "quality" feature (above 220 or 230 units). Individual 3D-rendered samples were then adjusted manually and verified for adherence to the unbiased brick-counting rules. Maximum projection images for presentation were produced using National Institutes of Health ImageJ software (version 1.46r).

2.6 Solutions (sections 3.1 – 3.3)

Artificial cerebrospinal fluid (ACSF) was used as the extracellular recording solution in all electrophysiology experiments. It contained (in mM): 120 NaCl, 3 or 4 KCl, 1.48 $MgCl_2$, 25.95 $NaHCO_3$, 1 $CaCl_2$, 1.23 NaH_2PO_4, and 10 D-glucose, at pH 7.35 and 297 - 299 mOsm/kg. The internal solution used in patch pipettes contained the following (in mM): 110 K-gluconate, 1 $MgCl_2$, 10 KCl, and 10 HEPES, pH 7.4 with KOH (282 mOsm/kg). A slightly higher concentration of external K^+ was used (4 mM) for extracellular recordings from hypothalamic slices than for whole-cell recordings in slices (3 mM) to facilitate spontaneous firing. Osmolality was adjusted as required by the addition of mannitol.

2.7 Extracellular recordings in hypothalamic explants (sections 3.1, 3.2)

Animals were decapitated, and the brain was removed from the cranial vault. Hypothalamic explants were prepared as described previously using a razor blade and pinned ventral side up to the Sylgard base of a perfusion chamber in which carbogenated (95% O_2, 5% CO_2) artificial CSF (ACSF; 32°C) was delivered over the region of interest at a rate of ~1–1.5 ml/min[519]. Osmolality was adjusted as required by the addition of mannitol. Extracellular recording microelectrodes pulled on a pipette puller (P-87; Sutter Instruments) and filled with 1 M NaCl (10–20 MΩ) were advanced using an IVM micromanipulator (Scientifica) at an advance rate of 0.4 μm/s. Voltage signals recorded via an Axoclamp-2A (Molecular Devices) were filtered at 0.5–1.2 kHz and amplified (500×) before capture using Clampex 10 software (Molecular Devices). When single-unit AP firing (signal-to-noise ratio >3) was detected, electrode advance was paused, and the average rate of basal AP firing was determined over a period of 1 min. Cells that completely stopped firing soon after being encountered were counted but were assigned a value of 0 Hz for basal firing. Cells that continued to fire for >1 min were selected for additional testing to a hyperosmotic stimulus. The effects of a 5 min hyperosmotic stimulation (+15 mOsm/kg) on OVLT neurons were assessed by subtracting the rate of basal firing from the average rate of AP firing recorded during the last 60 s of the stimulus. Neurons in the SON were tested for their osmoresponsiveness using a +20 mOsm/kg stimulus. Unlike the OVLT neurons which reliably fired at a maximal rate in the last minute of the stimulus (see Figure 3.1.3.2), SON neurons displayed a delayed response (see Figure 3.2.3.4). Therefore, in order to quantify the maximal firing rate of SON neurons in response to hyperosmotic stimulation, the basal firing rate was measured 30 s before the stimulus and then subtracted from the average firing rate observed during the 30 s interval that displayed the greatest increase in firing during the stimulus.

2.8 Whole-cell recordings in hypothalamic slices (sections 3.1, 3.2)

Angled hypothalamic slices were obtained as described previously[284,519,520]. Briefly, brains were placed in ice-cold (0 - 4°C) carbogenated ACSF. The dorsal surface of the brain was glued to an angled (35°) mounting block, and a single 400 μm slice just caudal to the optic chiasma was cut on a vibratome (VT1200; Leica), transferred to a recording chamber, and perfused at ~2 ml/min with carbogenated ACSF at 30 - 32°C.

Patch pipettes (3 - 5 MΩ) made using a P-87 puller (Sutter Instruments) were filled with internal solution. Series resistance was 5 - 15 MΩ. Whole-cell current and voltage recorded using an Axopatch-1D amplifier (Molecular Devices) was digitized using Clampex and analyzed using Clampfit 10 software (Molecular Devices). Resting membrane potential (RMP) was defined as the membrane voltage observed when zero current was being injected. AP threshold was defined arbitrarily as the voltage from which the rate of rise exceeded 50 V/s during the upstroke of the AP. Rheobase was defined as the steady-state voltage from which APs were observed to occur in response to slow current injection. Conductance was determined as the slope of a line fitted by linear regression to the current–voltage (*I–V*) relation in the voltage range below AP threshold (i.e., −70 to −50 mV for OVLT neurons and −60 to −40 mV for SON neurons). For SON neurons, a subset of the data was obtained from current-clamp recordings in which conductance was determined to be the inverse of input resistance ($\Delta I/\Delta V$) between −60 and −40 mV.

2.9 Cell-attached recordings in hypothalamic slices (section 3.3)

Cell-attached recordings of action potentials were performed in angled slices prepared from AVP-eGFP Wistar rats as described in section 2.8. MNCs in the SON were visualized using fluorescence and differential interference contrast microscopy using a BX-51 microscope equipped with a U-MNB2 mirror unit (Olympus Co.) and a 60x water immersion objective. Cells were approached with a glass pipette (6 – 12Ω) pulled on a P-87 pipette puller (Sutter Instruments) containing ACSF solution, and gently patched. Changes in voltage were captured from current (*I*)-clamped (I = 0) MNCs using an Axopatch-1D amplifier (Molecular Devices), and filtered (0.7 – 1.7 kHz), amplified (x 1) and digitized using Clampex 10 (Molecular Devices). MNCs that elicited single-unit action potentials (signal to noise ratio > 3) upon contact were considered spontaneously active if they fired for more than 20 sec. Cells that ceased to fire shortly after contact (< 20 s) were designated as non-spontaneously active neurons. MNCs that continued to fire for >1 min were selected for firing frequency (Hz) analysis, calculated as the average firing rate over an 80 sec period. Spontaneously active MNCs that did not fire for at least 1 min are excluded from frequency analysis. All recordings were analyzed using Clampfit 10 software (Molecular Devices).

2.10 AVP measurements (section 3.3)

Blood samples were collected by cardiac puncture as described in Stare and Bourque (2016)[515]. Rats were deeply anesthetized with 2 - 5% isoflurane at 0.4 – 1.5 L/min of O_2, and injected intravenously with 0.5 mL / 0.9% saline. Body temperature was monitored using a probe thermometer (VWR) and adjusted to 36 – 37°C using a heat lamp (Physitemp). Approximately 3 – 5 mL of blood was acquired and separated into two microcentrifuge tubes. One tube was placed on ice for 30 to 90 min to be used for serum osmolality measurements described above (section 2.4), and the other was placed in a cooling chamber (-20°C; Diversified Biotech) for 30 min. The latter samples were then centrifuged at 6000 g for 5 min. The resultant serum samples were then stored in fresh microcentrifuge tubes at -80°C to be used for AVP analysis.

Serum AVP was isolated and measured using the chromatography and radioimmunoassay techniques described previously[521-523]. Briefly, acidified serum (0.5 mL 1N HCl / 1 mL serum) was centrifuged for 4 min at 6000 g. The resulting supernatant was then loaded into to a C-18 chromatography column (Sep Pak, Fisher), which had been conditioned with 4 mL methanol and washed with 10 mL ddH_2O. The column was then washed by gravity-flow with 10 mL 4% acetic acid, followed by 4 mL elution solution (3 parts acetonitrile with 1 part 4% acetic acid). The AVP-containing eluates were dried (Speed Vac, Savant Instruments) and added to radioimmunoassay buffer containing 0.05 M phospho-buffered NaCl with 0.1% gelatin (ph 7.0), iodinated AVP (antigen), and rabbit polyclonal AVP antibody (code: 728-4), prior to incorporation in the assay[521,523]. Recovery efficiency (<8%) was determined as described previously[521,522].

2.11 Hematocrit (section 3.3)

Rats were deeply anaesthetized with 5% isoflurane with 0.4 – 1.5 L/min of O_2, then decapitated. Approximately 1 - 2 mL of trunk blood was collected in chilled microcentrifuge tubes, from which 2 – 4 aliquots were drawn into capillary tubes. The capillary tubes were then spun at 7500 g for 9 min in a centrifuge outfitted with a hematocrit rotor (LW Scientific). The hematocrit (% packed cell volume) was determined using a microhematocrit reader (LW Scientific). Reported values are averages of two to four measurements.

2.12 Urine osmolality measurements (section 3.3)

The bladder was exposed during the necropsy of the same rats described in the section above (section 2.11). Urine was collected into a 1 mL syringe by puncturing the bladder with a 25 G x ¾ in. needle, and placed on ice for 30 min to 9 hours prior to processing. Urine samples were then transferred into 1.5 mL microcentrifuge tubes, and centrifuged for 5 min at 6000 x g. The supernatant was collected into a fresh tube, and a small aliquot (8 – 80 μL) was diluted 1 part urine to 2 – 12 parts ddH_2O to bring up the volume and to ensure that the values fell within the linear range of the osmometer. Reported values are averages of three to seven replicates and corrected for dilution. Urine osmolalities are reported for CLP rats with hypo-osmotic serum. We defined hypo-osmotic as having a serum osmolality less than 3 times the standard deviation of the mean serum osmolality of sham rats (< 289.2 mOsm/kg).

2.13 Statistics (section 3.1 – 3.3)

All values are reported as mean ± SEM. Groups were compared for differences using SigmaPlot 12 (Systat Software; sections 3.1 and 3.2) or Prism 6 (GraphPad Software; section 3.3) by applying, as appropriate, Student's *t* test, paired *t* test, one-way ANOVA, or two-way ANOVA. When significant differences were reported by ANOVA ($p < 0.05$), *post hoc* Tukey's or Holm–Sidak tests were applied to identify the groups that differed and to compute *F* and *p* values. Proportions of responsive cells in sham and CLP animals were compared using the χ^2 test with Yate's correction performed with SigmaPlot 12 (sections 3.1 and 3.2), or Prism 6 (section 3.3). Slopes of *I–V* relations used to measure membrane conductance were obtained by linear regression, and were compared using Prism 5 (sections 3.1 and 3.2). Water intake for the first 18 h post-surgery was compared by calculating the slopes from linear regression analysis. Differences between values were considered significant at $p < 0.05$.

2.14 Figures

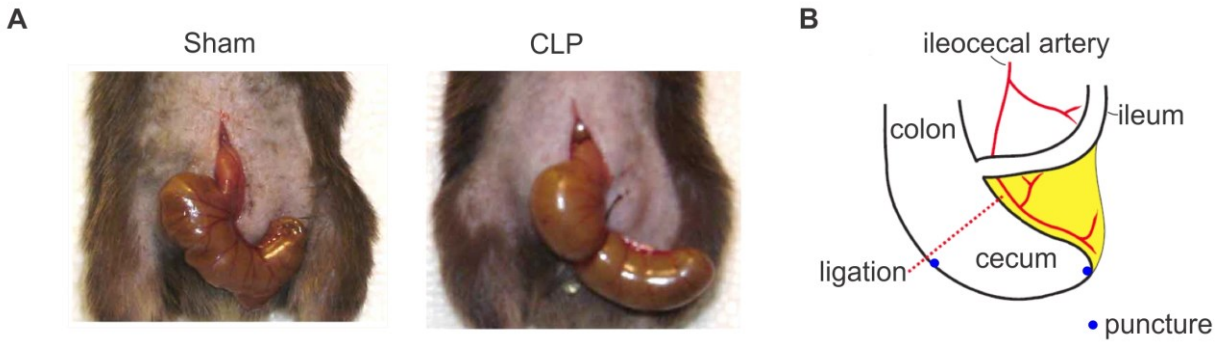

Figure 2.14.1 The CLP model used to induce sepsis in rats. **A**, Sample photos of the sham laparotomy (left) and the CLP ligation (right) in mice. **B**, Location of ligation and two punctures. This figure is modified and reprinted by permission from Ref. 379 © (2009) *Nature Publishing Group.*

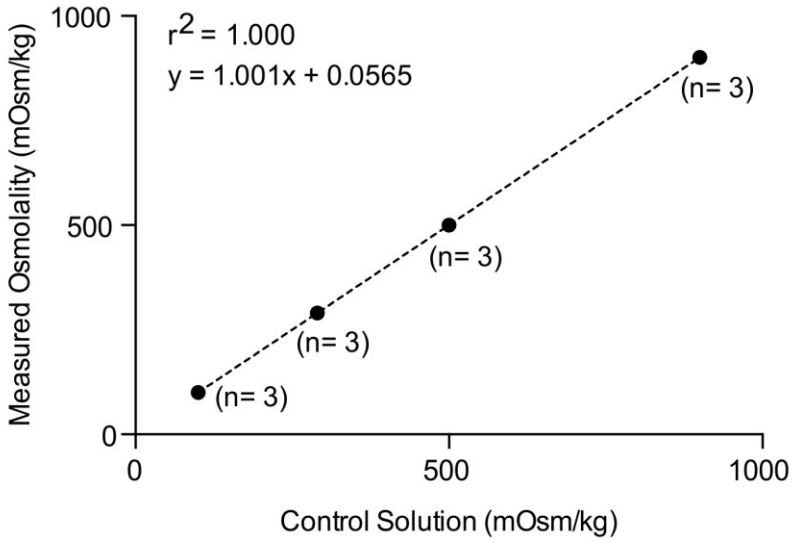

Figure 2.14.2 Standard curve used to test the calibration and performance of the osmometer used in this study. Dots represent the mean.

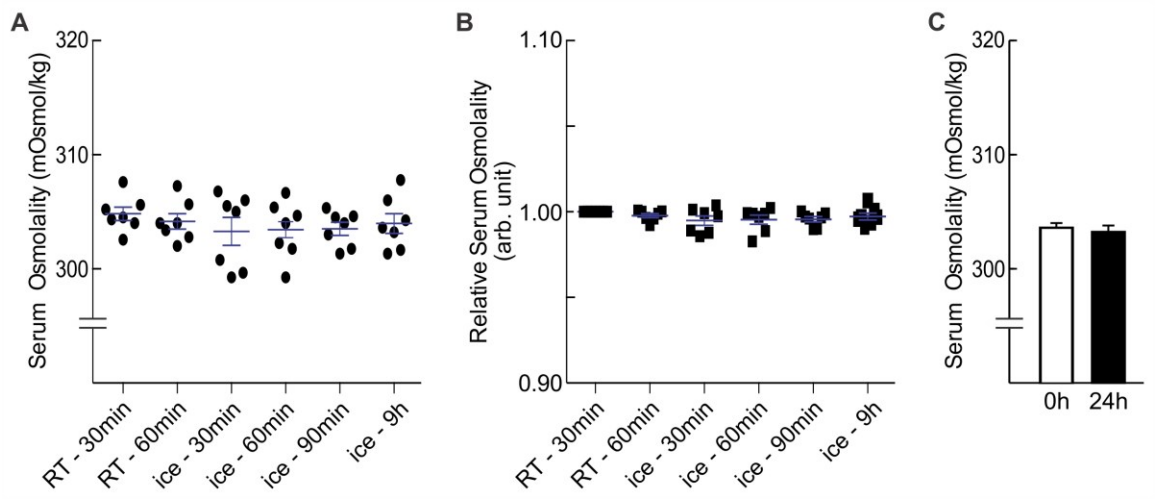

Figure 2.14.3 **A,** Serum osmolality values of blood samples (n = 7 each) allowed to coagulate for 30 min to 9 hours at RT (~22°C) or on ice (~0°C). There was no statistical difference between categories (One Way ANOVA, $F_{(5,\ 36)}$ = 0.472, p = 0.795). **B,** Osmolality values normalized to RT – 30min. **C,** Effect of one day of cold storage (4°C) on serum osmolality values. There was no statistical difference (paired *t*-test, p = 0.241) between samples that were measured immediately and samples that were stored at 4°C for one day (0h, 303.72 ± 0.44 mOsm/kg, *n* = 26; 24h, 303.146 ± 0.60 mOsm/kg, *n* = 26).

3.0 RESULTS

3.1 Effects of sepsis on OVLT osmosensory neurons mediating thirst

Osmoregulation is one of the key homeostatic systems perturbed during sepsis[471-474,477-480,483,485,487,489,524-527]. In healthy subjects, systemic hypertonicity normally excites osmoreceptor neurons in the OVLT, which then stimulate neural pathways to induce thirst sensation and AVP secretion from MNCs[20,38]. Studies have shown that osmotically-stimulated thirst is depressed in sepsis patients and animal models, and that this impairment may be due to a deficit in osmoreceptors of the OVLT[473,477-480]. We therefore measured osmotically-stimulated drinking behaviour in CLP rats, and the effects of direct osmotic stimulation of OVLT neurons *in vitro*. We further investigated the electrophysiological properties of OVLT neurons.

3.1.1 Results

3.1.1.1 Acute sepsis inhibits osmotic thirst

To examine the effects of peritoneal sepsis on thirst behavior, rats were subjected to CLP or sham surgery, returned to their cages, and provided *ad libitum* access to drinking fluid consisting of either water or 2% NaCl. Ingestion of 2% NaCl for a period of 24 h causes a rise in serum osmolality without affecting hematocrit (i.e., blood volume[528]), thus providing an effective procedure to stimulate central osmoreceptors (OVLT neurons). Basal serum osmolality measured 18–24 h after surgery was not significantly different when rats were given *ad libitum* access to water (sham, 295.9 ± 1.6 mOsm/kg, n = 7; CLP, 295.0 ± 2.2 mOsm/kg, n = 4; two-way ANOVA, Holm–Sidak test, p = 0.756; Fig. 3.1.3.1A). However, serum osmolality increased significantly in rats given access to 2% NaCl drinking solution ($F_{(1,19)}$ = 14.475, p = 0.001), and there was no statistical difference between the mean serum osmolality reached at end point for both types of surgery (sham, 302.0 ± 1.6 mOsm/kg, n = 7; CLP, 303.0 ± 1.9 mOsm/kg, n = 5; p = 0.698; Fig. 3.1.3.1A). Despite the increase in serum osmolality experienced by the CLP rats receiving 2% NaCl, cumulative fluid intake in this group was significantly lower than in shams (end values: sham, 16.4 ± 1.8 ml, n = 6; CLP, 4.2 ± 0.58 ml, n = 4; p = 0.00082; Fig. 3.1.3.1B).

3.1.1.2 Acute sepsis impairs osmotic detection in OVLT neurons

The results presented above indicate that osmotically induced thirst is impaired during

sepsis. Therefore, we examined the effects of CLP on the responsiveness of OVLT neurons to hyperosmotic stimulation in superfused hypothalamic explants. Increasing the osmolality of the ACSF by addition of 15 mM mannitol caused a reversible excitation of OVLT neurons in explants prepared from either unoperated (0.74 ± 0.19 Hz, n = 24 cells; p = 0.000809, paired t test) or sham (0.51 ± 0.18 Hz, n = 13 cells; p = 0.0154, paired t test) animals but not those from rats having undergone the CLP procedure (0.10 ± 0.05 Hz, n = 16 cells; p = 0.0719, paired t test; Fig. 3.1.3.2A–D). Because no significant difference was observed in the responsiveness of unoperated and sham animals (p = 0.611, t test), the datasets were pooled into a single "control" group. As shown in Figure 3.1.3.2D, the reduced osmoresponsiveness of OVLT neurons observed in CLP rats compared with controls was statistically significant both when all of the neurons tested were considered together (control, 0.66 ± 0.12 Hz vs CLP, 0.10 ± 0.18 Hz; p = 0.01) and when only OVLT neurons deemed osmoresponsive (i.e., those showing increases in firing >15%) were compared (control, 1.03 ± 0.14 Hz vs CLP, 0.22 ± 0.24 Hz; p = 0.004; $F_{(1,83)}$ = 15.598, two-way ANOVA and Holm–Sidak *post hoc* test). Moreover, there was a statistically significant reduction in the proportion of osmotically responsive neurons in the CLP group when compared with controls (controls, 67.6%; CLP, 37.5%; p = 0.04, χ^2 test; Fig. 3.1.3.2E).

3.1.1.3 Acute sepsis silences a subset of OVLT neurons

We next examined whether sepsis affected the overall density of spontaneously active neurons that could be detected in the OVLT of sham and CLP animals under isotonic conditions. To this end, a microelectrode was placed on the ventral surface of hypothalamic explants at one of seven predefined positions mapped over the core region of the OVLT (Fig. 3.1.3.3A). The electrode was then advanced from the surface of the tissue to a final depth of 300 μm, and the total number of spontaneously active neurons encountered along the track was registered for each position. As illustrated in Figure 3.1.3.3B, the density of spontaneously active neurons observed in CLP rats (0.75 ± 0.11 neurons/track; n = 56 tracks) was significantly lower than in shams (1.50 ± 0.23 neurons/track; n = 28 tracks; p = 0.00112, t test). Although the average basal firing rate of OVLT neurons recorded in CLP explants was slightly lower than that observed in shams, this effect was not statistically significant (sham, 2.8 ± 0.34 Hz, n = 54; CLP, 1.94 ± 0.28 Hz, n = 55; p = 0.0586; Fig. 3.1.3.3C).

Previous work has shown that sepsis induced by the CLP procedure can induce signs of

apoptosis in the MnPO[529], a structure that lies immediately dorsal to the OVLT. Therefore, we examined whether the reduced density of active neurons in the OVLT of septic rats might be attributable to neuronal loss. To test this hypothesis, we compared the density of OVLT cells stained with the neuronal marker NeuN in tissue sections (sham, n = 26 sections from 3 rats; CLP, n = 23 sections from 3 rats). However, as illustrated in Figure 3.1.3.3D, the average density of NeuN-stained neurons counted in the OVLT was not different between the two groups of animals (p = 0.333; Fig. 3.1.3.3E).

We next examined the membrane properties of OVLT neurons using whole-cell patch-clamp recordings in hypothalamic slices prepared from septic and sham animals. As illustrated in Figure 3.1.3.4A–C, steady-state *I–V* analysis showed that OVLT neurons from CLP animals display a significantly more hyperpolarized RMP than shams (sham, −40.1 ± 2.3 mV, n = 23; CLP, −47.8 ± 2.4 mV, n = 22; p = 0.0266, *t* test; Fig. 3.1.3.4C). Regression analysis in the linear region of the *I–V* indicated that membrane conductance was significantly lower in neurons from CLP animals compared with shams (sham, 1.299 ± 0.050 nS; CLP, 0.915 ± 0.069 nS; p = 0.0479; Fig. 3.1.3.4C).

Although the more negative resting potential of OVLT neurons in CLP rats could explain why fewer neurons display spontaneous electrical activity, this effect could also be caused by a relative decrease in the intrinsic excitability of the cells. To determine whether the excitability of OVLT neurons was affected by the CLP procedure, frequency–current (*F–I*) analysis was performed under current clamp. For this procedure, the baseline voltage of every cell was first adjusted to a subthreshold value near −60 mV by continuous current injection. We then examined the effects of superimposing a series of 1 s depolarizing pulses whose amplitude increased consecutively by 5 pA (pulse rate, 0.1 Hz). As illustrated in Figure 3.1.3.5A–C, there were no significant differences in the degree of current-induced firing or AP parameters between OVLT neurons from CLP and sham animals. Notably, the maximum firing frequency (F_{MAX}) achieved in each group was not significantly different (sham, 16.4 ± 1.6 Hz, n = 25 neurons; CLP, 18.8 ± 2.3 Hz, n = 24 neurons; p = 0.387), nor were maximal AP upstroke velocity (V_{MAX}: sham, 165.6 ± 13.1 V/s, n = 22 neurons; CLP, 180.8 ± 16.1 V/s, n = 21 neurons; p = 0.465), AP threshold (sham, −27.0 ± 1.2 mV, n = 22 neurons; CLP, −26.1 ± 1.7 mV, n = 21 neurons; p = 0.650), and rheobase (sham, −38.2 ± 0.95 mV, n = 22 neurons; CLP, −40.1 ± 0.97 mV, n = 21 neurons; p = 0.171; Fig. 3.1.3.5C).

3.1.2 Discussion

The effects of sepsis on brain function have a significant influence on the quality of life of patients that survive septic shock[316-318], yet little is known about how sepsis affects the electrical properties of central neurons. Here we used a rodent CLP model that approximates human peritoneal sepsis[379,530] to investigate how hypothalamic neurons responsible for hydromineral homeostasis are affected during this condition. Our experiments specifically examined the cellular and behavioral changes associated with the acute phase of untreated sepsis, which occurs 18–24 h after surgery in rats[379,454,461].

Previous studies showed that systemic infusion of endotoxins can inhibit thirst induced by water deprivation through an effect involving the preoptic area[473,474,526]. Because this region encompasses the central osmoreceptor (OVLT), these observations suggested that osmotic thirst might become impaired during sepsis. Indeed, our results reveal that thirst stimulated by systemic hypertonicity is impaired during acute sepsis. In agreement with the loss of this behavioral response *in vivo*, we observed that the responsiveness of OVLT neurons to hyperosmotic stimulation *in vitro* was severely compromised. This impaired osmoresponsiveness of OVLT neurons may be a causal factor in the loss of thirst and osmoregulatory defects associated with the acute phase of sepsis.

Approximately 65% of OVLT neurons are excited by exposure to hyperosmotic fluid, and this response is mediated in part by the activation of ion channels encoded by the transient receptor potential vanilloid type 1 (*trpv1*) gene[123,133,531]. The data reported here show that the proportion of osmoresponsive neurons declines significantly during sepsis and that the excitatory effect of hyperosmotic stimulation on osmosensitive OVLT neurons is impaired dramatically in explants prepared from septic animals. Moreover, the density of OVLT neurons displaying spontaneous AP firing under resting conditions was reduced significantly in explants prepared from CLP rats. This observation is significant because the excitatory drive to downstream osmoregulatory effector neurons, such as MNCs, is likely mediated by the global activity of afferent OVLT neurons. Indeed, the increase in firing rate observed in OVLT neurons exposed to hypertonicity is relatively small (e.g., ~1 Hz for a 15 mOsm/kg stimulus;. 3.1.3.2*D*), and many of the cells in this nucleus are silent under control conditions and become electrically active when stimulated by hypertonicity[123]. Therefore, the effect of hypertonicity on the firing rate of effector neurons is likely to reflect the integrated synaptic information provided by a large pool of OVLT

neurons.

Expression of the immediate early gene *c-fos* has been shown to be increased in the OVLT after systemic injections of lipopolysaccharide[457,532-534]. Although this suggests that OVLT neurons may be excited during sudden exposure to endotoxin, these data do not provide information regarding the time course or persistence of changes in electrical activity or excitability. Our results indicate that OVLT neurons are inhibited 18–24 h after sepsis is established by CLP. Additional studies are needed to examine the effect of CLP at earlier and later time points during electrophysiological recordings from OVLT neurons.

The effects of CLP on the electrophysiological properties of OVLT neurons and osmotically induced thirst are consistent with studies showing that global lesions of the OVLT abolish osmotic thirst in rats[96,98] and other mammals[67,97]. Although the basis for the loss of osmoresponsiveness in OVLT neurons remains to be determined, possible causes include a decrease in the expression of the Trpv1 channels required for osmoreception[123] or an impairment of elements that mechanically couple osmotically induced changes in cell volume to channel activation[133,229,231,535].

Our electrophysiological recordings from OVLT neurons in hypothalamic slices provide some insight into the mechanism responsible for the reduced density of electrically active neurons observed in the OVLT of septic rats. Although the ability to fire APs and the intrinsic excitability of OVLT neurons did not seem to differ in sham and CLP-treated rats, the RMP of the cells was significantly hyperpolarized in the septic condition. In fact, many of the cells were hyperpolarized to voltages well below rheobase, explaining why an increased fraction of OVLT neurons were silent under these conditions. *I–V* analysis indicated that the hyperpolarized RMP of OVLT neurons in septic rats was associated with a decrease in slope conductance. Moreover, the reversal potential for this effect, obtained by extrapolating the linear regressions, was near −24 mV (data not shown). These observations suggest that the hyperpolarization of septic OVLT neurons is attributable to the inhibition of a tonic nonselective cation current[228,536]. The identity of the ion channel or channels modulated during sepsis, as well as the mediators of these effects, remain to be established. It is interesting to note that the inhibitory effect of endotoxin on water intake can be mimicked by application of TNFα within the preoptic area[478]. Because the OVLT is a CVO that lacks a blood–brain barrier, circulating proinflammatory cytokines would likely have ready access to these neurons.

Our data provide insights into the cellular mechanisms that underlie defects in centrally mediated osmoregulatory behaviors and humoral responses during the acute phase of sepsis in rats. Further study is required to determine if these changes are physiological (appropriate) or pathological (inappropriate). Finally, a recent study showed that the post-acute phase of septic shock in humans that survive sepsis is commonly associated with a long-lasting deficit in both osmotically evoked thirst and AVP release[480]. It will be interesting to determine whether OVLT neurons are permanently compromised after CLP, and how this may contribute to long-term changes in AVP osmoregulation.

3.1.3 Figures

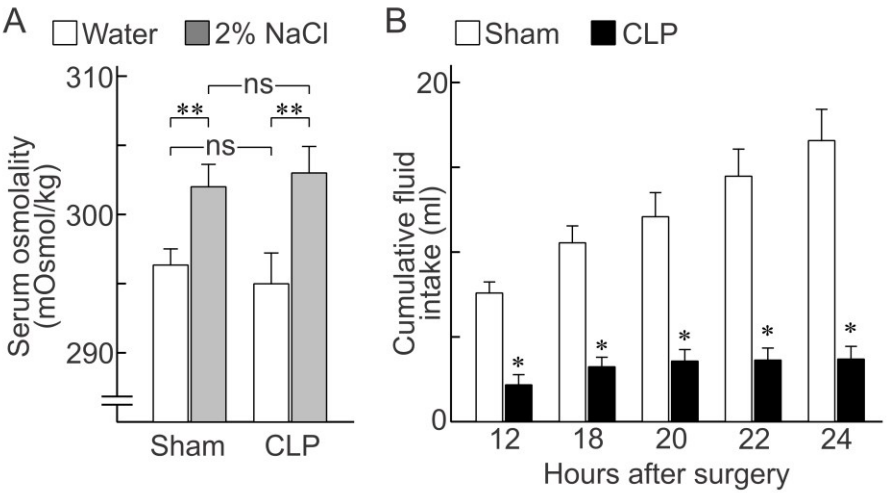

Figure 3.1.3.1 CLP-induced sepsis inhibits osmotic thirst. **A**, Bar graphs show mean ± SEM values of serum osmolality 18–24 h after sham or CLP surgery in rats provided with water (sham, n = 7 rats; CLP, n = 4 rats) or 2% NaCl as drinking fluid (sham, n = 7 rats; CLP, n = 5 rats). **B**, Bar graphs show mean ± SEM cumulative fluid intake in sham (n = 6 rats) and CLP (n = 4 rats) rats given access to 2% NaCl. *$p < 0.05$; ** $p < 0.01$; ns, not significant.

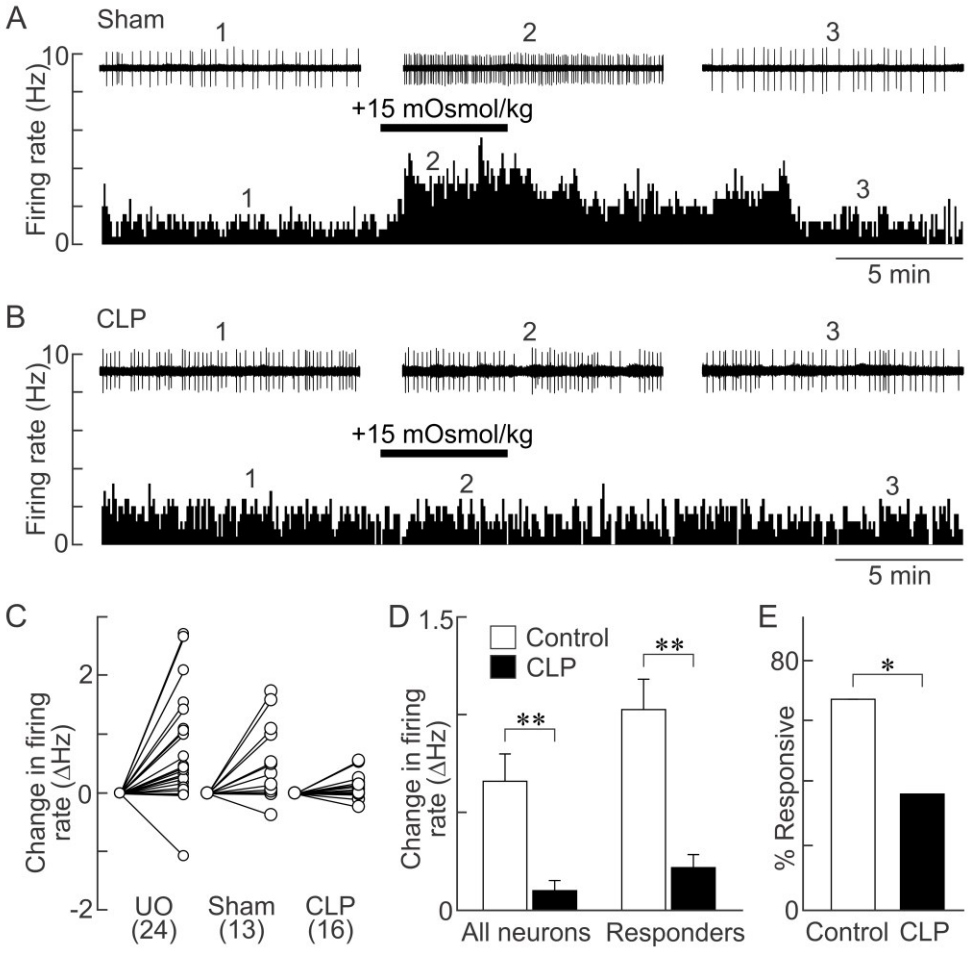

Figure 3.1.3.2 Osmosensitivity of OVLT neurons is lost in CLP rats. **A**, Ratemeter records (5 s bin width) showing the firing rate of a single OVLT neuron in an explant prepared from a sham rat. Note the reversible excitatory effect of the hyperosmotic stimulus (bar). Traces above the ratemeter plot show excerpts of the raw single-unit activity recorded at the time points indicated by the numbers. **B**, Response of an OVLT neuron recorded from a CLP rat (layout as in **A**). **C**, Two-point plots show the changes in firing rate induced by hyperosmotic stimulation in all of the OVLT neurons tested in unoperated (UO), sham and CLP rats (UO 24 neurons/9 rats; sham, 13 neurons/7 rats; CLP, 16 neurons/5 rats). **D**, Bar graphs show mean ± SEM changes in firing rate induced by hypertonicity in all of the OVLT neurons tested in control rats (UO + sham; 37 neurons/16 rats) and CLP (16 neurons/5 rats) or in the specific subset of OVLT neurons that showed at least a 15% increase in firing rate (responders: control, 25 neurons/16 rats; CLP, 6 neurons/5 rats). **E**, Bar graphs show proportions of responders in controls (25 neurons/16 rats) and CLP animals (6 neurons/5 rats). *$p < 0.05$; **$p < 0.01$.

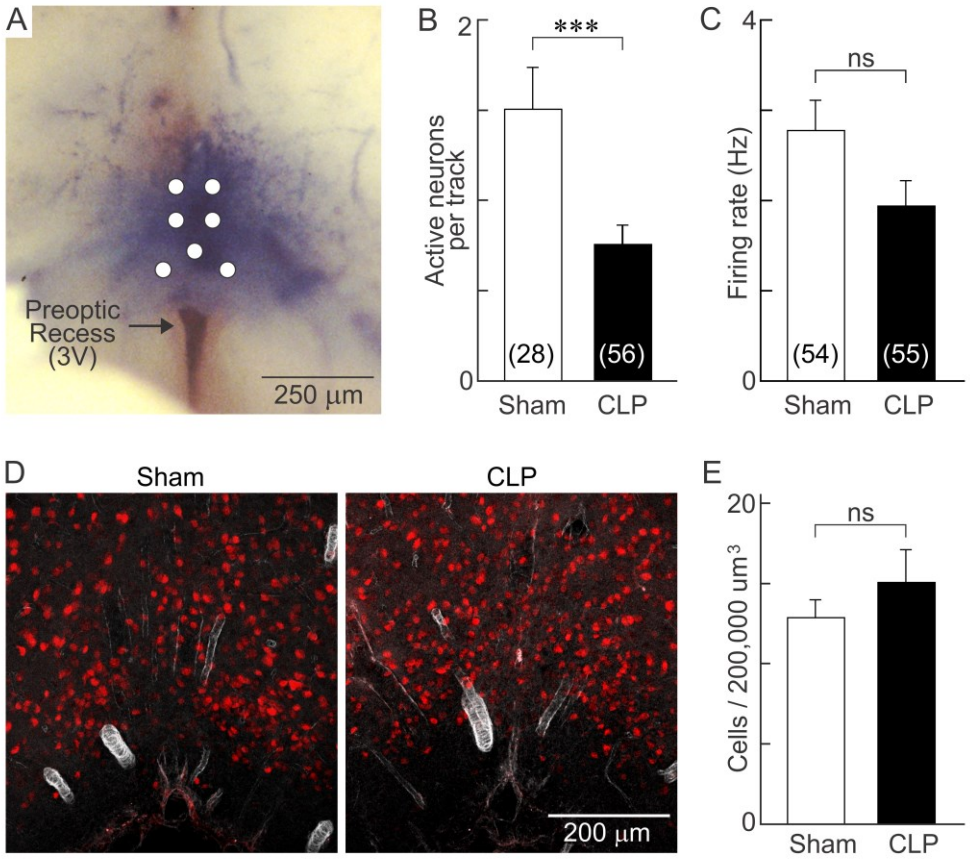

Figure 3.1.3.3 CLP reduces the density of cells showing spontaneous electrical activity but not neuron density in OVLT. **A**, Whole-mount photograph showing the ventral surface of the brain in a hypothalamic explant prepared from a rat injected intravenously with 1% Evans Blue. Top is rostral, and bottom is caudal. Note that the dye is localized to the parenchyma of the tissue that lies immediately rostral to the anterior edge of the preoptic recess of the third ventricle (3V). The white circles show the positions at which microelectrodes were inserted. **B**, Bar graphs show the mean ± SEM density of spontaneously active neurons observed in the OVLT of sham and CLP animals (n = 28 tracks/4 rats and n = 56 tracks/8 rats, respectively). **C**, Bar graphs show the mean ± SEM steady-state firing rate of spontaneously active OVLT neurons in sham (n = 54 neurons/10 rats) and CLP (n = 55 neurons/8 rats) animals. **D**, Immunofluorescence micrograph showing staining for the neuronal marker NeuN (red) in representative coronal sections through the OVLT of sham (n = 26 sections/3 rats) and CLP (n = 23 sections/3 rats) animals. **E**, Bar graphs show the mean ± SEM density of NeuN-positive cells counted per unit volume in the OVLT of sham and CLP rats. ***$p < 0.005$; ns, not significant.

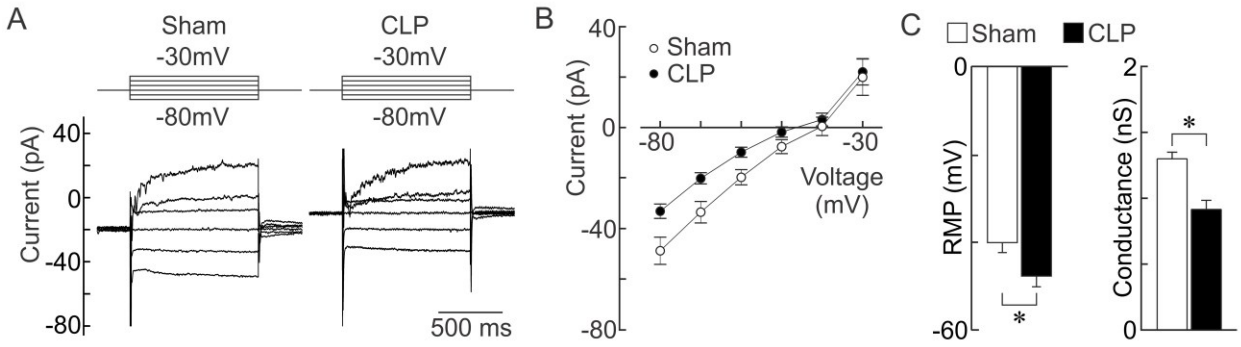

Figure 3.1.3.4 Effects of sepsis on the membrane properties of OVLT neurons. **A**, Whole-cell membrane current responses (bottom) to voltage commands (top) recorded from OVLT neurons in hypothalamic slices. Each set of traces shows the average responses generated by the entire set of sham (n = 23 neurons/3 rats) and CLP (n = 22 neurons/3 rats) neurons studied. **B**, Average I–V plots obtained from the corresponding cells. **C**, Bar graphs show mean ± SEM values of RMP (i.e., voltage at $I = 0$) and conductance measured from the same cells. *$p < 0.05$.

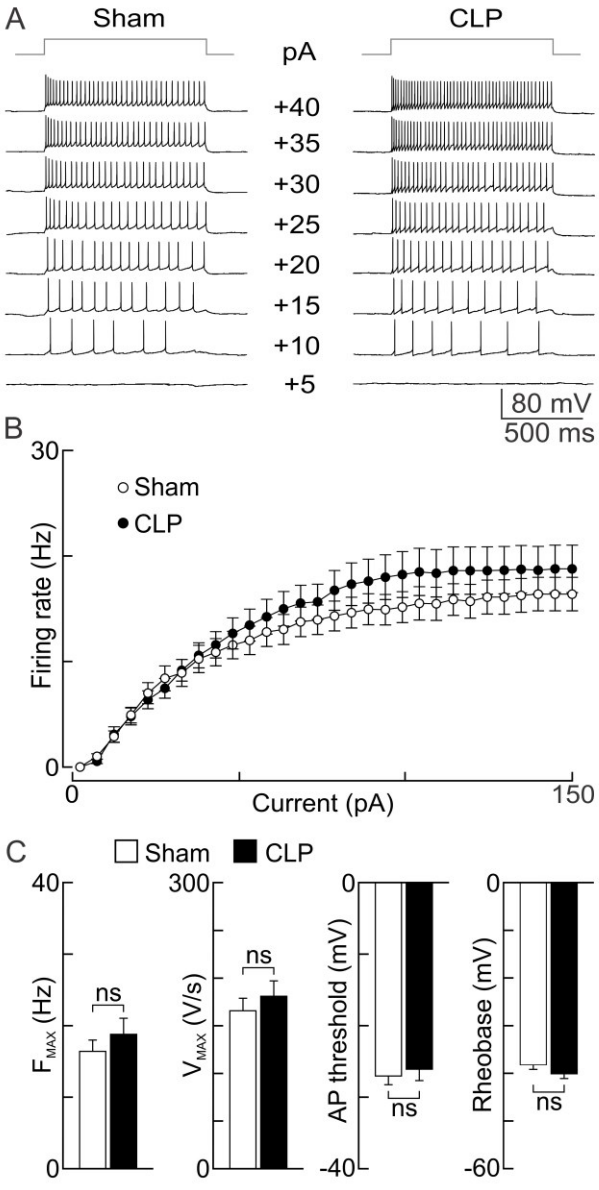

Figure 3.1.3.5 Effects of CLP on the intrinsic excitability of OVLT neurons. **A**, Traces show voltage responses (bottom) to current pulses (top; intensity indicated next to voltage sweeps) recorded in single OVLT neurons in slices from sham and CLP animals. **B**, Plots show mean ± SEM steady-state frequencies recorded during the second half of a 1 s pulse in the two groups of neurons (sham, n = 25 neurons/6 rats; CLP, n = 24 neurons/6 rats). **C**, Bar graphs show mean ± SEM values of F_{MAX} (sham, n = 25 neurons/6 rats; CLP, n = 24 neurons/6 rats), as well as V_{MAX}, AP threshold, and rheobase (sham, n = 22 neurons/3 rats; CLP, n = 21 neurons/3 rats for the latter 3 parameters) observed in the two groups of cells under current clamp. ns, not significant.

3.2 Effects of sepsis on magnocellular neurons (MNCs) of the supraoptic nucleus (SON)

In the first series of experiments, we show that the osmoresponsiveness of the OVLT is diminished. This means that the AVP-releasing MNCs in this sepsis rat model loses a major sensory input source. Loss of OVLT input would mean that MNCs would be less likely to fire action potentials, have a lower firing frequency, or both. This would translate to impaired secretion of AVP and low circulating AVP because MNC electrical activity regulates release of hormones from the neurohypophysis[169]. Yet paradoxically, AVP is high in early phase of human sepsis and rodent sepsis models[442,513,537]. We therefore examined the effects of sepsis induced by CLP on the electrophysiological properties of MNCs of the SON.

3.2.1 Results

3.2.1.1 MNCs are more spontaneously active in the septic condition

We compared the spontaneous electrical activity of MNCs in the SON of hypothalamic explants prepared from CLP and sham animals. Extracellular recordings of single-unit AP firing were obtained by advancing an electrode at a rate of 0.4 μm/s to a maximum depth of 200 μm along nine positions per rat within the core region of the SON (Fig. 3.2.3.1A). As shown in Figure 3.2.3.1B, the average density of spontaneously active neurons was significantly greater in the SON of CLP rats compared with shams (sham, 0.93 ± 0.17 neurons/track, n = 42 tracks; CLP, 1.57 ± 0.17 neurons/track, n = 54 tracks; p = 0.008, t test). The average firing rate of spontaneously active MNCs was slightly greater in CLP rats, but this effect was not statistically significant (sham, 3.7 ± 0.63 Hz, n = 26; CLP, 4.3 ± 0.7 Hz, n = 40; p = 0.551; Fig. 3.2.3.1C).

We next examined the effects of CLP on the membrane properties of SON MNCs using whole-cell voltage- and current-clamp recording in hypothalamic slices. As illustrated in Figure 3.2.3.2, A and B, MNCs from CLP animals displayed *I–V* relations that were similar to those in sham animals. Although the average RMP of MNCs was slightly more positive in CLP animals than in shams, this effect was not significant (sham, −49.9 ± 1.5 mV, n = 47 neurons; CLP, −47.2 ± 1.3 mV, n = 50 neurons; p = 0.177; Fig. 3.2.3.2C). The average conductance of the neurons was also unaffected by sepsis (sham, 2.04 ± 0.12 nS, n = 42; CLP, 2.09 ± 0.17 nS, n = 48; p = 0.831; Fig. 3.2.3.2C). *F–I* analysis performed under current clamp revealed that MNCs from CLP animals (n = 22) were significantly less responsive than shams (n = 23) in a narrow range of current injection (25–45 pA; $p < 0.05$, t test; Fig. 3.2.3.3A,B). Consistent with this finding, we

found that rheobase was significantly more positive in MNCs from septic rats (sham, −47.6 ± 0.56 mV, n = 24; CLP, −45.6 ± 0.60 mV, n = 22; p = 0.0219, t test; Fig. 3.2.3.3C). However, none of the other parameters analyzed were significantly different: F_{max} (sham, 46.8 ± 1.8 Hz, n = 22; CLP, 44.5 ± 1.8 Hz, n = 21; p = 0.372); V_{max} (sham, 193.04 ± 12.8 V/s, n = 25; CLP, 208.48 ± 12.6 V/s, n = 26; p = 0.394), and AP threshold (sham, −33.9 ± 0.82 mV, n = 25; CLP, −33.8 ± 0.77 mV, n = 26; p = 0.963; Fig. 3.2.3.3C).

3.2.1.2 Acute sepsis reduces osmoresponsiveness of MNCs

Last, we examined whether the osmoresponsiveness of MNCs was affected using extracellular recordings from SON neurons in hypothalamic explants. As illustrated in Figure 3.2.3.4A–D, MNCs recorded in explants from sham and CLP rats both displayed significant increases in firing rate in response to a hyperosmotic stimulus (sham, from 2.96 ± 0.64 to 6.77 ± 0.94 Hz, n = 19, t = 4.662, p < 0.001; CLP, from 2.15 ± 0.56 to 4.58 ± 0.82 Hz, n = 20; t = 3.092, p < 0.016; one-way repeated measures ANOVA and Holm–Sidak *post hoc* test). Although the proportion of osmoresponsive cells was not different in the two groups (sham, 94.7%; CLP, 80%; p = 0.342, χ^2 test; Fig. 3.2.3.4E), the average firing rate observed in the hyperosmotic condition was significantly lower in MNCs from CLP animals compared with shams (t = 2.697, p = 0.028, Holm–Sidak test; Fig. 3.2.3.4D).

3.2.2 Discussion

Because the acute phase of CLP is associated with an increase in circulating AVP levels, we examined the effects of CLP on the properties of hypothalamic MNCs whose electrical activity determines secretion by the neurohypophysis[169,538]. However, whole-cell recordings from MNCs in slices did not reveal any changes in membrane properties that could explain an increase in the electrical excitability of these neurons in animals subjected to CLP. In fact, MNCs were slightly less excitable because of a depolarization of rheobase. Moreover, the AP firing rate of osmotically stimulated MNCs was significantly lower in preparations from CLP animals than in shams. Although the basis for the latter changes remains to be determined, it can be concluded that changes in the intrinsic properties of MNCs do not appear to be responsible for enhanced AVP release during the acute phase of sepsis.

Interestingly, extracellular recordings from MNCs in hypothalamic explants indicated

that a significantly greater proportion of these cells are electrically active under basal conditions in CLP animals compared with shams, and that this increase was not associated with a significant difference in the proportion of phasically firing MNCs (Sham, 19.2%; CLP, 31.0%; $p = 0.582$, Fisher's exact test; data not shown). This observation suggests that substances released by astrocytes, microglia, or other neurons may enhance the proportion of electrically active neurons in this preparation. Although an increase in the density of spontaneously active MNCs could suffice to increase circulating AVP[187,188], it remains possible that CLP provokes an increase in the firing rate of these neurons *in vivo* because of the presence of additional factors. For example, AVP-releasing MNCs are intrinsically thermosensitive[539-541], and it is possible that this property mediates an additional excitatory influence during the febrile response associated with CLP-mediated sepsis[454]. MNCs are also excited by afferents that relay the hypovolemic condition[542], and previous studies have shown that hypovolemia can induce long-lasting changes in network properties that affect MNCs[543]. Thus, additional studies are required to define the mediators of CLP-induced changes in osmoregulatory neurons and networks.

It is worth mentioning that a previous study reported that sepsis reduces immunohistochemical expression of AVP in SON MNCs, whereas expression is increased in MNCs of the PVN[544]. These differences were not accompanied by changes in AVP mRNA assessed by *in situ* hybridization, suggesting that they reflect differences in posttranscriptional peptide processing. Whether CLP causes differences in AVP synthesis, degradation, transport, or local release within the SON and PVN remains to be determined.

It is presently unclear why the osmosensitivity of MNCs is preserved while that of OVLT neurons is compromised during the early stages of sepsis. However, as noted previously, the OVLT lacks a blood–brain barrier and neurons in this area may receive immediate exposure to the systemic signals that mediate the deleterious effects of CLP. It will be interesting to determine whether the properties and osmoresponsiveness of MNCs are ultimately affected to impair AVP release during the post-acute phase of sepsis.

3.2.3 Figures

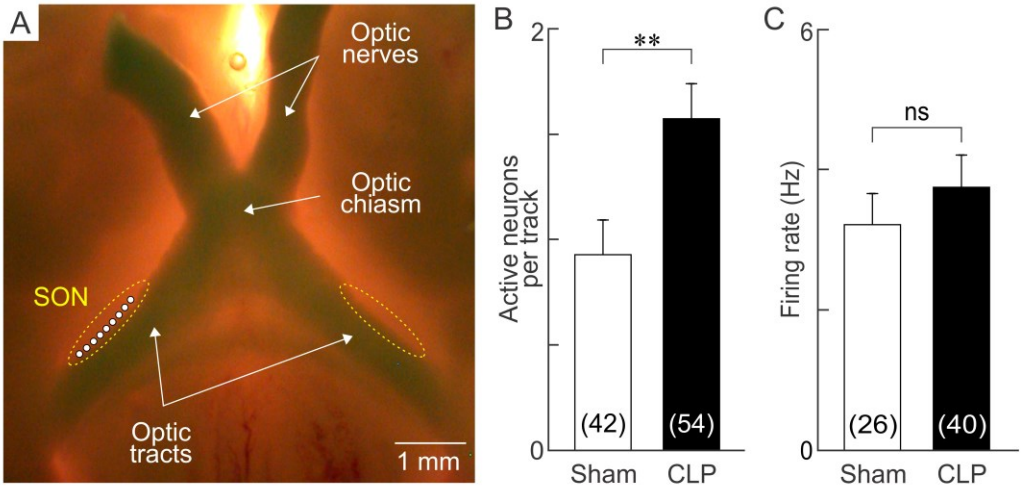

Figure 3.2.3.1 Sepsis increases spontaneous electrical activity of MNCs in the SON. **A**, Whole-mount photograph showing the ventral surface of a hypothalamic explant configured for extracellular recording from MNCs in the SON (dotted lines). Top is rostral, and bottom is caudal. All recordings were made immediately lateral to the optic tract at the positions indicated by the white circles. **B**, Bar graph shows the mean ± SEM density of spontaneously active MNCs recorded in the SON of explants prepared from sham (n = 42 tracks/5 rats) and CLP (n = 54 tracks/6 rats) animals. **C**, Bar graph shows the mean ± SEM values of firing frequency of spontaneously firing MNCs (sham, n = 26 neurons/5 rats; CLP, n = 40 neurons/6 rats). $^{**}p < 0.01$; ns, not significant.

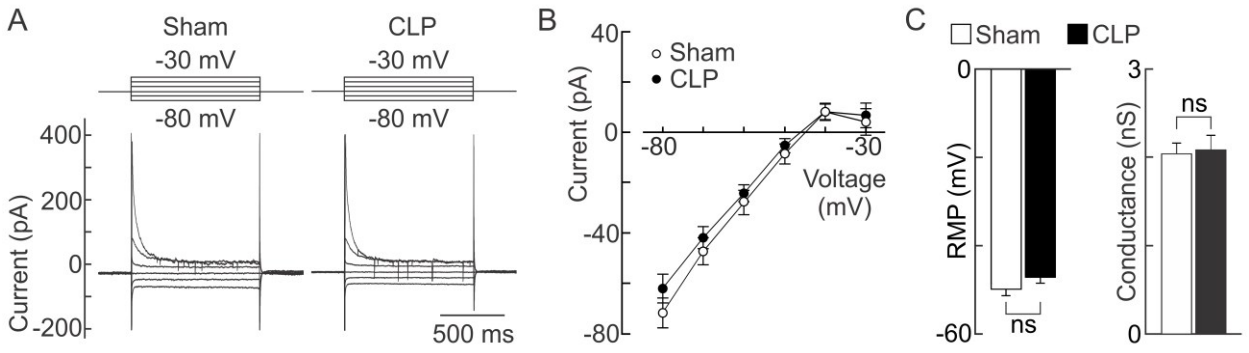

Figure 3.2.3.2 Effects of sepsis on the membrane properties of SON neurons. **A**, Whole-cell membrane current responses (bottom) to voltage commands (top) recorded from MNCs in the SON of hypothalamic slices. Each set of traces shows the average responses generated by the entire set of sham (n = 23 neurons/2 rats) and CLP (n = 28 neurons/3 rats) neurons studied. **B**, Average *I–V* plots obtained from the corresponding cells. **C**, Bar graphs show mean ± SEM values of RMP and conductance (sham, n = 47 neurons/6 rats; CLP, n = 50 neurons/6 rats) in the two groups of animals. ns, not significant.

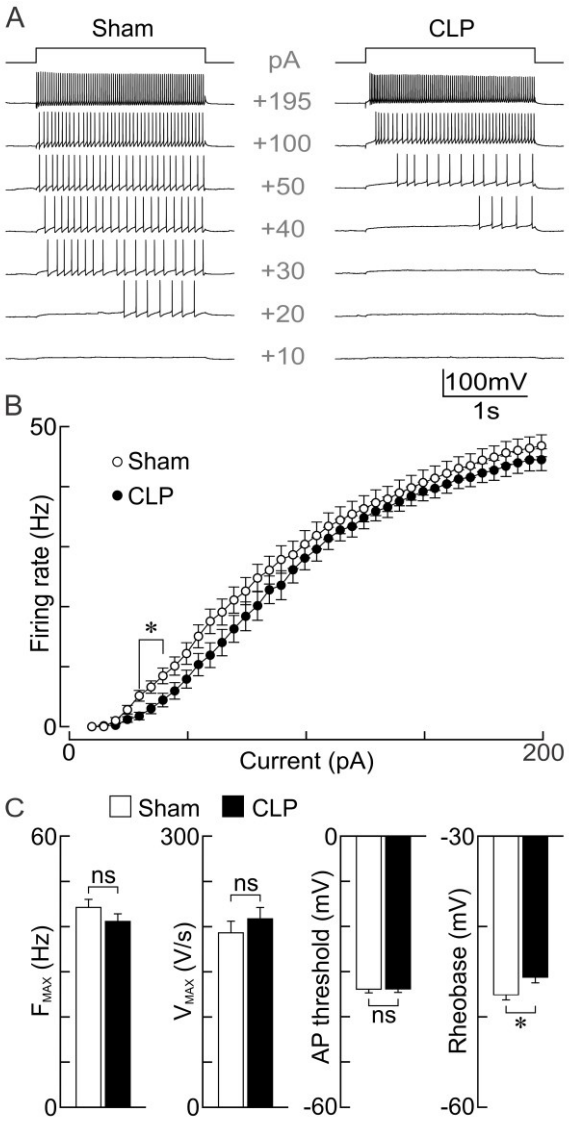

Figure 3.2.3.3 Effects of sepsis on the intrinsic excitability of MNCs. **A**, Voltage responses of single MNCs (bottom traces) to current steps (top; intensity indicated next to each voltage sweep) in the SON of slices from sham (left) and CLP (right) animals. **B**, Plots show mean ± SEM steady-state firing rate induced as a function of current in MNCs from shams (n = 22 neurons/4 rats) and CLP (n = 21 neurons/3 rats) animals. **C**, Bar graphs show the mean ± SEM values of various parameters related to intrinsic excitability in MNCs from sham and CLP rats: F_{MAX} (sham, n = 22 neurons/4 rats; CLP, n = 21 neurons/3 rats), V_{MAX} (sham, n = 25 neurons/4 rats; CLP, n = 26 neurons/4 rats), AP threshold (sham, n = 25 neurons/4 rats; CLP, n = 26 neurons/4 rats), and rheobase (sham, n = 24 neurons/4 rats; CLP, n = 22 neurons/3 rats). *p < 0.05; ns, not significant.

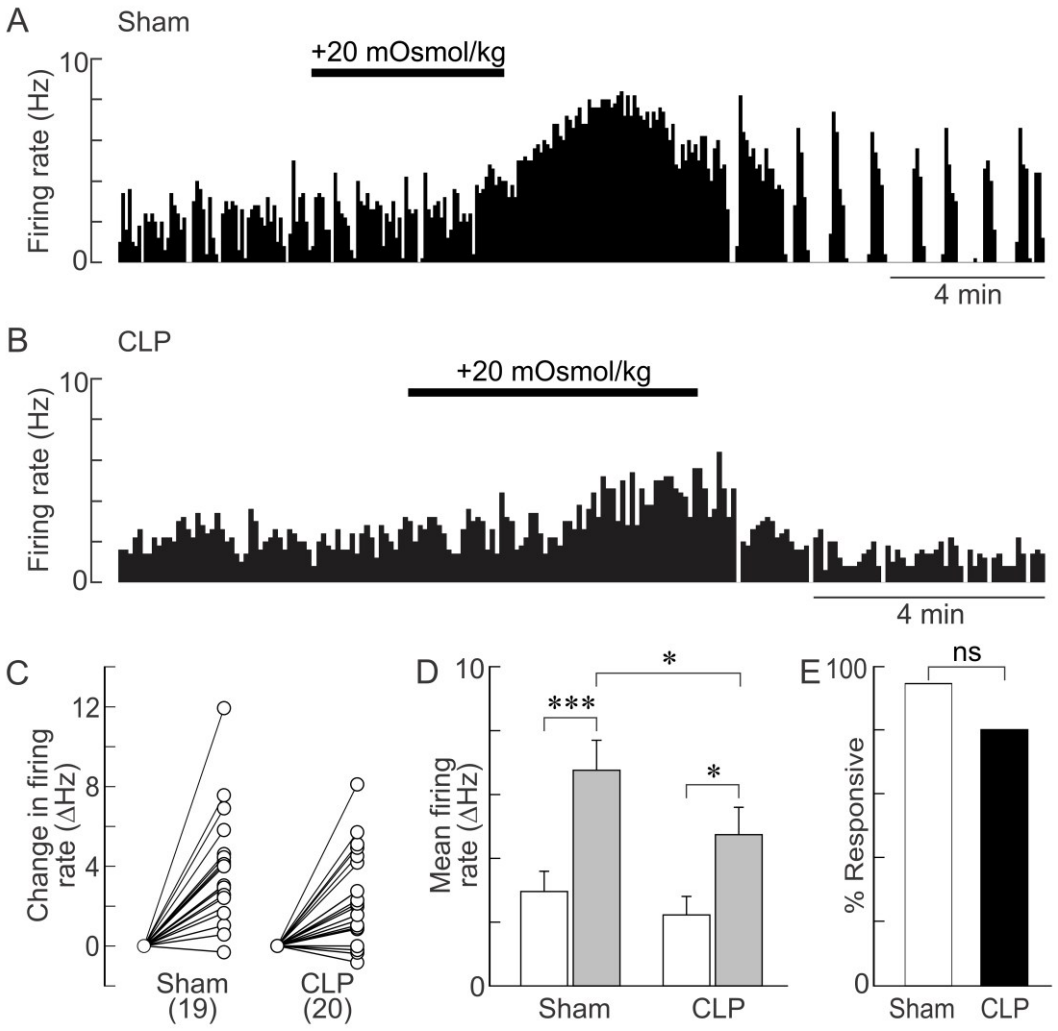

Figure 3.2.3.4 Effects of sepsis on osmoresponsiveness in MNCs. **A**, Ratemeter record showing the firing rate of a single SON neuron subjected to hyperosmotic stimulation (bar) in an explant prepared from a sham rat. **B**, Representative response of an SON neuron recorded from an explant obtained from a CLP rat. **C**, Two-point plots show the changes in firing rate induced by hyperosmotic stimulation in all of the SON neurons tested in sham (n = 19 neurons/5 rats) and CLP (n = 20 neurons/5 rats) rats. **D**, Bar graphs show mean ± SEM values of firing rate observed before (white bars) and during (gray bars) a hyperosmotic stimulus for the cells plotted in **C**. **E**, Bar graph shows percentage of responders in sham and CLP animals. *$p < 0.05$; ***$p < 0.001$; ns, not significant.

3.3 Vasopressin and water balance in septic rats

Circulating AVP levels are high during the early phase of sepsis and septic shock[513,537]. In the previous experiments (section 3.2), we observed that an increased proportion of unidentified MNCs display spontaneous electrical activity in the SON of septic rats. Recruitment of AVP-releasing MNCs could contribute to the high circulating AVP levels seen in the early stages of sepsis in both humans and rats. Therefore in this study we specifically evaluated firing behaviour of identified AVP-secreting MNCs. The primary function of AVP is to regulate water retention in the kidney in response to osmotic shifts of the extracellular fluid, thereby contributing to hydromineral homeostasis[3,166]. We therefore also measured circulating AVP levels and physiological parameters that are indicative of body fluid balance.

3.3.1 Results

3.3.1.1 Effects of sepsis on identified AVP-secreting neurons

Non-invasive cell-attached recordings were obtained from the somata of identified AVP-releasing MNCs in the SON of acute hypothalamic slices prepared from adult transgenic rats expressing eGFP driven by the AVP gene promoter[514]. Recordings of spontaneous single-unit action potential (AP) firing were obtained 18 to 24 hours after sham or CLP surgery. As illustrated in Figure 3.3.3.1A, a higher proportion of AVP-MNCs from CLP rats were spontaneously active compared to sham controls (sham 12.5%, n = 7 / 56 neurons, 4 rats; CLP 33.8%, n = 26 / 77 neurons, 5 rats: χ^2, p = 0.009). However, the mean firing frequency of spontaneously active neurons did not differ between CLP and sham MNCs (sham 2.8 ± 0.45 Hz, n = 7 neurons / 4 rats; CLP 3.5 ± 0.74 Hz, n = 23 neurons / 5 rats: Mann-Whitney U = 66.0, p = 0.492; Fig. 3.3.3.1B).

Hormone release from the neurohypophysis into the circulation is triggered directly by the electrical activity of MNCs which promotes Ca^{2+}−dependent exocytosis from neurosecretory terminals in the neurohypophysis[169,545,546]. A larger proportion of spontaneously active AVP-MNCs could therefore mediate an increase in circulating levels of AVP[547]. To examine if the increased density of electrically active AVP-secreting MNCs observed 18-24 hours post CLP correlates with an increase in hormone levels, we compared the concentration of peripheral AVP measured in CLP and sham rats. As shown in Figure 3.3.3.1C, plasma AVP was significantly

higher in CLP rats compared to controls (sham 24.05 ± 2.86 pg/mL, n = 13 rats; CLP 55.17 ± 8.01 pg/mL, n = 12 rats: t-test, p = 0.001).

3.3.1.2 Osmoregulation in the acute phase of CLP

Although a greater proportion of AVP-MNCs are spontaneously active 18-24 h after CLP, previous work has shown that MNCs are less responsive to hyperosmolality under these conditions[548]. Therefore the increased level of circulating AVP observed 18-24 hours post CLP is unlikely to be caused by systemic hyperosmolality, and renal water reabsorption promoted by elevated AVP might actually promote hypo-osmolality. Indeed, we found that the osmolality of serum collected from a large cohort of septic rats was slightly but significantly lower than shams (sham 297.6 ± 0.4 mOsm/kg, n = 47; CLP 290.9 ± 0.9 mOsm/kg, n = 48: t-test, $p < 0.0001$; Fig. 3.3.3.2A).

A decrease in serum osmolality could also be caused by an increase in water intake[549]. Although osmotically-induced saline ingestion is suppressed 18-24 hours post-CLP[548], it remains possible that basal water intake could be elevated during this period. However contrary to this hypothesis we found that CLP rats drank less than shams (two-way RM ANOVA with Sidak's *post-hoc*, $F_{(1,45)} = 147.9$, $p < 0.0001$) across all time points ($F_{(3,135)} = 249.8$, $p < 0.0001$; Fig. 3.3.3.2B). Although cumulative water consumption progressively increased over time in both groups of rats as calculated by linear regression analysis (sham 1.09 ± 0.08, CLP 0.16 ± 0.03; $F_{(1,137)} = 108.545$, $p < 0.0001$), overall intake was reduced in CLP rats (end values: sham 18.99 ± 1.15 mL, n = 24; CLP 2.996 ± 0.48 mL, n = 23). As shown in Figure 3.3.3.2C, the alteration in drinking behaviour was not associated with a change in blood hematocrit (sham 39.64 ± 1.21%, n = 7; CLP 43.45 ±1.98%, n = 11: t-test, p = 0.1749).

The lowering of serum osmolality and absence of a change in hematocrit despite reduced water intake in CLP rats suggests that water reabsorption is enhanced by the higher levels of circulating AVP. To test this hypothesis, we examined if CLP rats displaying overt serum hypo-osmolality, defined as serum osmolality that fell well outside of the normal range of sham rats, were excreting urine that was more concentrated than sham rats. For this purpose, urine osmolality values were compared between shams and CLP rats that displayed serum osmolality values <289.2 mOsm/kg (more than 3x SD below the mean serum osmolality of shams). As illustrated in Figure 3.3.3.3, urine osmolality was significantly higher in overtly hypo-osmotic

CLP rats than in shams (sham 995 ± 88 mOsm/kg, n = 18; CLP 1544 ± 245 mOsm/kg, n = 6; t-test, p = 0.0137).

3.3.2 Discussion

Previous studies on rats have shown that circulating levels of AVP are elevated during the early stages of sepsis (or after injection of endotoxin) and that this correlates with an increase in the density of Fos-labeled cells (a marker of enhanced neuronal activity) in the SON[457]. In our particular cohort, rats studied 18-24 hours after CLP also displayed a significant increase in circulating AVP levels compared to shams, indicating that this time point represents an effective model of early sepsis under our experimental conditions.

In principle, the increase in circulating AVP that we observed during early sepsis could have been caused by an increase in the firing frequency of individual AVP-MNCs[546,550], or by an increase in the fraction of the cells which displayed spontaneous action potential firing[547]. Our electrophysiological experiments, the first on identified AVP-MNCs in sepsis, showed that there is an increase in the density of spontaneously active AVP-MNCs in the SON of hypothalamic slices taken from CLP rats compared to shams. However, there was no significant difference in the average firing rate among spontaneously active neurons in the two groups. These data are comparable to our earlier findings on unidentified MNCs[551], and are consistent with studies showing an increased density of c-Fos staining in the SON of rodents during early sepsis[457,486,552].

While many factors are likely to contribute to the elevation of circulating AVP during early sepsis *in vivo*, our data suggest this effect is mediated in part by an increase in the overall proportion of electrically active AVP MNCs. The mechanisms responsible for this effect remain to be defined. However the fact that a higher density of neurons display activity *in vitro,* in preparations that have been disconnected from peripheral inputs and super fused with aseptic artificial cerebrospinal fluid, suggests that long-lasting forms of neural or glial plasticity are involved. Mediators of such plastic changes could include inflammatory signals produced by the local vasculature and associated glial cells, as well as neurotransmitters released onto AVP-MNCs by afferent axons carrying information related to the pathological state of the animal. For example, MNCs in the SON receive afferent projections from circumventricular organs that could relay information regarding humoral or immune status because they lack a blood-brain

barrier (e.g. OVLT)[553,554], or from central nuclei that signal cardiovascular stress (e.g. hypotension), such as the NTS and LPBN[542]. Interestingly, ablation of the AV3V, which includes the OVLT, attenuates endotoxin-induced AVP secretion[486]. Furthermore, in an aggressive version of the CLP rat model, Fos-like immunoreactivity increased in OVLT and SON neurons at a time point that correlated with a significant increase in plasma AVP[457]. Because the hypothalamic slices used in our experiments retain functional connectivity between the OVLT and SON[555-557], it is possible that a long-lasting enhancement of communication between these nuclei is responsible for promoting electrical activity in silent AVP-MNCs *in vitro*. Additional studies are required to address this possibility and establish the mechanisms by which CLP stimulates AVP-MNCs in the SON.

Apart from revealing a population based excitation of identified AVP-MNCs and enhanced circulating AVP levels, our experiments showed that water intake is significantly reduced 18-24 hours after CLP. This observation is consistent with our previous findings of reduced osmotic thirst in the same model[551], and also the general adipsia commonly reported in various models of endotoxemia and CLP in rodents[482,558,559]. Recent studies have shown that reducing the electrical activity of neurons in the OVLT and AV3V can suppress water intake in mice[560,561]. The membrane hyperpolarization and reduced basal electrical activity of OVLT neurons observed during early sepsis[548] may therefore underlie this effect.

The early stages of sepsis are associated with hypotension[457], therefore the increase in circulating AVP that occurs at this time may defend the body against this condition by promoting vasoconstriction and water reabsorption by the kidney at a time when water intake is suppressed. Moreover, since the osmotic control of AVP secretion is attenuated under these conditions[548], the potential inhibitory effect of serum dilution on AVP release may be sufficiently weakened to allow the maintenance of high AVP levels that are inappropriate vis-à-vis the normal osmotic regulation of this antidiuretic hormone. In a previous study[548] we found no significant differences in the average serum osmolality of a small number of CLP rats (n = 4) compared to shams. However analysis of the large dataset collected in the present study revealed a small but significant reduction of serum osmolality 18-24 hours post CLP compared to sham controls. These results are in line with the findings of others using a CLP rat model of sepsis[454,513]. Since water intake is suppressed at this time, it is likely that the hypo-osmotic state results specifically from enhanced renal water reabsorption that is stimulated by high levels of AVP. Indeed we

found that urine osmolality was significantly higher in the severely hypo-osmotic subset of CLP rats compared to shams.

Our study suggests that the early stages of sepsis in rats are associated with serum osmolality that results from AVP mediated water reabsorption. Since elevated circulating AVP levels in this case are not associated with hyperosmolality or hypovolemia (as indicated by the absence of a change in hematocrit), this condition is akin to SIADH[562,563]. SIADH is a water metabolism disorder marked by high AVP levels despite low circulating solute levels, and accompanied by increasing urine osmolality[292,300]. SIADH presents with euvolemic hyponatremia, in which AVP is released despite plasma osmolality falling well below the threshold for AVP stimulation[564]. It has been documented to co-occur with various infections, including meningitis and pneumonia[300,565-567], and has recently been linked to inflammation. Our experiments therefore suggest that a form of SIADH occurs transiently during the early stages of sepsis and that this effect arises due to an increase in the proportion of electrically active AVP releasing MNCs in the hypothalamus.

3.3.3 Figures

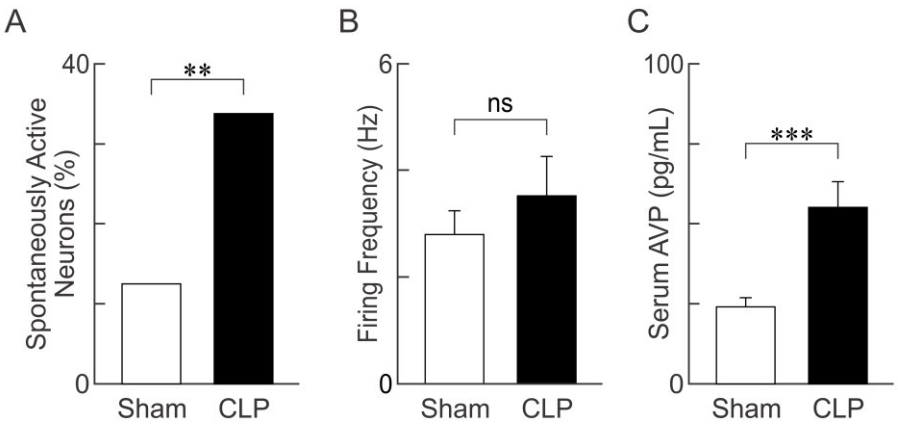

Figure 3.3.3.1 Early phase CLP causes an increase in the proportion of electrically active AVP-MNCs and circulating AVP**. A**, Bar graph shows the percentages of identified AVP-MNCs displaying spontaneous electrical activity in the SON of hypothalamic slices prepared from sham animals (open bar) and rats sacrificed 18-24 hours post CLP (black bar). **B**, Bar graph shows mean ± SEM firing rates observed in the spontaneously active AVP-MNCs recorded in the SON of hypothalamic slices prepared from sham animals (open bar) and rats sacrificed 18-24 hours post CLP (black bar). **C**, bar graphs show mean ± SEM values of plasma AVP in rats 18-24 post-CLP (black) or in shams (white). ** p <0.01; *** p <0.001; ns, not significant.

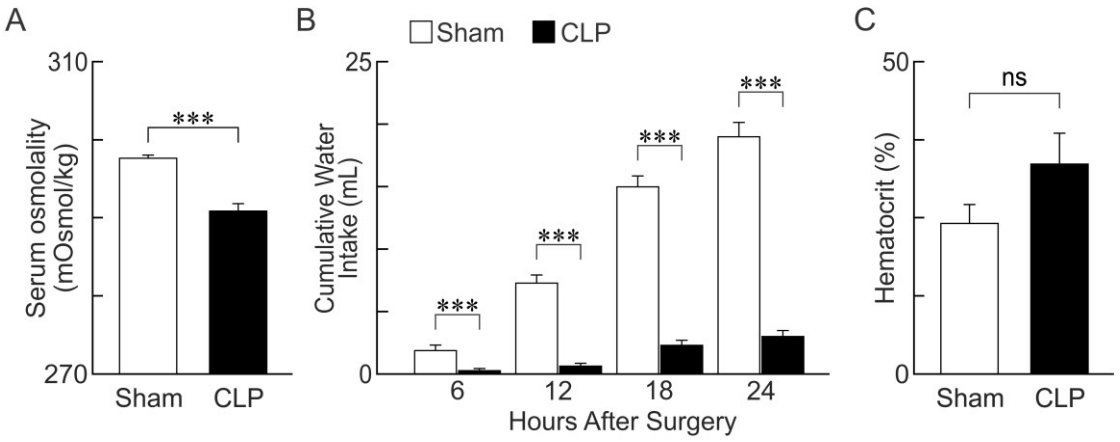

Figure 3.3.3.2 Early phase CLP decreases serum osmolality and inhibits water intake without affecting blood volume. **A**, graph shows mean ± SEM. values of serum osmolality from Sham and CLP rats collected at experimental endpoint (18 – 24 hours post surgery). **B**, bar graphs show mean ± SEM. values of cumulative water intake measured at different time points after CLP or sham surgery. **C**, bar graphs show mean ± SEM. values of hematocrit measured 18-24 hours after CLP or sham surgery. *** p <0.001; ns, not significant

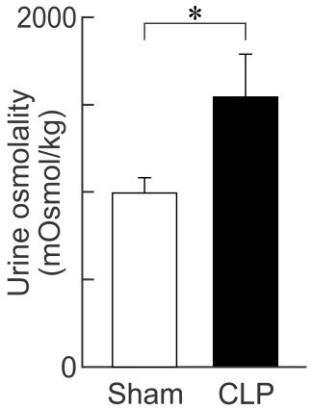

Figure 3.3.3.3 Hypo-osmolar rats in early stage CLP show increased urine osmolality compared to shams. Bar graphs show mean ± SEM. values of urine osmolality in rats sampled 18-24 hours after CLP or sham surgery. The CLP cohort includes only overtly hypo-osmolar rats. * p <0.05.

4.0 DISCUSSION

In sepsis, both human and animal model studies demonstrate an inability of subjects to launch an appropriate thirst sensation and increase in AVP in response to a hypertonic stimulation, indicating a disruption in osmoregulation. This is an important finding because (1) osmoregulation is critical to mammalian survival and disruption of this homeostatic process does not favour survivability (section 1.1.1), (2) AVP is important in both the etiology and treatment of sepsis (section 1.2.7), and (3) osmotic control of AVP is the most potent regulator of the hormone, yet the least studied in sepsis (section 1.3.4). The work described herein (section 3.0) provides the necessary description of the electrophysiological alterations of OVLT neurons and MNCs that may contribute to the disruption of hydromineral homeostasis, including the first evidence to directly implicate cerebral osmoreceptors in dysregulated osmotic thirst and AVP secretion seen in septic patients[480,487], as evidenced by the behavioural and systemic osmoregulatory changes described in during the acute, or early, phase of experimental sepsis.

4.1 Summary of findings

In section 3.1, we demonstrate that the osmotic thirst and associated osmoresponsiveness of the OVLT is ablated by CLP-induced sepsis. While some previous studies have reported dipsogenic effects of LPS[526,527], the majority of the literature, including our contribution, indicates that sepsis causes adipsia despite osmotic challenge[471,472,480,482,489] (section 1.3.3.1). We further demonstrate that unstimulated water intake is depressed but not abolished in the CLP rat model (section 3.3). Most studies have used *c-fos* to investigate the role of OVLT neurons in sepsis animal models[457,512,568-573]. We are the first to use electrophysiological techniques, which provide a high resolution of changes in neuron electrical behaviour, to investigate electrical changes in the OVLT. Individually, OVLT neurons are less spontaneously active, with a trend towards reduced firing frequency, likely caused by membrane hyperpolarization mediated by a decrease in conductance, which silences a portion of these neurons. These neurons do not differ from those of shams in other parameters, including F_{max}, V_{max}, rheobase, or AP threshold. The findings presented in section 3.1 suggest the loss of a nonselective cation current contributes to the hyperpolarization, but the findings on their own do not provide an obvious candidate for further pursuit. Further electrophysiological investigation is required to determine what ion channel or channels contribute to the silencing of OVLT neurons.

Conversely, SON MNCs are more spontaneously active in both explant and slice preparations, with no appreciable differences in firing frequency or firing pattern between CLP and control preparations (sections 3.1 and 3.2). Interestingly, MNCs from septic rats expressed a depolarized rheobase indicating more current was necessary to induce AP firing despite a statistically similar RMP compared with sham MNCs, suggesting that MNC activity was extrinsically driven in the septic condition (section 3.2). While the spontaneous firing frequency in explant preparations could be attributed to increased excitatory synaptic input from other nuclei, hypothalamic slices do not retain as an extensive synaptic network. Additionally, patch-clamp recordings in section 3.3 were conducted in identified AVP-MNCs whereas the recordings in slice preparations in section 3.2 were performed on unidentified MNCs, thus the unintentional inclusion of recordings from OT-MNCs could potentially confound the findings presented in this section. The synaptic inputs would thus need to be further investigated, as would the role of gliotransmission. Interestingly, a recent study has demonstrated that OVLT neurons express $V1_a$ receptors[574] thereby providing a potential means for circulating AVP to regulate OVLT activity and modulate its own secretion via a potential feedback loop through the OVLT-SON pathway (see section 1.1.3).

The systemic context of the electrophysiological findings summarized above provide an even more interesting picture of the osmoregulatory changes occurring in acute sepsis. Whereas we and others[461,526,551] have reported that serum osmolality is unaltered in sepsis rodent models, a more expansive investigation revealed that serum osmolality is, in fact, reduced in the CLP rat (section 3.3). This finding is surprising, but could explain in some part the lack of OVLT responsiveness to osmotic stimuli. Systemic hypo-osmolality inhibits OVLT electrical discharge (as discussed in section 1.1.3), and though the recordings presented in this thesis were conducted in iso-osmotic conditions (unless otherwise noted), hypo-osmolality in the living organism may have left an imprint that was strong enough to continually counter hyperosmotic stimulation in our artificial conditions. Hemorrhage, for example, has been demonstrated to leave a long lasting imprint on the networks that regulate rat MNCs[543]. Alternatively, the dissociation between ECF osmolality and both MNC activity and circulating AVP in the absence of blood volume change or water intake (section 3.3) could be indicative of a water-electrolyte disorder. A decrease or leftward shift in the AVP osmostat in association with increased urine osmolality is known to be pathogenic in SIADH[79] (section 1.1.6.2), and though there is little evidence of this, recent

hypothesis propose that inflammatory mediators contribute to the pathogenesis of electrolyte-water disorders[301], thus we suggest that SIADH may be secondary to sepsis in the acute phase (section 3.3.2).

Although we and others function under the assumption that a loss of osmoregulation of AVP in sepsis and other types of shock is *pathological* (inappropriate), it could also very well be *physiological* (appropriate). In the late phase of sepsis, enhanced input from any source - including from peripheral and cerebral osmoreceptors - is desirable to maintain blood pressure in light of AVP-deficiency in sepsis and there is evidence that increasing AVP is beneficial to the patient (section 1.2.7.1). A lack of input from the OVLT from this perspective is pathological, particularly if it persists after recovery from sepsis[480,487] or causes cell death[381,575]. However, increased baroreceptor input in the early phase of sepsis as a result of hypotension would be a strong stimulus for MNC activity-dependent AVP secretion (section 1.1.4.3). As sepsis progress in to the late phase, any further input could overwhelm MNCs and cause excitotoxicity[492]. Thus, reducing the contribution of osmotic stimuli would be protective but also delay the exhaustion of AVP stores (section 1.2.7.1). Further studies are necessary to determine the cellular mechanisms that result in changes in OVLT and MNC function, and what impact reversing or preventing such mechanistic changes has on the neurons and organism as a whole. Furthermore, investigating the long term effects of CLP on osmoregulatory thirst and AVP secretion would provide insight into the permanency of changes we observed in the OVLT and MNCs.

4.2 Osmosensitivity

According to the data presented in this thesis (section 3.1), NaCl-stimulated osmotic thirst is decreased in CLP rats. Changes in systemic Na^+ can be detected in parallel by both Na^{+}[576] and osmolality sensors[9], however we chose to focus on the latter. The OVLT's capacity to respond to an osmotic stimulus is abolished while that of the SON is only diminished. Speculatively, the osmoresponsiveness of SON MNCs would probably also become abolished as sepsis progresses because it is likely that the mechanisms that affected OVLT osmoresponsiveness also impact the SON. Whereas the OVLT is bombarded by the cytokine storm occurring in the circulation during sepsis by virtue of being a CVO, the MNC bodies of the SON are behind a blood-brain-barrier. The barrier can delay the effects of peripheral inflammation, however, MNC axon terminals are located in the posterior pituitary (another

CVO[40]). Therefore diffusion of circulating inflammatory mediators can modify electrical activity of MNCs either directly or indirectly through local glia. For example, IL-1β has been shown to depolarize and excite rat MNCs via an activation of an osmosensory cation current in rat hypothalamic slices[577,578]. The blood-brain-barrier will also lose integrity as sepsis progresses, allowing inflammatory mediators passage into the brain or more potently activating local microglia[492]. While there are many possible mechanisms that could contribute to changes in OVLT and MNC neurons differentially or synergistically, the remainder of this discussion will focus on a select few.

4.3 TRPV1 function and intrinsic osmosensitivity

OVLT and SON osmoresponsive neurons are intrinsic osmosensors, whose function can be altered by extrinsic factors, such as astrocyte contributions or other neural inputs, or intrinsic factors[9,210,230,234]. As discussed in sections 1.1.3.1 and 1.1.4.1.2, TRPV1 confers intrinsic osmosensitivity on both OVLT and SON osmosensory neurons. It begs the question, is *intrinsic* osmosensitivity of these neurons affected and is this mediated by a functional change in TRPV1? There are several possible mechanisms by which TRPV1 can be affected, including reduced membrane expression or by reducing the probability of channel opening. The cytoskeleton plays an important role in translating cell volume changes into the mechanical force necessary to activate TRPV1 channels, a phenomenon that has been well described[230,234]. Artificially destabilizing either actin or microtubules eliminates intrinsic osmoresponsiveness in acutely isolated MNCs[229,231]. Many inflammatory mediators, including TNFα, have been demonstrated to alter the cytoskeletal arrangements of cells[579-583], thus potentially providing a link between inflammation and presumptive loss of intrinsic osmosensation in OVLT neurons and MNCs. The next steps would then be to investigate the electrophysiological responses of acutely isolated OVLT neurons and identified AVP-MNCs from CLP rats to osmotic and mechanical stimuli, followed by confocal microscopy to investigate actin and microtubule density and organization. It is expected that stabilizing the cytoskeleton would then partially or prevent the decline of osmotic responsiveness of the tested neurons.

4.4 Nitric oxide (NO): a common denominator

Hyperproduction of NO is an important contributor to sepsis pathophysiology[402,584] (section 1.2.6.1) and another potential mechanistic contributor to altered function of osmosensory neurons in sepsis. Because NO is a gas and was historically considered to be difficult to measure directly, experimental measure and manipulation commonly used indirect means, including measuring NOS messenger RNA or protein quantity, and use of NOS inhibitors and NO precursors or donors. Interestingly, NO has been implicated in the abnormal pituitary response in sepsis[491] and body fluid homeostasis by altering drinking behaviour and circulating AVP[186,585-588].

The majority of what is known of NO function in the OVLT comes from staining experiments, particularly in the context of fever. The OVLT has been shown to express NOS[121] and LPS administration increases NOS in this CVO[568]. Whether NO is pyretic or antipyretic is controversial: some studies have reported that pretreatment with NOS inhibitors reduce NO-mediated fever, yet others report that NOS inhibitors induce or enhance fever[589-596]. Nonetheless, it remains to be determined whether NO enhances or inhibits the membrane properties and firing behaviour of the OVLT and what role it may play in osmosensation.

In the context of thirst, studies investigating the role of NO in the drinking behaviour of rats found that increasing NO activity by injecting an NO donor either systemically or directly into the preoptic area inhibited thirst induced by 24 hour water deprivation, an affect that was reversed by pre-treatment with NOS inhibitors[585,586]. However, others have reported that intracerebroventricular injections of NOS inhibitors attenuated water intake in rats stimulated by dehydration, NaCl injections, and hemorrhage[587,597,598]. Interestingly, one study found that pretreatment with a NOS inhibitor attenuated LPS-induced sickness behaviour in mice[599]. Furthermore, systemic LPS treatment at doses that did not cause adipsia in water deprived rats (as discussed in section 1.3.3.1) became potently antidipsogenic in combination with injection of an NO donor into the preoptic area[478]. The sum of the aforementioned findings suggests that the site of action of NO and the physiological context (i.e. healthy versus ill) determines, in part, the role the gaseous mediator plays in thirst.

It is now well established that NO functions as an atypical neurotransmitter and exerts a predominantly inhibitory effect on centrally-mediated sympathetic activity and neurosecretion from the SON[186]. NO is tonically produced in the SON in iso-osmotic and euvolemic rats, and

serves to inhibit AVP secretion[587,597,600], decreasing the firing rate of SON MNCs in rat hypothalamic slices[587]. Electrophysiological recordings *in vivo* and *in vitro* of identified AVP-releasing SON neurons in rat demonstrated that the inhibitory effect of NO was related to increased GABAergic pre-synaptic activity[601]. NO is an established inhibitor of MNC activity, yet paradoxically, experiments wherein endogenous NO production was blocked by intracerebroventricular injections of a NOS inhibitor decreased serum AVP in healthy rats whereas injections of a NO donor increased serum AVP[588], suggesting that NO facilitates AVP secretion. A generalist explanation for the paradoxical effects of NO on AVP secretion involves an appreciation for the fact that NO function is dependent on context. The data mentioned imply that *physiological* stimuli within moderate levels, including osmolality, blood volume, and vascular tension, remove the tonic inhibition of NO on SON neurons to facilitate AVP release and promote homeostasis but higher physiological levels inhibits AP release. For example, NO may differentially affect K^+ currents in the nerve terminals of MNCs, acting as a switch to both enhance and inhibit AVP secretion. Initially, NO can facilitate AVP release by rapidly suppressing the transient voltage-mediated outward K^+ current (a.k.a. A-current), resulting in the broadening of APs (see section 1.1.4.1.1) and increased Ca^{2+} entry[602,603]. NO then enhances the whole-terminal K^+ current by activating large-conductance Ca^{2+}-activated K^+ (BK) channel activity in a delayed manner, which affects a decrease in membrane excitability and therefore suppresses neuropeptide release. Higher degrees of stimuli are inhibitory and potentially toxic to the MNCs, and most likely fall in the pathological range. In other words, NO at basal levels has minimal effects in physiological conditions and is neuroprotective, but it is a potent inhibitor in states of stress and raised sympathetic activity, and neurotoxic at high concentrations[604,605].

In the context of sepsis, one hypothesis proposes that an initial increase in NO due to increased hypothalamic iNOS activity contributes to the increased AVP secretion during the early phase of sepsis[455,606], but that this excess of NO blunts AVP release in the late phase and contributes to AVP-deficiency (section 1.2.7.1)[453,455,457,607,608]. In support of this proposal, evidence in the CLP rat model indicated an initial increase followed by a progressive decline in *c-fos* expression in the SON during increasing severity of sepsis in the presence of high systemic NO[457]. Furthermore, *in vivo* experiments in iNOS knockout mice elicited a sustained increase in serum AVP and mean arterial blood pressure when challenged with LPS, like aligning with the early phase of sepsis[607]. Yet, increased iNOS expression was correlated with higher levels of

apoptosis in the SON and PVN of patients that died from hypotensive septic shock (2 – 9 days post-onset) compared to patients that died from other forms of shock[575]. This evidence was corroborated in a rat CLP model that demonstrated elevated cleaved caspase-3 expression in SON and PVN MNCs[381], providing support that AVP reduction in the late phase of sepsis is caused by MNC neurotoxicity. It would be interesting to determine what specific dose, if any, can inhibit osmotically-induced water intake and concomitantly facilitate AVP secretion, what dosage ranges of NO would effect a facilitative versus inhibitory effect on AVP secretion in MNCs, if the location of action (i.e. perikarya versus axon terminal) results in distinctive effects of NO, and if the origin of NO (i.e. circulatory versus cerebral, neuronal versus glial) has a differential impact on the function of osmosensory neurons (i.e. neuronal NO may cause inhibition and peripheral NO may cause facilitation). Directly measuring NO levels using the fluorescent indicator diaminofluorescein-2[609] would provide more reliable results than quantifying NO-surrogates. Interestingly, NO disrupts actin polymerization in other non-neuronal cell types[610,611] providing a link to potential disruption of intrinsic osmosensitivity as discussed in section 4.3.

4.5 Preliminary evidence of circadian effects

Altered osmoregulatory gain of AVP secretion can be part of normal, daily fluctuations of the osmostat, as occurs during sleep (section 1.1.5.1). Preliminary evidence indicates that in the early sleep phase of CLP rats, serum hypo-osmolality is associated with a trend towards decreased AVP-MNC firing frequency and an increase in spontaneous activity, in comparison to shams (Fig 6.1.1). Yet in the middle phase of the sleep cycle, AVP-MNC firing rate is enhanced while spontaneous activity remains high in CLPs compared with data from CLP rats in the early sleep phase. These findings are insufficient to reach any meaningful conclusion: increasing the sample size or better design of experimental procedures to include different time frames (i.e. early sleep phase versus late sleep phase, awake phase versus sleep phase) would provide a better experimental paradigm. However, they do point to altered osmoregulation of AVP secretion in septic rats during different sleep phases that is not seen in control rats. Inflammation is known to affect circadian rhythms, and circadian rhythms are known to regulate inflammatory processes[612,613]. For example, sleep deprivation in CLP mice increases mortality[614] and circadian variation in hormone concentrations is flattened in sepsis[491]. Direct injection of LPS or cytokines

into the SCN phase shifts the circadian rhythm, and inflammatory mediators have been demonstrated to regulate SCN firing activity[615,616]. There is thus likely a role for altered SCN activity in sepsis that is independent of cell death[617]. Further investigation would be necessary to determine the nature of neuron activity in the SCN, OVLT, and AVP-MNCs, and the effects of systemic osmoregulation. Finally, the preliminary data also represent different surgery times. If CLP is induced in the early sleep phase, serum osmolality is more hypo-osmotic and MNC firing rate is depressed in comparison to rats who underwent the CLP surgery in the middle of their sleep phase. CLP rats from the latter group had serum osmolalities statistically equivalent to that of shams, and MNC firing frequencies slightly enhanced compared to shams. If the time of sepsis induction affects AVP secretion or neuronal activity, and potentially sepsis progression and mortality rates or long-term cognitive function, this could be clinically relevant for hospital-acquired sepsis.

4.6 Conclusion

The data presented in this thesis provide insights into the cellular mechanisms that underlie defects in centrally mediated osmoregulatory behaviours and humoral responses during the acute phase of sepsis in rats. The electrophysiological properties of OVLT neurons are altered to functionally silence a subset of neurons, and reduce osmoresponsiveness to hyperosmotic stimuli. MNCs on the other hand are more functionally active, a feature that enhances circulating AVP in the early phase of sepsis, but still somewhat responsive to osmotic stimuli. The changes in neuron function described herein translate to changes indicative of systemic osmoregulation alteration. Acute sepsis causes adipsia in both osmotically stimulated and unstimulated rats, in association with high AVP and serum hypo-osmolality the early phase of the syndrome. The mechanism of dissociation of osmotic control of AVP from serum osmolality remains to be determined, but data suggests that the early phase of sepsis is transiently associated with a disorder in osmoregulation. Our findings support the shift in dogma that considers centrally-mediated alterations in homeostasis to contribute to the pathophysiology of sepsis.

5.0 REFERENCES

1 Maggio, A., Zhu, J.-K., Hasegawa, P. M. & Bressan, R. A. Osmogenetics: aristotle to arabidopsis. *The Plant Cell* **18**, 1542-1557 (2006).

2 Schopf, J. W. Microfossils of the Early Archean Apex chert: new evidence of the antiquity of life. *Science* **260**, 640-646 (1993).

3 de Souza Mecawi, A., Ruginsk, S. G., Elias, L. L. K., Varanda, W. A. & Antunes-Rodrigues, J. Neuroendocrine regulation of hydromineral homeostasis. *Comprehensive Physiology* (2015).

4 Rossier, B. C., Baker, M. E. & Studer, R. A. Epithelial sodium transport and its control by aldosterone: the story of our internal environment revisited. *Physiological reviews* **95**, 297-340 (2015).

5 Tessera, M. Life began when evolution began: A lipidic vesicle-based scenario. *Origins of Life and Evolution of Biospheres* **39**, 559-564 (2009).

6 Hammel, H. Forum on osmosis. I. Osmosis: diminished solvent activity or enhanced solvent tension? *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* **237**, R95-R107 (1979).

7 Norton, A. C. Using Osmometry.

8 Dufour, D. in *American Association for Clinical Chemistry Meeting.* (Advanced Instruments, Inc.).

9 Bourque, C. W. Central mechanisms of osmosensation and systemic osmoregulation. *Nat Rev Neurosci* **9**, 519-531 (2008).

10 Suescún-Bolívar, L. P. & Thomé, P. E. Osmosensing and osmoregulation in unicellular eukaryotes. *World Journal of Microbiology and Biotechnology* **31**, 435-443 (2015).

11 Strange, K. Cellular volume homeostasis. *Adv. Physiol. Educ.* **28**, 155-159 (2004).

12 Smith, H. W. *From fish to philosopher*. (Little, Brown, 1953).

13 Bradley, T. J. *Animal osmoregulation*. (Oxford University Press, 2009).

14 Zerbe, R. L. & Robertson, G. L. Osmoregulation of thirst and vasopressin secretion in human subjects: effect of various solutes. *Am. J. Physiol.* **244**, E607-E614 (1983).

15 Dunn, F. L., Brennan, T. J., Nelson, A. E. & Robertson, G. L. The role of blood osmolality and volume in regulating vasopressin secretion in the rat. *J. Clin. Invest.* **52**, 3212-3219 (1973).

16 Shirreffs, S. M., Merson, S. J., Fraser, S. M. & Archer, D. T. The effects of fluid restriction on hydration status and subjective feelings in man. *Br. J. Nutr.* **91**, 951-958 (2004).

17 McKinley, M. J. & Johnson, A. K. The physiological regulation of thirst and fluid intake. *Physiology* **19**, 1-6 (2004).

18 Johnson, A. K. The sensory psychobiology of thirst and salt appetite. *Med. Sci. Sports Exerc.* **39**, 1388-1400 (2007).

19 Lee, H.-J., Macbeth, A. H., Pagani, J. H. & Young, W. S. Oxytocin: the great facilitator of life. *Progress in neurobiology* **88**, 127-151 (2009).

20 McKinley, M. J., Denton, D. A., Oldfield, B. J., De Oliveira, L. B. & Mathai, M. L. Water intake and the neural correlates of the consciousness of thirst. *Semin. Nephrol.* **26**, 249-257 (2006).

21 Andersson, B. & McCann, S. A Farther Study of Polydipsia Evoked by Hypothalamic Stimulation in the Goat. *Acta Physiologica Scandinavica* **33**, 333-346 (1955).

22 Andersson, B. Polydipsia caused by intrahypothalamic injections of hypertonic NaCl-solutions. *Experientia* **8**, 157-158 (1952).
23 Montemurro, D. & Stevenson, J. The localization of hypothalamic structures in the rat influencing water consumption. *The Yale journal of biology and medicine* **28**, 396 (1955).
24 Teitelbaum, P. & Epstein, A. N. The lateral hypothalamic syndrome: recovery of feeding and drinking after lateral hypothalamic lesions. *Psychological review* **69**, 74 (1962).
25 McKinley, M. *et al.* The median preoptic nucleus: front and centre for the regulation of body fluid, sodium, temperature, sleep and cardiovascular homeostasis. *Acta Physiologica* **214**, 8-32 (2015).
26 Johnson, A. K. The periventricular anteroventral third ventricle (AV3V): its relationship with the subfornical organ and neural systems involved in maintaining body fluid homeostasis. *Brain research bulletin* **15**, 595-601 (1985).
27 Camacho, A. & Phillips, M. I. Horseradish peroxidase study in rat of the neural connections of the organum vasculosum of the lamina terminalis. *Neuroscience letters* **25**, 201-204 (1981).
28 Saper, C. B. & Levisohn, D. Afferent connections of the median preoptic nucleus in the rat: anatomical evidence for a cardiovascular integrative mechanism in the anteroventral third ventricular (AV3V) region. *Brain research* **288**, 21-31 (1983).
29 Gutman, M. B., Ciriello, J. & Mogenson, G. J. Electrophysiological identification of forebrain connections of the subfornical organ. *Brain research* **382**, 119-128 (1986).
30 Miselis, R. R., Shapiro, R. E. & Hand, P. J. Subfornical organ efferents to neural systems for control of body water. *Science* **205**, 1022-1025 (1979).
31 Zardettosmith, A. M., Thunhorst, R. L., Cicha, M. Z. & Kim Johnson, A. Afferent Signaling and Forebrain Mechanisms in the Behavioral Control of Extracellular Fluid Volumea. *Annals of the New York Academy of Sciences* **689**, 161-176 (1993).
32 Thompson, R. H. & Swanson, L. W. Structural characterization of a hypothalamic visceromotor pattern generator network. *Brain Research Reviews* **41**, 153-202 (2003).
33 Nakamura, K. & Morrison, S. F. A thermosensory pathway that controls body temperature. *Nature neuroscience* **11**, 62-71 (2008).
34 Nakamura, K. & Morrison, S. F. Preoptic mechanism for cold- defensive responses to skin cooling. *The Journal of physiology* **586**, 2611-2620 (2008).
35 Nakamura, K. & Morrison, S. F. A thermosensory pathway mediating heat-defense responses. *Proceedings of the National Academy of Sciences* **107**, 8848-8853 (2010).
36 Olivares, E. *et al.* Effect of electrolytic lesion of the dorsal raphe nucleus on water intake and sodium appetite. *Brazilian journal of medical and biological research* **36**, 1709-1716 (2003).
37 Barofsky, A.-L., Grier, H. C. & Pradhan, T. K. Evidence for regulation of water intake by median raphe serotonergic neurons. *Physiology & behavior* **24**, 951-955 (1980).
38 Hollis, J. H., McKinley, M. J., D'Souza, M., Kampe, J. & Oldfield, B. J. The trajectory of sensory pathways from the lamina terminalis to the insular and cingulate cortex: a neuroanatomical framework for the generation of thirst. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* **294**, R1390-R1401 (2008).
39 Saker, P. *et al.* Regional brain responses associated with drinking water during thirst and after its satiation. *Proceedings of the National Academy of Sciences* **111**, 5379-5384 (2014).

40 McKinley, M. J. *The sensory circumventricular organs of the mammalian brain: subfornical organ, OVLT and area postrema*. Vol. 172 (Springer Science & Business Media, 2003).
41 Miselis, R. R. The efferent projections of the subfornical organ of the rat: a circumventricular organ within a neural network subserving water balance. *Brain research* **230**, 1-23 (1981).
42 Saper, C. & Loewy, A. Efferent connections of the parabrachial nucleus in the rat. *Brain research* **197**, 291-317 (1980).
43 Berk, M. & Finkelstein, J. Afferent projections to the preoptic area and hypothalamic regions in the rat brain. *Neuroscience* **6**, 1601-1624 (1981).
44 Clark, J., Clark, A., Bartle, A. & Winn, P. The regulation of feeding and drinking in rats with lesions of the lateral hypothalamus made byN-methyl-d-aspartate. *Neuroscience* **45**, 631-640 (1991).
45 Saper, C. B. Convergence of autonomic and limbic connections in the insular cortex of the rat. *Journal of Comparative Neurology* **210**, 163-173 (1982).
46 Denton, D. *et al.* Neuroimaging of genesis and satiation of thirst and an interoceptor-driven theory of origins of primary consciousness. *Proceedings of the National Academy of Sciences* **96**, 5304-5309 (1999).
47 Egan, G. *et al.* Neural correlates of the emergence of consciousness of thirst. *Proc. Natl Acad. Sci. USA* **100**, 15241-15246 (2003).
48 Grill, H. J. & Miselis, R. R. Lack of ingestive compensation to osmotic stimuli in chronic decerebrate rats. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* **240**, R81-R86 (1981).
49 Robinson, B. W. & Mishkin, M. Alimentary responses to forebrain stimulation in monkeys. *Experimental brain research* **4**, 330-366 (1968).
50 Farrell, M. J. *et al.* Cortical activation and lamina terminalis functional connectivity during thirst and drinking in humans. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* **301**, R623-R631 (2011).
51 Craig, A. Interoception: the sense of the physiological condition of the body. *Current opinion in neurobiology* **13**, 500-505 (2003).
52 Danziger, J. & Zeidel, M. L. Osmotic homeostasis. *Clinical Journal of the American Society of Nephrology* **10**, 852-862 (2015).
53 Thrasher, T. N. Baroreceptor regulation of vasopressin and renin secretion: low-pressure versus high-pressure receptors. *Frontiers in neuroendocrinology* **15**, 157-196, doi:10.1006/frne.1994.1007 (1994).
54 Fitzsimons, J. T. *The physiology of thirst and sodium appetite*. Vol. 35 (CUP Archive, 1979).
55 Johnson, A. K., Cunningham, J. T. & Thunhorst, R. L. Integrative role of the lamina terminalis in the regulation of cardiovascular and body fluid homeostasis. *Clinical and Experimental Pharmacology and Physiology* **23**, 183-191 (1996).
56 Contreras, R. J., Beckstead, R. M. & Norgren, R. The central projections of the trigeminal, facial, glossopharyngeal and vagus nerves: an autoradiographic study in the rat. *Journal of the autonomic nervous system* **6**, 303-322 (1982).
57 Kalia, M. & Sullivan, J. M. Brainstem projections of sensory and motor components of the vagus nerve in the rat. *Journal of Comparative Neurology* **211**, 248-264 (1982).

58 Johnson, A. K. & Thunhorst, R. L. The neuroendocrinology of thirst and salt appetite: visceral sensory signals and mechanisms of central integration. *Frontiers in neuroendocrinology* **18**, 292-353 (1997).
59 Oka, Y., Ye, M. & Zuker, C. S. Thirst driving and suppressing signals encoded by distinct neural populations in the brain. *Nature* **520**, 349-352 (2015).
60 Baylis, P. H. & Thompson, C. J. Osmoregulation of vasopressin secretion and thirst in health and disease. *Clinical endocrinology* **29**, 549-576 (1988).
61 Baylis, P. H. Osmoregulation and control of vasopressin secretion in healthy humans. *Am. J. Physiol.* **253**, R671-R678 (1987).
62 McKinley, M., Denton, D. & Weisinger, R. Sensors for antidiuresis and thirst—osmoreceptors or CSF sodium detectors? *Brain research* **141**, 89-103 (1978).
63 Thrasher, T. N., Brown, C. J., Keil, L. C. & Ramsay, D. J. Thirst and vasopressin release in the dog: an osmoreceptor or sodium receptor mechanism? *Am. J. Physiol.* **238**, R333-R339 (1980).
64 Thornborough, J. R., Passo, S. S. & Rothballer, A. B. Receptors in cerebral circulation affecting sodium excretion in the cat. *Am. J. Physiol.* **225**, 138-141 (1973).
65 Blaine, E. H., Denton, D. A., McKinley, M. J. & Weller, S. A central osmosensitive receptor for renal sodium excretion. *J. Physiol.* **244**, 497-509 (1975).
66 Emmeluth, C. Natriuresis caused by increased carotid Na+ concentration after renal denervation. *Am. J. Physiol.* **270**, F510-F517 (1996).
67 McKinley, M. *et al.* Efferent neural pathways of the lamina terminalis subserving osmoregulation. *Progress in brain research* **91**, 395-402 (1992).
68 Andersen, L. J., Jensen, T. U., Bestle, M. H. & Bie, P. Gastrointestinal osmoreceptors and renal sodium excretion in humans. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **278**, R287-R294 (2000).
69 Weisinger, R. S., Denton, D. A. & McKinley, M. J. Self-administered intravenous infusion of hypertonic solutions and sodium appetite of sheep. *Behav. Neurosci.* **97**, 433-444 (1983).
70 Huang, W., Lee, S.-L. & Sjoquist, M. Natriuretic role of endogenous oxytocin in male rats infused with hypertonic NaCl. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* **268**, R634-R640 (1995).
71 Huang, W., Lee, S.-L., Arnason, S. S. & Sjoquist, M. Dehydration natriuresis in male rats is mediated by oxytocin. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* **270**, R427-R433 (1996).
72 Blackburn, R. E., Samson, W. K., Fulton, R. J., Stricker, E. M. & Verbalis, J. G. Central oxytocin inhibition of salt appetite in rats: evidence for differential sensing of plasma sodium and osmolality. *Proc. Natl Acad. Sci. USA* **90**, 10380-10384 (1993).
73 Blackburn, R. E., Samson, W. K., Fulton, R. J., Stricker, E. M. & Verbalis, J. G. Central oxytocin and ANP receptors mediate osmotic inhibition of salt appetite in rats. *Am. J. Physiol.* **269**, R245-R251 (1995).
74 Thompson, C., Bland, J., Burd, J. & Baylis, P. The osmotic thresholds for thirst and vasopressin release are similar in healthy man. *Clinical science (London, England: 1979)* **71**, 651-656 (1986).
75 Robertson, G. L., Shelton, R. L. & Athar, S. The osmoregulation of vasopressin. *Kidney Int.* **10**, 25-37 (1976).

76 Geelen, G. Inhibition of plasma vasopressin after drinking in dehydrated humans. *Am. J. Physiol.* **247**, R968-R971 (1984).
77 Thompson, C., Burd, J. & Baylis, P. Acute suppression of plasma vasopressin and thirst after drinking in hypernatremic humans. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* **252**, R1138-R1142 (1987).
78 Thrasher, T. N., Nistal-Herrera, J. F., Keil, L. C. & Ramsay, D. J. Satiety and inhibition of vasopressin secretion after drinking in dehydrated dogs. *Am. J. Physiol.* **240**, E394-E401 (1981).
79 Smith, D., Moore, K., Tormey, W., Baylis, P. H. & Thompson, C. J. Downward resetting of the osmotic threshold for thirst in patients with SIADH. *Am. J. Physiol. Endocrinol. Metab.* **287**, E1019-E1023 (2004).
80 Seckl, J., Williams, T. & Lightman, S. Oral hypertonic saline causes transient fall of vasopressin in humans. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* **251**, R214-R217 (1986).
81 Stricker, E. M. & Hoffmann, M. L. Presystemic signals in the control of thirst, salt appetite, and vasopressin secretion. *Physiol. Behav.* **91**, 404-412 (2007).
82 Lechner, S. G. *et al.* The molecular and cellular identity of peripheral osmoreceptors. *Neuron* **69**, 332-344 (2011).
83 Liedtke, W. *et al.* Vanilloid receptor–related osmotically activated channel (VR-OAC), a candidate vertebrate osmoreceptor. *Cell* **103**, 525-535 (2000).
84 Strotmann, R., Harteneck, C., Nunnenmacher, K., Schultz, G. & Plant, T. D. OTRPC4, a nonselective cation channel that confers sensitivity to extracellular osmolarity. *Nature cell biology* **2**, 695-702 (2000).
85 Boschmann, M. *et al.* Water drinking induces thermogenesis through osmosensitive mechanisms. *The Journal of Clinical Endocrinology & Metabolism* **92**, 3334-3337 (2007).
86 Lipp, A. *et al.* Osmosensitive mechanisms contribute to the water drinking-induced pressor response in humans. *Neurology* **65**, 905-907 (2005).
87 Jordan, J. *et al.* The pressor response to water drinking in humans a sympathetic reflex? *Circulation* **101**, 504-509 (2000).
88 McHugh, J. *et al.* Portal osmopressor mechanism linked to transient receptor potential vanilloid 4 and blood pressure control. *Hypertension* **55**, 1438-1443 (2010).
89 Scott, E. M., Greenwood, J. P., Stoker, J. B., Gilbey, S. G. & Mary, D. A. Water drinking and sympathetic activation. *The Lancet* **356**, 2013 (2000).
90 Mckinley, M. J. *et al.* Vasopressin secretion: osmotic and hormonal regulation by the lamina terminalis. *Journal of neuroendocrinology* **16**, 340-347 (2004).
91 Verney, E. B. The antidiuretic hormone and the factors which determine its release. *Proc. R. Soc. Lond. B Biol. Sci.* **135**, 25-106 (1947).
92 Jewell, P. A. & Verney, E. An experimental attempt to determine the site of the neurohypophysial osmoreceptors in the dog. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, 197-324 (1957).
93 Andersson, B., Leksell, L. & Lishajko, F. Perturbations in fluid balance induced by medially placed forebrain lesions. *Brain research* **99**, 261-275 (1975).
94 Bie, P. Osmoreceptors, vasopressin, and control of renal water excretion. *Physiological reviews* **60**, 961-1048 (1980).

95 Buggy, J. & Jonhson, A. K. Preoptic-hypothalamic periventricular lesions: thirst deficits and hypernatremia. *Am. J. Physiol.* **233**, R44-R52 (1977).
96 Johnson, A. K. & Buggy, J. Periventricular preoptic-hypothalamus is vital for thirst and normal water economy. *AM J Physiol* **203**, 449 - 458 (1978).
97 Thrasher, T. N., Keil, L. C. & Ramsay, D. J. Lesions of the organum vasculosum of the lamina terminalis (OVLT) attenuate osmotically-induced drinking and vasopressin secretion in the dog. *Endocrinology* **110**, 1837-1839 (1982).
98 Thrasher, T. N. & Keil, L. C. Regulation of drinking and vasopressin secretion: role of organum vasculosum laminae terminalis. *Am. J. Physiol.* **253**, R108-R120 (1987).
99 McKinley, M. *et al.* Osmoregulatory thirst in sheep is disrupted by ablation of the anterior wall of the optic recess. *Brain research* **236**, 210-215 (1982).
100 Sibbald, J. R., Hubbard, J. I. & Sirett, N. E. Responses from osmosensitive neurons of the rat subfornical organ in vitro. *Brain Res.* **461**, 205-214 (1988).
101 Anderson, J. W., Washburn, D. L. & Ferguson, A. V. Intrinsic osmosensitivity of subfornical organ neurons. *Neuroscience* **100**, 539-547 (2000).
102 Mangiapane, M. L., Thrasher, T. N., Keil, L. C., Simpson, J. B. & Ganong, W. F. Role for the subfornical organ in vasopressin release. *Brain research bulletin* **13**, 43-47 (1984).
103 Thrasher, T., Simpson, J. & Ramsay, D. Lesions of the subfornical organ block angiotensin-induced drinking in the dog. *Neuroendocrinology* **35**, 68-72 (1982).
104 Lind, R. W., Thunhorst, R. L. & Johnson, A. K. The subfornical organ and the integration of multiple factors in thirst. *Physiology & behavior* **32**, 69-74 (1984).
105 Ramsay, D., Thrasher, T. & Keil, L. The organum vasculosum laminae terminalis: a critical area for osmoreception. *Progress in brain research* **60**, 91-98 (1983).
106 Prager-Khoutorsky, M. & Bourque, C. W. Anatomical organization of the rat organum vasculosum laminae terminalis. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* **309**, R324-R337 (2015).
107 Krisch, B., Leonhardt, H. & Oksche, A. Compartments in the organum vasculosum laminae terminalis of the rat and their delineation against the outer cerebrospinal fluid-containing space. *Cell and tissue research* **250**, 331-347 (1987).
108 Moreno, M. L. *et al.* Activation of organum vasculosum of lamina terminalis, median preoptic nucleus, and medial preoptic area in anticipation of nursing in rabbit pups. *Chronobiology international* **30**, 1272-1282 (2013).
109 Yamaguchi, K., Morimoto, A. & Murakami, N. Organum vasculosum laminae terminalis (OVLT) in rabbit and rat: topographic studies. *Journal of Comparative Neurology* **330**, 352-362 (1993).
110 Ballabh, P., Braun, A. & Nedergaard, M. The blood–brain barrier: an overview: structure, regulation, and clinical implications. *Neurobiology of disease* **16**, 1-13 (2004).
111 Laflamme, N. & Rivest, S. Toll-like receptor 4: the missing link of the cerebral innate immune response triggered by circulating gram-negative bacterial cell wall components. *The FASEB Journal* **15**, 155-163 (2001).
112 Nadeau, S. & Rivest, S. Effects of circulating tumor necrosis factor on the neuronal activity and expression of the genes encoding the tumor necrosis factor receptors (p55 and p75) in the rat brain: a view from the blood–brain barrier. *Neuroscience* **93**, 1449-1464 (1999).
113 Vallières, L. & Rivest, S. Regulation of the genes encoding interleukin- 6, its receptor, and gp130 in the rat brain in response to the immune activator lipopolysaccharide and the

proinflammatory cytokine interleukin- 1β. *Journal of neurochemistry* **69**, 1668-1683 (1997).
114 Lacroix, S., Feinstein, D. & Rivest, S. The bacterial endotoxin lipopolysaccharide has the ability to target the brain in upregulating its membrane CD14 receptor within specific cellular populations. *Brain pathology* **8**, 625-640 (1998).
115 Ericsson, A., Kovacs, K. & Sawchenko, P. A functional anatomical analysis of central pathways subserving the effects of interleukin-1 on stress-related neuroendocrine neurons. *The Journal of neuroscience* **14**, 897-913 (1994).
116 Somponpun, S. J., Johnson, A. K., Beltz, T. & Sladek, C. D. Estrogen receptor-α expression in osmosensitive elements of the lamina terminalis: regulation by hypertonicity. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* **287**, R661-R669 (2004).
117 Miller, R. L., Wang, M. H., Gray, P. A., Salkoff, L. B. & Loewy, A. D. ENaC-expressing neurons in the sensory circumventricular organs become c-Fos activated following systemic sodium changes. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* **305**, R1141-R1152 (2013).
118 Sunn, N., McKinley, M. & Oldfield, B. Circulating angiotensin II activates neurones in circumventricular organs of the lamina terminalis that project to the bed nucleus of the stria terminalis. *Journal of neuroendocrinology* **15**, 725-731 (2003).
119 Vivas, L., Pastuskovas, C. V. & Tonelli, L. Sodium depletion induces Fos immunoreactivity in circumventricular organs of the lamina terminalis. *Brain research* **679**, 34-41 (1995).
120 Thompson, S. A. Localization of immunoreactive prolactin in ependyma and circumventricular organs of rat brain. *Cell and tissue research* **225**, 79-93 (1982).
121 Bhat, G. *et al.* Histochemical localization of nitric oxide neurons in the hypothalamus: association with gonadotropin-releasing hormone neurons and co-localization with N-methyl-D-aspartate receptors. *Neuroendocrinology* **62**, 187-197 (1995).
122 Liedtke, W. & Friedman, J. M. Abnormal osmotic regulation in trpv4-/- mice. *Proc. Natl Acad. Sci. USA* **100**, 13698-13703 (2003).
123 Ciura, S. & Bourque, C. W. Transient Receptor Potential Vanilloid 1 Is Required for Intrinsic Osmoreception in Organum Vasculosum Lamina Terminalis Neurons and for Normal Thirst Responses to Systemic Hyperosmolality. *The Journal of Neuroscience* **26**, 9069-9075, doi:10.1523/jneurosci.0877-06.2006 (2006).
124 Mannari, T., Morita, S., Furube, E., Tominaga, M. & Miyata, S. Astrocytic TRPV1 ion channels detect blood- borne signals in the sensory circumventricular organs of adult mouse brains. *Glia* **61**, 957-971 (2013).
125 Blatteis, C. M. Role of the OVLT in the febrile response to circulating pyrogens. *Progress in brain research* **91**, 409-412 (1992).
126 Hosoi, T., Okuma, Y. & Nomura, Y. The mechanisms of immune-to-brain communication in inflammation as a drug target. *Current Drug Targets-Inflammation & Allergy* **1**, 257-262 (2002).
127 Harden, L. M. *et al.* Interleukin-10 modulates the synthesis of inflammatory mediators in the sensory circumventricular organs: implications for the regulation of fever and sickness behaviors. *Journal of neuroinflammation* **10**, 1 (2013).

128 Toney, G. M. & Stocker, S. D. Hyperosmotic activation of CNS sympathetic drive: implications for cardiovascular disease. *The Journal of physiology* **588**, 3375-3384 (2010).
129 Toney, G. M., Chen, Q. H., Cato, M. J. & Stocker, S. D. Central osmotic regulation of sympathetic nerve activity. *Acta Physiol. Scand.* **177**, 43-55 (2003).
130 Stocker, S. D., Osborn, J. L. & Carmichael, S. P. Forebrain osmotic regulation of the sympathetic nervous system. *Clinical and Experimental Pharmacology and Physiology* **35**, 695-700 (2008).
131 Veerasingham, S. J. & Leenen, F. H. Excitotoxic lesions of the ventral anteroventral third ventricle and pressor responses to central sodium, ouabain and angiotensin II. *Brain research* **749**, 157-160 (1997).
132 Sayer, R. J., Hubbard, J. I. & Sirett, N. E. Rat organum vasculosum laminae terminalis in vitro: responses to transmitters. *Am. J. Physiol.* **247**, R374-R379 (1984).
133 Ciura, S., Liedtke, W. & Bourque, C. W. Hypertonicity Sensing in Organum Vasculosum Lamina Terminalis Neurons: A Mechanical Process Involving TRPV1 But Not TRPV4. *The Journal of Neuroscience* **31**, 14669-14676, doi:10.1523/jneurosci.1420-11.2011 (2011).
134 Nissen, R., Bourque, C. W. & Renaud, L. Membrane properties of organum vasculosum lamina terminalis neurons recorded in vitro. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* **264**, R811-R815 (1993).
135 Grob, M., Trottier, J.-F., Drolet, G. & Mouginot, D. Characterization of the neurochemical content of neuronal populations of the lamina terminalis activated by acute hydromineral challenge. *Neuroscience* **122**, 247-257 (2003).
136 Bisley, J., Rees, S., McKinley, M., Hards, D. & Oldfield, B. Identification of osmoresponsive neurons in the forebrain of the rat: a Fos study at the ultrastructural level. *Brain research* **720**, 25-34 (1996).
137 Shi, P. *et al.* Intra- carotid hyperosmotic stimulation increases Fos staining in forebrain organum vasculosum laminae terminalis neurones that project to the hypothalamic paraventricular nucleus. *The Journal of physiology* **586**, 5231-5245 (2008).
138 Oldfield, B., Bicknell, R., McAllen, R., Weisinger, R. & McKinley, M. Intravenous hypertonic saline induces Fos immunoreactivity in neurons throughout the lamina terminalis. *Brain research* **561**, 151-156 (1991).
139 Oldfield, B. J., Badoer, E., Hards, D. K. & McKinley, M. J. Fos production in retrogradely labelled neurons of the lamina terminalis following intravenous infusion of either hypertonic saline or angiotensin II. *Neuroscience* **60**, 255-262 (1994).
140 Sunn, N., McKinley, M. & Oldfield, B. Identification of efferent neural pathways from the lamina terminalis activated by blood- borne relaxin. *Journal of neuroendocrinology* **13**, 432-437 (2001).
141 Richard, D. & Bourque, C. W. Synaptic control of rat supraoptic neurones during osmotic stimulation of the organum vasculosum lamina terminalis in vitro. *J. Physiol.* **489**, 567-577 (1995).
142 Antunes, V., Yao, S., Pickering, A., Murphy, D. & Paton, J. A spinal vasopressinergic mechanism mediates hyperosmolality- induced sympathoexcitation. *The Journal of physiology* **576**, 569-583 (2006).
143 Johnson, R. F., Beltz, T. G., Thunhorst, R. L. & Johnson, A. K. Investigations on the physiological controls of water and saline intake in C57BL/6 mice. *American Journal of*

Physiology-Regulatory, Integrative and Comparative Physiology **285**, R394-R403 (2003).
144 McKinley, M., Mathai, M., Pennington, G., Rundgren, M. & Vivas, L. Effect of individual or combined ablation of the nuclear groups of the lamina terminalis on water drinking in sheep. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* **276**, R673-R683 (1999).
145 McKinley, M. *et al.* Physiological and pathophysiological influences on thirst. *Physiology & behavior* **81**, 795-803 (2004).
146 Sladek, C. & Kim Johnson, A. Effect of anteroventral third ventricle lesions on vasopressin release by organ-cultured hypothalamo-neurohypophyseal explants. *Neuroendocrinology* **37**, 78-84 (1983).
147 Shi, P., Stocker, S. D. & Toney, G. M. Organum vasculosum laminae terminalis contributes to increased sympathetic nerve activity induced by central hyperosmolality. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **293**, R2279-R2289 (2007).
148 Honda, K., Negoro, H., Dyball, R., Higuchi, T. & Takano, S. The osmoreceptor complex in the rat: evidence for interactions between the supraoptic and other diencephalic nuclei. *The Journal of Physiology* **431**, 225-241 (1990).
149 Vivas, L., Chiaraviglio, E. & Carrer, H. F. Rat organum vasculosum laminae terminalis in vitro: responses to changes in sodium concentration. *Brain Res.* **519**, 294-300 (1990).
150 Zaelzer, C. *et al.* ΔN-TRPV1: A Molecular Co-detector of Body Temperature and Osmotic Stress. *Cell reports* **13**, 23-30 (2015).
151 Mizuno, A., Matsumoto, N., Imai, M. & Suzuki, M. Impaired osmotic sensation in mice lacking TRPV4. *American Journal of Physiology-Cell Physiology* **285**, C96-C101 (2003).
152 Sharif Naeini, R., Witty, M. F., Seguela, P. & Bourque, C. W. An N-terminal variant of Trpv1 channel is required for osmosensory transduction. *Nature Neurosci.* **9**, 93-98 (2006).
153 Taylor, A. C., McCarthy, J. J. & Stocker, S. D. Mice lacking the transient receptor vanilloid potential 1 (TRPV1) channel display normal thirst responses and central Fos activation to hypernatremia. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **294**, R1285-R1293 (2008).
154 Kinsman, B. *et al.* Osmoregulatory thirst in mice lacking the transient receptor potential vanilloid type 1 (TRPV1) and/or type 4 (TRPV4) receptor. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* **307**, R1092-R1100 (2014).
155 Oliver, G. & Schäfer, E. On the physiological action of extracts of pituitary body and certain other glandular organs. *The Journal of physiology* **18**, 277-279 (1895).
156 Russell, J. A. & Leng, G. Veni, vidi, vici: the neurohypophysis in the twentieth century. *Experimental physiology* **85**, 1-6 (2000).
157 Von den Velden, R. Die nierenwirkung von hypophysenextrakten beim menschen. *Berl Klin Wochenscgr* **50**, 2083-2086 (1913).
158 Farini, F. Diabete insipido ed opoterapia. *Gazz Osped Clin* **34**, 1135-1139 (1913).
159 Blumgart, H. L. The antidiuretic effect of pituitary extract applied intranasally in a case of diabetes insipidus. *Archives of Internal Medicine* **29**, 508-514 (1922).
160 Starling, E. H. & Verney, E. The secretion of urine as studied on the isolated kidney. *Proceedings of the Royal Society of London. Series B, Containing Papers of a Biological Character* **97**, 321-363 (1925).

161 Laszlo, F., Laszlo, F. & De Wied, D. Pharmacology and clinical perspectives of vasopressin antagonists. *Pharmacological Reviews* **43**, 73-108 (1991).
162 Holmes, C. L., Patel, B. M., Russell, J. A. & Walley, K. R. Physiology of vasopressin relevant to management of septic shock. *CHEST Journal* **120**, 989-1002 (2001).
163 Turner, R. A., Pierce, J. G. & du Vigneaud, V. The purification and the amino acid content of vasopressin preparations. *Journal of Biological Chemistry* **191**, 21-28 (1951).
164 Vigneaud, V. d., Lawler, H. C. & Popenoe, E. A. Enzymatic cleavage of glycinamide from vasopressin and a proposed structure for this pressor-antidiuretic hormone of the posterior pituitary. *Journal of the American Chemical Society* **75**, 4880-4881 (1953).
165 Acher, R., Chauvet, J., Chauvet, M. T., Michel, G. & Rouillé, Y. Molecular evolution of neurohypophysial hormones in relation to osmoregulation: the two fish options. *Fish Physiology and Biochemistry* **17**, 325-332, doi:10.1023/a:1007720909113 (1997).
166 Koshimizu, T.-a. *et al.* Vasopressin V1a and V1b receptors: from molecules to physiological systems. *Physiological reviews* **92**, 1813-1864 (2012).
167 McEwen, B. B. in *Advances in Pharmacology* Vol. Volume 50 1-50 (Academic Press, 2004).
168 Sachs, H., FAwcETT, P., Takabatake, Y. & Portanova, R. Biosynthesis and release of vasopressin and neurophysin. *Astwood Recent progress in hormone Research* **25**, 447-484 (2013).
169 Dreifuss, J., Kalnins, I., Kelly, J. & Ruf, K. Action potentials and release of neurohypophysial hormones in vitro. *The Journal of physiology* **215**, 805 (1971).
170 Fisher, T. E. & Bourque, C. W. The function of Ca2+ channel subtypes in exocytotic secretion: new perspectives from synaptic and non-synaptic release. *Prog. Biophys. Mol. Biol.* **77**, 269-303 (2001).
171 Fisher, T. E. & Bourque, C. W. Voltage-gated calcium currents in the magnocellular neurosecretory cells of the rat supraoptic nucleus. *The Journal of Physiology* **486**, 571 (1995).
172 Lim, N. F., Nowycky, M. C. & Bookman, R. J. Direct measurement of exocytosis and calcium currents in single vertebrate nerve terminals. *Nature* **344**, 449-451 (1990).
173 Bicknell, R. Optimizing release from peptide hormone secretory nerve terminals. *Journal of Experimental Biology* **139**, 51-65 (1988).
174 Leng, G., Brown, C. H. & Russell, J. A. Physiological pathways regulating the activity of magnocellular neurosecretory cells. *Progress in neurobiology* **57**, 625-655 (1999).
175 Brown, C. H., Bains, J. S., Ludwig, M. & Stern, J. E. Physiological regulation of magnocellular neurosecretory cell activity: integration of intrinsic, local and afferent mechanisms. *Journal of neuroendocrinology* **25**, 678-710 (2013).
176 Brown, C. H., Han, S. Y., Moaddab, M., Scott, V. & Schwenke, D. O. Peptidergic Control of Oxytocin and Vasopressin Neurons and Its Role in Reproductive and Hypertension- Associated Plasticity. *Neurophysiology of Neuroendocrine Neurons*, 63-84 (2014).
177 Gross, P., Sposito, N., Pettersen, S. & Fenstermacher, J. Differences in function and structure of the capillary endothelium in the supraoptic nucleus and pituitary neural lobe of rats. *Neuroendocrinology* **44**, 401-407 (1986).
178 Swaab, D., Pool, C. & Nijveldt, F. Immunofluorescence of vasopressin and oxytocin in the rat hypothalamo-neurohypophyseal system. *Journal of neural transmission* **36**, 195-215 (1975).

179 Rhodes, C., Morriell, J. & Pfaff, D. Immunohistochemical analysis of magnocellular elements in rat hypothalamus: distribution and numbers of cells containing neurophysin, oxytocin, and vasopressin. *Journal of Comparative Neurology* **198**, 45-64 (1981).
180 Oliet, S. H. Neuronal–Glia Remodeling of the Magnocellular System. *Neurophysiology of Neuroendocrine Neurons*, 187-205 (2015).
181 Ludwig, M. Dendritic Release of Vasopressin and Oxytocin. *Journal of neuroendocrinology* **10**, 881-896 (1998).
182 Brown, C. Rhythmogenesis in vasopressin cells. *Journal of neuroendocrinology* **16**, 727-739 (2004).
183 da Silva, M. P., de Almeida Moraes, D. J., de Souza Mecawi, A., Rodrigues, J. A. & Varanda, W. A. Nitric Oxide Modulates HCN Channels in Magnocellular Neurons of the Supraoptic Nucleus of Rats by an S-Nitrosylation-Dependent Mechanism. *Journal of Neuroscience* **36**, 11320-11330 (2016).
184 Liu, Q.-S., Jia, Y.-S. & Ju, G. Nitric oxide inhibits neuronal activity in the supraoptic nucleus of the rat hypothalamic slices. *Brain research bulletin* **43**, 121-125 (1997).
185 Gui, L.-N., Inenaga, K., Nagatomo, T. & Yamashita, H. Sodium nitroprusside modulates NMDA response in the rat supraoptic neurons in vitro. *Brain research bulletin* **35**, 253-260 (1994).
186 Stern, J. E. Nitric oxide and homeostatic control: an intercellular signalling molecule contributing to autonomic and neuroendocrine integration? *Progress in Biophysics and Molecular Biology* **84**, 197-215, doi:http://dx.doi.org/10.1016/j.pbiomolbio.2003.11.015 (2004).
187 Walters, J. K. & Hatton, G. I. Supraoptic neuronal activity in rats during five days of water deprivation. *Physiol. Behav.* **13**, 661-667 (1974).
188 Brimble, M. J. & Dyball, R. E. Characterization of the responses of oxytocin- and vasopressin-secreting neurones in the supraoptic nucleus to osmotic stimulation. *J. Physiol.* **271**, 253-271 (1977).
189 Wakerley, J. B., Poulain, D. A. & Brown, D. Comparison of firing patterns in oxytocin- and vasopressin-releasing neurones during progressive dehydration. *Brain Res.* **148**, 425-440 (1978).
190 Bourque, C. W. Osmoregulation of vasopressin neurons: a synergy of intrinsic and synaptic processes. *Prog. Brain Res.* **119**, 59-76 (1998).
191 Poulain, D. & Wakerley, J. Electrophysiology of hypothalamic magnocellular neurones secreting oxytocin and vasopressin. *Neuroscience* **7**, 773-808 (1982).
192 Wakerley, J., Poulain, D., Dyball, R. & Cross, B. Activity of phasic neurosecretory cells during haemorrhage. *Nature* **258**, 82-84 (1975).
193 Dutton, A. & Dyball, R. Phasic firing enhances vasopressin release from the rat neurohypophysis. *The Journal of Physiology* **290**, 433-440 (1979).
194 Knepel, W. & Meyer, D. The effect of naloxone on vasopressin release from rat neurohypophysis incubated in vitro. *The Journal of physiology* **341**, 507 (1983).
195 Bondy, C., Gainer, H. & Russell, J. Effects of stimulus frequency and potassium channel blockade on the secretion of vasopressin and oxytocin from the neurohypophysis. *Neuroendocrinology* **46**, 258-267 (1987).
196 Bourque, C. & Renaud, L. Activity dependence of action potential duration in rat supraoptic neurosecretory neurones recorded in vitro. *The Journal of physiology* **363**, 429-439 (1985).

197 Hobbach, H., Hurth, S., Jost, D. & Racké, K. Effects of tetraethylammonium ions on frequency- dependent vasopressin release from the rat neurohypophysis. *The Journal of physiology* **397**, 539-554 (1988).
198 Bicknell, R. & Leng, G. Relative efficiency of neural firing patterns for vasopressin release in vitro. *Neuroendocrinology* **33**, 295-299 (1981).
199 Bicknell, R., Brown, D., Chapman, C., Hancock, P. & Leng, G. Reversible fatigue of stimulus- secretion coupling in the rat neurohypophysis. *The Journal of physiology* **348**, 601-613 (1984).
200 Mason, W. T. Supraoptic neurones of rat hypothalamus are osmosensitive. *Nature* **287**, 154-157 (1980).
201 Bourque, C. W. Ionic basis for the intrinsic activation of rat supraoptic neurones by hyperosmotic stimuli. *J. Physiol.* **417**, 263-277 (1989).
202 Oliet, S. H. & Bourque, C. W. Properties of supraoptic magnocellular neurones isolated from the adult rat. *J. Physiol.* **455**, 291-306 (1992).
203 Leng, G., Dyball, R. & Mason, W. in *Vasopressin* 333-342 (Raven Press New York, 1985).
204 Honda, K., Negoro, H., Higuchi, T. & Tadokoro, Y. Activation of neurosecretory cells by osmotic stimulation of anteroventral third ventricle. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* **252**, R1039-R1045 (1987).
205 Bourque, C. & Renaud, L. Electrophysiology of mammalian magnocellular vasopressin and oxytocin neurosecretory neurons. *Frontiers in neuroendocrinology* **11**, 183-212 (1990).
206 Baertschi, A. J. & Vallet, P. G. Osmosensitivity of the hepatic portal vein area and vasopressin release in rats. *J. Physiol.* **315**, 217-230 (1981).
207 Bourque, C. W., Oliet, S. H. & Richard, D. Osmoreceptors, osmoreception, and osmoregulation. *Front. Neuroendocrinol.* **15**, 231-274 (1994).
208 Kobashi, M. & Adachi, A. Convergence of hepatic osmoreceptive inputs on sodium-responsive units within the nucleus of the solitary tract of the rat. *Journal of neurophysiology* **54**, 212-219 (1985).
209 King, M. S. & Baertschi, A. J. Ventral pontine catecholaminergic pathway mediates the vasopressin response to splanchnic osmostimulation in conscious rats. *Brain research* **580**, 81-91 (1992).
210 Choe, K. Y. & Bourque, C. W. The Osmotic Control of Vasopressin- Releasing Neurons. *Neurophysiology of Neuroendocrine Neurons*, 85-104 (2014).
211 Renaud, L. P., Cunningham, J. T., Nissen, R. & Yang, C. R. Electrophysiology of central pathways controlling release of neurohypophysial hormones. *Annals of the New York Academy of Sciences* **689**, 122-132 (1993).
212 Russell, J., Blackburn, R. & Leng, G. The role of the AV3V region in the control of magnocellular oxytocin neurons. *Brain research bulletin* **20**, 803-810 (1988).
213 Richard, D. & Bourque, C. Synaptic activation of rat supraoptic neurons by osmotic stimulation of the organum vasculosum lamina terminalis. *Neuroendocrinology* **55**, 609-611 (1992).
214 Thrasher, T. Role of forebrain circumventricular organs in body fluid balance. *Acta physiologica Scandinavica. Supplementum* **583**, 141 (1989).

215 Hussy, N., Deleuze, C., Pantaloni, A., Desarmenien, M. G. & Moos, F. Agonist action of taurine on glycine receptors in rat supraoptic magnocellular neurones: possible role in osmoregulation. *J. Physiol.* **502**, 609-621 (1997).
216 Deleuze, C., Duvoid, A. & Hussy, N. Properties and glial origin of osmotic-dependent release of taurine from the rat supraoptic nucleus. *J. Physiol.* **507**, 463-471 (1998).
217 Pasantes-Morales, H., Franco, R., Torres-Marquez, M. E., Hernandez-Fonseca, K. & Ortega, A. Amino acid osmolytes in regulatory volume decrease and isovolumetric regulation in brain cells: contribution and mechanisms. *Cell. Physiol. Biochem.* **10**, 361-370 (2000).
218 Massieu, L., Montiel, T., Robles, G. & Quesada, O. Brain amino acids during hyponatremia in vivo: clinical observations and experimental studies. *Neurochem. Res.* **29**, 73-81 (2004).
219 Hosoi, M., Takeuchi, K., Sawada, H. & Toyohara, H. Expression and functional analysis of mussel taurine transporter, as a key molecule in cellular osmoconforming. *J. Exp. Biol.* **208**, 4203-4211 (2005).
220 Lang, F. Mechanisms and significance of cell volume regulation. *J. Am. Coll. Nutr.* **26**, 613S-623S (2007).
221 Bres, V. Pharmacological characterization of volume-sensitive, taurine permeable anion channels in rat supraoptic glial cells. *Br. J. Pharmacol.* **130**, 1976-1982 (2000).
222 Choe, K. Y., Olson, J. E. & Bourque, C. W. Taurine release by astrocytes modulates osmosensitive glycine receptor tone and excitability in the adult supraoptic nucleus. *The Journal of Neuroscience* **32**, 12518-12527 (2012).
223 Deleuze, C., Alonso, G., Lefevre, I. A., Duvoid-Guillou, A. & Hussy, N. Extrasynaptic localization of glycine receptors in the rat supraoptic nucleus: further evidence for their involvement in glia-to-neuron communication. *Neuroscience* **133**, 175-183 (2005).
224 Armstrong, W. E. Morphological and electrophysiological classification of hypothalamic supraoptic neurons. *Progress in neurobiology* **47**, 291-339 (1995).
225 Choe, K. Y., Prager- Khoutorsky, M., Farmer, W. T., Murai, K. K. & Bourque, C. W. Effects of Salt Loading on the Morphology of Astrocytes in the Ventral Glia Limitans of the Rat Supraoptic Nucleus. *Journal of neuroendocrinology* **28** (2016).
226 Qiu, D. L. Effect of hypertonic saline on rat hypothalamic paraventricular nucleus magnocellular neurons in vitro. *Neurosci. Lett.* **355**, 117-120 (2004).
227 Oliet, S. H. & Bourque, C. W. Mechanosensitive channels transduce osmosensitivity in supraoptic neurons. *Nature* **364**, 341-343 (1993).
228 Oliet, S. H. & Bourque, C. W. Steady-state osmotic modulation of cationic conductance in neurons of rat supraoptic nucleus. *Am. J. Physiol.* **265**, R1475-R1479 (1993).
229 Zhang, Z., Kindrat, A. N., Sharif Naeini, R. & Bourque, C. W. Actin filaments mediate mechanical gating during osmosensory transduction in rat supraoptic nucleus neurons. *J. Neurosci.* **27**, 4008-4013 (2007).
230 Prager- Khoutorsky, M. & Bourque, C. Mechanical basis of osmosensory transduction in magnocellular neurosecretory neurones of the rat supraoptic nucleus. *Journal of neuroendocrinology* **27**, 507-515 (2015).
231 Prager-Khoutorsky, M., Khoutorsky, A. & Bourque, C. W. Unique interweaved microtubule scaffold mediates osmosensory transduction via physical interaction with TRPV1. *Neuron* **83**, 866-878 (2014).

232 Han, J., Gnatenco, C., Sladek, C. D. & Kim, D. Background and tandem-pore potassium channels in magnocellular neurosecretory cells of the rat supraoptic nucleus. *J. Physiol.* **546**, 625-639 (2003).
233 Liu, X. H., Zhang, W. & Fisher, T. E. A novel osmosensitive voltage gated cation current in rat supraoptic neurones. *J. Physiol.* **568**, 61-68 (2005).
234 Prager-Khoutorsky, M. & Bourque, C. W. Osmosensation in vasopressin neurons: changing actin density to optimize function. *Trends in neurosciences* **33**, 76-83 (2010).
235 Czaczkes, J., Kleeman, C., Koenig, M. & Boston, R. Physiologic studies of antidiuretic hormone by its direct measurement in human plasma. *Journal of Clinical Investigation* **43**, 1625 (1964).
236 Claybaugh, J. R., Sato, A. K., Crosswhite, L. K. & Hassell, L. H. Effects of time of day, gender, and menstrual cycle phase on the human response to a water load. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* **279**, R966-R973 (2000).
237 Levinsky, N. G., Davidson, D. G. & Berliner, R. W. Changes in urine concentration during prolonged administration of vasopressin and water. *American Journal of Physiology--Legacy Content* **196**, 451-456 (1959).
238 Chan, W. Effects of prolonged administration of vasopressin on plasma sodium and on renal excretion of electrolytes and water. *Journal of Pharmacology and Experimental Therapeutics* **178**, 141-151 (1971).
239 Smith, M., Cowley, M., Guyton, A. & Manning, R. Acute and chronic effects of vasopressin on blood pressure, electrolytes, and fluid volumes. *American Journal of Physiology-Renal Physiology* **237**, F232-F240 (1979).
240 Leaf, A., Bartter, F. C., Santos, R. F. & Wrong, O. Evidence in man that urinary electrolyte loss induced by pitressin is a function of water retention. *Journal of Clinical Investigation* **32**, 868 (1953).
241 Goodwin, F., Ledingham, J. & Laragh, J. The effects of prolonged administration of vasopressin and oxytocin on renin, aldosterone and sodium balance in normal man. *Clinical science* **39**, 641-651 (1970).
242 Ginsburg, M. & Heller, H. The clearance of injected vasopressin from the circulation and its fate in the body. *Journal of Endocrinology* **9**, 283-291 (1953).
243 Robertson, G. L., Mahr, E. A., Athar, S. & Sinha, T. Development and clinical application of a new method for the radioimmunoassay of arginine vasopressin in human plasma. *Journal of Clinical Investigation* **52**, 2340 (1973).
244 Verbalis, J. G. Disorders of body water homeostasis. *Best practice & research clinical endocrinology & metabolism* **17**, 471-503 (2003).
245 Bisset, G. & Chowdrey, H. Control of release of vasopressin by neuroendocrine reflexes. *Quarterly Journal of Experimental Physiology* **73**, 811-872 (1988).
246 Day, T. A. & Sibbald, J. R. Solitary nucleus excitation of supraoptic vasopressin cells via adrenergic afferents. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* **254**, R711-R716 (1988).
247 Raby, W. N. & Renaud, L. Dorsomedial medulla stimulation activates rat supraoptic oxytocin and vasopressin neurones through different pathways. *The Journal of physiology* **417**, 279 (1989).

248 Sawchenko, P. & Swanson, L. The organization of noradrenergic pathways from the brainstem to the paraventricular and supraoptic nuclei in the rat. *Brain Research Reviews* **4**, 275-325 (1982).
249 Chan, R. & Sawchenko, P. Spatially and temporally differentiated patterns of c- fos expression in brainstem catecholamilriergic cell groups induced by cardiovascular challenges in the rat. *Journal of Comparative Neurology* **348**, 433-460 (1994).
250 Jones, B. E. & Moore, R. Y. Ascending projections of the locus coeruleus in the rat. II. Autoradiographic study. *Brain research* **127**, 23-53 (1977).
251 Share, L. Role of vasopressin in cardiovascular regulation. *Physiological Reviews* **68**, 1248-1284 (1988).
252 Morel, A., O'Carroll, A.-M., Brownstein, M. J. & Lolaft, S. J. Molecular cloning and expression of a rat Via arginine vasopressin receptor. (1992).
253 Wakatsuki, T., Nakaya, Y. & Inoue, I. Vasopressin modulates K (+)-channel activities of cultured smooth muscle cells from porcine coronary artery. *American Journal of Physiology-Heart and Circulatory Physiology* **263**, H491-H496 (1992).
254 Nelson, M. T. & Quayle, J. M. Physiological roles and properties of potassium channels in arterial smooth muscle. *American Journal of Physiology-Cell Physiology* **268**, C799-C822 (1995).
255 Russ, R. & Walker, B. R. Role of nitric oxide in vasopressinergic pulmonary vasodilatation. *American Journal of Physiology-Heart and Circulatory Physiology* **262**, H743-H747 (1992).
256 Sai, Y., Okamura, T., Amakata, Y. & Toda, N. Comparison of responses of canine pulmonary artery and vein to angiotensin II, bradykinin and vasopressin. *European journal of pharmacology* **282**, 235-241 (1995).
257 Evora, P., Pearson, P. J. & Schaff, H. V. Arginine vasopressin induces endothelium-dependent vasodilatation of the pulmonary artery. V1-receptor-mediated production of nitric oxide. *CHEST Journal* **103**, 1241-1245 (1993).
258 Okamura, T., Toda, M., Ayajiki, K. & Toda, N. Receptor subtypes involved in relaxation and contraction by arginine vasopressin in canine isolated short posterior ciliary arteries. *Journal of vascular research* **34**, 464-472 (1997).
259 Okamura, T., Ayajiki, K., Fujioka, H. & Toda, N. Mechanisms underlying arginine vasopressin-induced relaxation in monkey isolated coronary arteries. *Journal of hypertension* **17**, 673-678 (1999).
260 Schwartz, J. & Reid, I. Role of vasopressin in blood pressure regulation in conscious water-deprived dogs. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* **244**, R74-R77 (1983).
261 Abboud, F. *et al.* Role of vasopressin in cardiovascular and blood pressure regulation. *Journal of Vascular Research* **27**, 106-115 (1990).
262 Luk, J. *et al.* Role of V1 receptors in the action of vasopressin on the baroreflex control of heart rate. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* **265**, R524-R529 (1993).
263 Cowley, A. W., Monos, E. & Guyton, A. C. Interaction of vasopressin and the baroreceptor reflex system in the regulation of arterial blood pressure in the dog. *Circulation Research* **34**, 505-514 (1974).
264 Cowley Jr, A. W., Switzer, S. J. & Guinn, M. M. Evidence and quantification of the vasopressin arterial pressure control system in the dog. *Circ Res* **46**, 58-67 (1980).

265 Möhring, J. *et al.* Greatly enhanced pressor response to antidiuretic hormone in patients with impaired cardiovascular reflexes due to idiopathic orthostatic hypotension. *Journal of cardiovascular pharmacology* **2**, 367-376 (1980).
266 Vincent, J.-L. & Su, F. Physiology and pathophysiology of the vasopressinergic system. *Best practice & research Clinical anaesthesiology* **22**, 243-252 (2008).
267 Schrier, R., Berl, T. & Anderson, R. Osmotic and nonosmotic control of vasopressin release. *American Journal of Physiology-Renal Physiology* **236**, F321-F332 (1979).
268 Johnson, J. A., Zehr, J. E. & Moore, W. W. Effects of separate and concurrent osmotic and volume stimuli on plasma ADH in sheep. *American Journal of Physiology--Legacy Content* **218**, 1273-1280 (1970).
269 Schrier, R. W. & Berl, T. Nonosmolar factors affecting renal water excretion. *New England Journal of Medicine* **292**, 81-88 (1975).
270 Schwartz, J. & Reid, I. A. Effect of vasopressin blockade on blood pressure regulation during hemorrhage in conscious dogs. *Endocrinology* **109**, 1778-1780 (1981).
271 Robertson, G. L. & Athar, S. The interaction of blood osmolality and blood volume in regulating plasma vasopressin in man. *The Journal of Clinical Endocrinology & Metabolism* **42**, 613-620 (1976).
272 Robertson, G., Athar, S. & Shelton, R. Osmotic control of vasopressin function. *Disturbances in body fluid osmolality*, 125-148 (1977).
273 Quillen, E. & Cowley, A. Influence of volume changes on osmolality-vasopressin relationships in conscious dogs. *American Journal of Physiology-Heart and Circulatory Physiology* **244**, H73-H79 (1983).
274 Trudel, E. & Bourque, C. Circadian modulation of osmoregulated firing in rat supraoptic nucleus neurones. *Journal of neuroendocrinology* **24**, 577-586 (2012).
275 GEORGE, C. P. *et al.* Diurnal Variation of Plasma Vasopressin in Man*. *The Journal of Clinical Endocrinology & Metabolism* **41**, 332-338 (1975).
276 Gordon, C. & Lavie, P. Day-night variations in urine excretions and hormones in dogs: role of autonomic innervation. *Physiology & behavior* **35**, 175-181 (1985).
277 Granda, T., Velasco, A. & Rausch, A. Variations and interrelation between vasopressin and plasma osmolality in diabetic rats with insulin treatment. *Life sciences* **63**, 1305-1313 (1998).
278 Windle, R., Forsling, M. & Guzek, J. Daily rhythms in the hormone content of the neurohypophysial system and release of oxytocin and vasopressin in the male rat: effect of constant light. *Journal of endocrinology* **133**, 283-290 (1992).
279 Forsling, M. L. Diurnal rhythms in neurohypophysial function. *Experimental physiology* **85** (2000).
280 Greeley, G., Morris, M., Eldridge, J. & Kizer, J. A diurnal plasma vasopressin rhythm in rats. *Life sciences* **31**, 2843-2846 (1982).
281 Cui, L. N., Saeb- Parsy, K. & Dyball, R. Neurones in the supraoptic nucleus of the rat are regulated by a projection from the suprachiasmatic nucleus. *The Journal of physiology* **502**, 149-159 (1997).
282 Okamura, H. in *Cold Spring Harbor symposia on quantitative biology.* 551-556 (Cold Spring Harbor Laboratory Press).
283 Brown, T. M. & Piggins, H. D. Electrophysiology of the suprachiasmatic circadian clock. *Progress in neurobiology* **82**, 229-255 (2007).

284 Trudel, E. & Bourque, C. W. Central clock excites vasopressin neurons by waking osmosensory afferents during late sleep. *Nat Neurosci* **13**, 467-474 (2010).
285 Loh, J. A. & Verbalis, J. G. Disorders of water and salt metabolism associated with pituitary disease. *Endocrinology and metabolism clinics of North America* **37**, 213-234 (2008).
286 Verbalis, J. G., Robinson, A. G. & Moses, A. in *Diabetes insipidus in man* 247-265 (Karger Publishers, 1985).
287 Miller, W. Molecular genetics of familial central diabetes insipidus. *The Journal of Clinical Endocrinology & Metabolism* **77**, 592-595 (1993).
288 Nemergut, E. C., Zuo, Z., Jane Jr, J. A. & Laws Jr, E. R. Predictors of diabetes insipidus after transsphenoidal surgery: a review of 881 patients. *Journal of neurosurgery* **103**, 448-454 (2005).
289 Brooks, B. S., El Gammal, T., Allison, J. D. & Hoffman, W. H. Frequency and variation of the posterior pituitary bright signal on MR images. *American journal of neuroradiology* **10**, 943-948 (1989).
290 Rudelli, R. & Deck, J. H. Selective traumatic infarction of the human anterior hypothalamus: Clinical anatomical correlation. *Journal of neurosurgery* **50**, 645-654 (1979).
291 Liamis, G. *et al.* Clinical and laboratory characteristics of hypernatraemia in an internal medicine clinic. *Nephrology Dialysis Transplantation* **23**, 136-143 (2008).
292 Schwartz, W. B., Bennett, W., Curelop, S. & Bartter, F. C. A syndrome of renal sodium loss and hyponatremia probably resulting from inappropriate secretion of antidiuretic hormone. *The American journal of medicine* **23**, 529-542 (1957).
293 Verbalis, J. Brain volume regulation in response to changes in osmolality. *Neuroscience* **168**, 862-870 (2010).
294 Hannon, M. & Thompson, C. J. The syndrome of inappropriate antidiuretic hormone: prevalence, causes and consequences. *European Journal of Endocrinology* **162**, S5-S12 (2010).
295 Hawkins, R. C. Age and gender as risk factors for hyponatremia and hypernatremia. *Clinica Chimica Acta* **337**, 169-172 (2003).
296 Upadhyay, A., Jaber, B. L. & Madias, N. E. Incidence and prevalence of hyponatremia. *The American journal of medicine* **119**, S30-S35 (2006).
297 Anderson, R. J., Chung, H.-M., Kluge, R. & Schrier, R. W. Hyponatremia: a prospective analysis of its epidemiology and the pathogenetic role of vasopressin. *Annals of Internal Medicine* **102**, 164-168 (1985).
298 Schrier, R. W. Does' asymptomatic hyponatremia'exist? *Nature Reviews Nephrology* **6**, 185-185 (2010).
299 Decaux, G. Is asymptomatic hyponatremia really asymptomatic? *The American journal of medicine* **119**, S79-S82 (2006).
300 Ellison, D. H. & Berl, T. The syndrome of inappropriate antidiuresis. *New England Journal of Medicine* **356**, 2064-2072 (2007).
301 Swart, R. M., Hoorn, E. J., Betjes, M. G. & Zietse, R. Hyponatremia and inflammation: the emerging role of interleukin-6 in osmoregulation. *Nephron Physiology* **118**, p45-p51 (2010).
302 Robertson, G. L. & Aycinena, P. Neurogenic disorders of osmoregulation. *The American journal of medicine* **72**, 339-353 (1982).

303 Cinel, I. & Opal, S. M. Molecular biology of inflammation and sepsis: A primer*. *Critical care medicine* **37**, 291-304 (2009).
304 Angus, D. C. & Van der Poll, T. Severe sepsis and septic shock. *New England Journal of Medicine* **369**, 840-851 (2013).
305 Sands, K. E. *et al.* Epidemiology of sepsis syndrome in 8 academic medical centers. *The Journal of the American Medical Association* **278**, 234-240 (1997).
306 Martin, G. S., Mannino, D. M., Eaton, S. & Moss, M. The epidemiology of sepsis in the United States from 1979 through 2000. *New England Journal of Medicine* **348**, 1546-1554 (2003).
307 Vincent, J.-L. *et al.* International study of the prevalence and outcomes of infection in intensive care units. *The Journal of the American Medical Association* **302**, 2323-2329 (2009).
308 Angus, D. C. *et al.* Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care. *Critical Care Medicine-Baltimore-* **29**, 1303-1310 (2001).
309 Mayr, F. B. *et al.* Infection rate and acute organ dysfunction risk as explanations for racial differences in severe sepsis. *Jama* **303**, 2495-2503 (2010).
310 Iskander, K. N. *et al.* Sepsis: Multiple Abnormalities, Heterogeneous Responses, and Evolving Understanding. *Physiological Reviews* **93**, 1247-1288, doi:10.1152/physrev.00037.2012 (2013).
311 Bone, R. C. *et al.* Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. The ACCP/SCCM Consensus Conference Committee. American College of Chest Physicians/Society of Critical Care Medicine. *Chest* **101**, 1644-1655 (1992).
312 Singer, M. *et al.* The third international consensus definitions for sepsis and septic shock (Sepsis-3). *Jama* **315**, 801-810 (2016).
313 Seymour, C. W. & Rosengart, M. R. Septic shock: advances in diagnosis and treatment. *JAMA* **314**, 708-717 (2015).
314 Melville, J., Ranjan, S. & Morgan, P. ICU mortality rates in patients with sepsis before and after the Surviving Sepsis Campaign. *Critical Care* **19**, P15-P15, doi:10.1186/cc14095 (2015).
315 Linde-Zwirble, W. T. & Angus, D. C. Severe sepsis epidemiology: sampling, selection, and society. *Critical Care* **8**, 222 (2004).
316 Lazosky, A., Young, G. B., Zirul, S. & Phillips, R. Quality of life after septic illness. *Journal of Critical Care* **25**, 406-412 (2009).
317 Sonneville, R. *et al.* Understanding brain dysfunction in sepsis. *Ann Intensive Care* **3**, 15 (2013).
318 Ziaja, M. Septic encephalopathy. *Curr Neurol Neurosci Rep* **13**, 383 (2013).
319 Deutschman, C. S. & Tracey, K. J. Sepsis: current dogma and new perspectives. *Immunity* **40**, 463-475 (2014).
320 Funk, D. J., Parrillo, J. E. & Kumar, A. Sepsis and Septic Shock: A History. *Critical Care Clinics* **25**, 83-101 (2009).
321 Baron, R. M., Baron, M. J. & Perrella, M. A. Pathobiology of Sepsis. *American Journal of Respiratory Cell and Molecular Biology* **34**, 129-134, doi:10.1165/rcmb.F308 (2006).
322 Beutler, B. & Rietschel, E. T. Innate immune sensing and its roots: the story of endotoxin. *Nat Rev Immunol* **3**, 169-176 (2003).

323 Barua, D. in *Cholera Current Topics in Infectious Disease* (eds Dhiman Barua & W.B. Greenough III) 1-36 (Springer US, 1992).
324 Finkelstein, R. A. in *Cholera* (eds D. Barua & W.B. Greenough III) 155-187 (Springer US, 1992).
325 Roth, J. & Blatteis, C. M. Mechanisms of fever production and lysis: lessons from experimental LPS fever. *Comprehensive Physiology* **4**, 1563-1604, doi:10.1002/cphy.c130033 (2014).
326 Osler, W. The evolution of modern medicine. (1904).
327 Bone, R. C., Sibbald, W. J. & Sprung, C. L. The ACCP-SCCM Consensus Conference on Sepsis and Organ Failure. *Chest* **101**, 1481-1483 (1992).
328 Blalock, A. Experimental shock: cause of low blood pressure produced by muscle injury. *Archives of Surgery* **20**, 959-996 (1930).
329 Randall, H. T. The shifts of fluid and electrolytes in shock *NYAS Annals of the New York Academy of Sciences* **55**, 412-428 (1952).
330 Hinshaw, L. *The Fundamental Mechanisms of Shock: Proceedings of a Symposium Held in Oklahoma City, Oklahoma, October 1–2, 1971*. Vol. 23 (Springer Science & Business Media, 2013).
331 Krishnagopalan, S., Kumar, A., Parrillo, J. E. & Kumar, A. Myocardial dysfunction in the patient with sepsis. *Current opinion in critical care* **8**, 376-388 (2002).
332 Rabuel, C. & Mebazaa, A. Septic shock: a heart story since the 1960s. *Intensive care medicine* **32**, 799-807 (2006).
333 Bone, R. C. The pathogenesis of sepsis. *Annals of Internal Medicine* **115**, 457-469 (1991).
334 Wilson, R. F., Thal, A. P., Kindling, P. H., Grifka, T. & Ackerman, E. Hemodynamic measurements in septic shock. *Archives of Surgery* **91**, 121 (1965).
335 Clowes Jr, G., Vucinic, M. & Weidner, M. G. Circulatory and metabolic alterations associated with survival or death in peritonitis: clinical analysis of 25 cases. *Annals of surgery* **163**, 866 (1966).
336 MacLean, L. D., Mulligan, W. G., McLean, A. & Duff, J. H. Patterns of septic shock in man--a detailed study of 56 patients. *Annals of surgery* **166**, 543 (1967).
337 Swan, H. *et al.* Catheterization of the heart in man with use of a flow-directed balloon-tipped catheter. *New England Journal of Medicine* **283**, 447-451 (1970).
338 Calvin, J., Driedger, A. & Sibbald, W. An assessment of myocardial function in human sepsis utilizing ECG gated cardiac scintigraphy. *CHEST Journal* **80**, 579-586 (1981).
339 Parker, M. M. *et al.* Profound but reversible myocardial depression in patients with septic shock. *Annals of internal medicine* **100**, 483-490 (1984).
340 Bouhemad, B. *et al.* Isolated and reversible impairment of ventricular relaxation in patients with septic shock*. *Critical care medicine* **36**, 766-774 (2008).
341 Dellinger, R. P. *et al.* Surviving Sepsis Campaign: international guidelines for management of severe sepsis and septic shock, 2012. *Intensive care medicine* **39**, 165-228 (2013).
342 Carswell, E. *et al.* An endotoxin-induced serum factor that causes necrosis of tumors. *Proceedings of the National Academy of Sciences* **72**, 3666-3670 (1975).
343 Beutler, B. *et al.* Identity of tumour necrosis factor and the macrophage-secreted factor cachectin. (1985).

344 Tracey, K. J. *et al.* Shock and Tissue Injury Induced by Recombinant Human Cachectin. *Science* **234**, 470-474 (1986).
345 Nawroth, P. P., Handley, D. A., Esmon, C. T. & Stern, D. M. Interleukin 1 induces endothelial cell procoagulant while suppressing cell-surface anticoagulant activity. *Proceedings of the National Academy of Sciences* **83**, 3460-3464 (1986).
346 Levy, M. M. *et al.* 2001 sccm/esicm/accp/ats/sis international sepsis definitions conference. *Intensive care medicine* **29**, 530-538 (2003).
347 Vincent, J.-L., Opal, S. M., Marshall, J. C. & Tracey, K. J. Sepsis definitions: time for change. *Lancet (London, England)* **381**, 774 (2013).
348 Shankar-Hari, M. *et al.* Developing a new definition and assessing new clinical criteria for septic shock: for the third international consensus definitions for sepsis and septic shock (sepsis-3). *Jama* **315**, 775-787 (2016).
349 Munford, R. S. in *Harrison's principles of internal medicine* (eds Dennis L. Kasper *et al.*) Ch. 325, 1751 - 1759 (2015).
350 Garnacho-Montero, J. *et al.* Determinants of outcome in patients with bacteraemic pneumococcal pneumonia: importance of early adequate treatment. *Scandinavian journal of infectious diseases* **42**, 185-192 (2010).
351 Mathevon, T., Souweine, B., Traoré, O., Aublet, B. & Caillaud, D. ICU-acquired nosocomial infection: impact of delay of adequate antibiotic treatment. *Scandinavian journal of infectious diseases* **34**, 831-835 (2002).
352 Gaieski, D. F. *et al.* Impact of time to antibiotics on survival in patients with severe sepsis or septic shock in whom early goal-directed therapy was initiated in the emergency department*. *Critical care medicine* **38**, 1045-1053 (2010).
353 Marik, P. E. Early management of severe sepsis: concepts and controversies. *CHEST Journal* **145**, 1407-1418 (2014).
354 Kumar, A. *et al.* Duration of hypotension before initiation of effective antimicrobial therapy is the critical determinant of survival in human septic shock. *Critical care medicine* **34**, 1589-1596 (2006).
355 Gelinas, J. P. & Russell, J. A. Vasopressors During Sepsis: Selection and Targets. *Clinics in Chest Medicine* **37**, 251-262, doi:http://dx.doi.org/10.1016/j.ccm.2016.01.008 (2016).
356 Abraham, E. New Definitions for Sepsis and Septic Shock: Continuing Evolution but With Much Still to Be Done. *JAMA* **315**, 757-759 (2016).
357 Vincent, J. L. & De Backer, D. Circulatory shock. *The New England journal of medicine* **369**, 1726-1734, doi:10.1056/NEJMra1208943 (2013).
358 Winters, B. D. *et al.* Long-term mortality and quality of life in sepsis: A systematic review*. *Critical care medicine* **38**, 1276-1283 (2010).
359 Buras, J. A., Holzmann, B. & Sitkovsky, M. Animal models of sepsis: setting the stage. *Nat. Rev. Drug Discov.* **4**, 854-865 (2005).
360 Wichterman, K. A., Baue, A. E. & Chaudry, I. H. Sepsis and septic shock[mdash]a review of laboratory models and a proposal. *J. Surg. Res.* **29**, 189-201 (1980).
361 Fink, M. P. & Heard, S. O. Laboratory models of sepsis and septic shock. *Journal of Surgical Research* **49**, 186-196 (1990).
362 Deitch, E. A. Animal models of sepsis and shock: a review and lessons learned. *Shock* **9**, 1-11 (1998).

363 Ayala, A., Herdon, C. D., Lehman, D. L., Ayala, C. A. & Chaudry, I. H. Differential induction of apoptosis in lymphoid tissues during sepsis: variation in onset, frequency, and the nature of the mediators. *Blood* **87**, 4261-4275 (1996).
364 van der Poll, T. & van Deventer, S. J. Cytokines and anticytokines in the pathogenesis of sepsis. *Infectious disease clinics of North America* **13**, 413-426 (1999).
365 Morris, C. F. M., Tahir, M., Arshid, S., Castro, M. S. & Fontes, W. Reconciling the IPC and two-hit models: dissecting the underlying cellular and molecular mechanisms of two seemingly opposing frameworks. *Journal of Immunology Research* **Article ID 697193**, 11 pages (2015).
366 Garrison, R. N., Spain, D. A., Wilson, M. A., Keelen, P. A. & Harris, P. D. Microvascular changes explain the" two-hit" theory of multiple organ failure. *Annals of surgery* **227**, 851 (1998).
367 Rittirsch, D., Hoesel, L. M. & Ward, P. A. The disconnect between animal models of sepsis and human sepsis. *Journal of leukocyte biology* **81**, 137-143 (2007).
368 Marshall, J. C. Such stuff as dreams are made on: mediator-directed therapy in sepsis. *Nat Rev Drug Discov* **2**, 391-405 (2003).
369 Parrillo, J. E. *et al.* Septic shock in humans: advances in the understanding of pathogenesis, cardiovascular dysfunction, and therapy. *Annals of internal medicine* **113**, 227-242 (1990).
370 Cohen, J. The immunopathogenesis of sepsis. *Nature* **420**, 885-891 (2002).
371 Rittirsch, D., Flierl, M. A. & Ward, P. A. Harmful molecular mechanisms in sepsis. *Nature Reviews Immunology* **8**, 776-787 (2008).
372 Michie, H. R. *et al.* Detection of circulating tumor necrosis factor after endotoxin administration. *New England Journal of Medicine* **318**, 1481-1486 (1988).
373 Hesse, D. *et al.* Cytokine appearance in human endotoxemia and primate bacteremia. *Surgery, gynecology & obstetrics* **166**, 147-153 (1988).
374 Remick, D. G., Newcomb, D. E., Bolgos, G. L. & Call, D. R. Comparison of the mortality and inflammatory response of two models of sepsis: lipopolysaccharide vs. cecal ligation and puncture. *Shock* **13**, 110-116 (2000).
375 Osuchowski, M. F., Welch, K., Siddiqui, J. & Remick, D. G. Circulating cytokine/inhibitor profiles reshape the understanding of the SIRS/CARS continuum in sepsis and predict mortality. *The Journal of Immunology* **177**, 1967-1974 (2006).
376 Yilmaz, M. S. *et al.* Lipopolysaccharide-inducedhypotension is mediated by a neural pathway involving the vagus nerve, the nucleus tractus solitarius and alpha-adrenergic receptors in the preoptic anterior hypothalamic area. *Journal of neuroimmunology* **203**, 39 - 49 (2008).
377 Feleder, C., Sertac Yilmaz, M., Peng, J., Göktalay, G. & Millington, W. R. The OVLT initiates the fall in arterial pressure evoked by high dose lipopolysaccharide: Evidence that dichotomous, dose-related mechanisms mediate endotoxic hypotension. *Journal of Neuroimmunology* **285**, 94-100, doi:10.1016/j.jneuroim.2015.05.023 (2015).
378 Freise, H., Brückner, U. & Spiegel, H. Animal models of sepsis. *Journal of Investigative Surgery* **14**, 195-212 (2001).
379 Rittirsch, D., Huber-Lang, M. S., Flierl, M. A. & Ward, P. A. Immunodesign of experimental sepsis by cecal ligation and puncture. *Nat. Protocols* **4**, 31-36 (2009).
380 Hubbard, W. J. Cecal ligation and puncture. *Shock* **24**, 52-57 (2005).

381 Oliveira-Pelegrin, G. R. *et al.* Cleaved caspase-3 expression in hypothalamic magnocellular neurons may affect vasopressin secretion during experimental polymicrobial sepsis. *Journal of Neuroimmunology* **258**, 10-16, doi:http://dx.doi.org/10.1016/j.jneuroim.2013.02.007 (2013).
382 Remick, D. G., Bolgos, G., Copeland, S. & Siddiqui, J. Role of interleukin-6 in mortality from and physiologic response to sepsis. *Infection and immunity* **73**, 2751-2757 (2005).
383 Chaudry, I. H., Wichterman, K. A. & Baue, A. E. Effect of sepsis on tissue adenine nucleotide levels. *YMSY</cja:jid> Surgery* **85**, 205-211 (1979).
384 Russell, J. A. *et al.* Vasopressin versus norepinephrine infusion in patients with septic shock. *New England Journal of Medicine* **358**, 877-887 (2008).
385 Takeuchi, O. & Akira, S. Pattern Recognition Receptors and Inflammation. *Cell* **140**, 805-820, doi:http://dx.doi.org/10.1016/j.cell.2010.01.022 (2010).
386 Cinel Ismail, I. & Dellinger, R. P. Advances in pathogenesis and management of sepsis. *Current Opinion in Infectious Diseases* **20**, 345-352.
387 Hotchkiss, R. S., Monneret, G. & Payen, D. Sepsis-induced immunosuppression: from cellular dysfunctions to immunotherapy. *Nature reviews. Immunology* **13**, 862-874 (2013).
388 Kindt, T. J., Goldsby, R. A., Osborne, B. A., Kuby, J. & Kuby, J. *Kuby immunology*. (W.H. Freeman, 2007).
389 Albelda, S. M., Smith, C. W. & Ward, P. Adhesion molecules and inflammatory injury. *The FASEB Journal* **8**, 504-512 (1994).
390 Kimmoun, A., Ducrocq, N. & Levy, B. Mechanisms of vascular hyporesponsiveness in septic shock. *Current vascular pharmacology* **11**, 139-149 (2013).
391 Ahrens, T. Hemodynamics in sepsis. *AACN advanced critical care* **17**, 435-445 (2006).
392 Cavazzoni, S. Z. & Dellinger, R. P. Hemodynamic optimization of sepsis-induced tissue hypoperfusion. *Critical Care* **10**, S2 (2006).
393 Semeraro, N., Ammollo, C. T., Semeraro, F. & Colucci, M. Sepsis-Associated Disseminated Intravascular Coagulation and Thromboembolic Disease. *Mediterranean Journal of Hematology and Infectious Diseases* **2**, e2010024, doi:10.4084/MJHID.2010.024 (2010).
394 Obritsch, M. D., Bestul, D. J., Jung, R., Fish, D. N. & MacLaren, R. The role of vasopressin in vasodilatory septic shock. *Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy* **24**, 1050-1063 (2004).
395 Rangel-Frausto, M. S. *et al.* The natural history of the systemic inflammatory response syndrome (SIRS): a prospective study. *Jama* **273**, 117-123 (1995).
396 Dünser, M. W., Wenzel, V., Mayr, A. J. & Hasibeder, W. R. Management of Vasodilatory Shock. *Drugs* **63**, 237-256 (2003).
397 Reid, I. A. Role of Vasopressin Deficiency in the Vasodilation of Septic Shock. *Circulation* **95**, 1108-1110, doi:10.1161/01.cir.95.5.1108 (1997).
398 Matsuda, N. & Hattori, Y. Vascular biology in sepsis: pathophysiological and therapeutic significance of vascular dysfunction. *Journal of smooth muscle research = Nihon Heikatsukin Gakkai kikanshi* **43**, 117-137 (2007).
399 Schrier, R. W. & Wang, W. Acute renal failure and sepsis. *New England Journal of Medicine* **351**, 159-169 (2004).
400 Landry, D. W. & Oliver, J. A. The pathogenesis of vasodilatory shock. *New England Journal of Medicine* **345**, 588-595 (2001).

401 De Cruz, S. J., Kenyon, N. J. & Sandrock, C. E. Bench-to-bedside review: the role of nitric oxide in sepsis. *Expert Review of Respiratory Medicine* **3**, 511-521, doi:10.1586/ers.09.39 (2009).
402 Kirkebøen, K. A. & Strand, Ø. A. The role of nitric oxide in sepsis – an overview. *Acta Anaesthesiologica Scandinavica* **43**, 275-288, doi:10.1034/j.1399-6576.1999.430307.x (1999).
403 Rees, D. Role of nitric oxide in the vascular dysfunction of septic shock. *Biochemical Society Transactions* **23**, 1025-1029 (1995).
404 Doughty, L. A., Kaplan, S. S. & Carcillo, J. A. Inflammatory cytokine and nitric oxide responses in pediatric sepsis and organ failure. *Critical care medicine* **24**, 1137-1143 (1996).
405 Groeneveld, P., Kwappenberg, K., Langermans, J., Nibbering, P. & Curtis, L. Nitric oxide (NO) production correlates with renal insufficiency and multiple organ dysfunction syndrome in severe sepsis. *Intensive care medicine* **22**, 1197-1202 (1996).
406 Loscalzo, J. & Welch, G. Nitric oxide and its role in the cardiovascular system. *Progress in cardiovascular diseases* **38**, 87-104 (1995).
407 Hauser, B., Bracht, H., Matejovic, M., Radermacher, P. & Venkatesh, B. Nitric oxide synthase inhibition in sepsis? Lessons learned from large-animal studies. *Anesthesia & Analgesia* **101**, 488-498 (2005).
408 Kilbourn, R. G. *et al.* Reversal of endotoxin-mediated shock by N G-methyl-L-arginine, an inhibitor of nitric oxide synthesis. *Biochemical and biophysical research communications* **172**, 1132-1138 (1990).
409 Thiemermann, C. & Vane, J. Inhibition of nitric oxide synthesis reduces the hypotension induced by bacterial lipopolysaccharides in the rat in vivo. *European journal of pharmacology* **182**, 591-595 (1990).
410 Grover, R. *et al.* An open-label dose escalation study of the nitric oxide synthase inhibitor, N (G)-methyl-L-arginine hydrochloride (546C88), in patients with septic shock. Glaxo Wellcome International Septic Shock Study Group. *Critical care medicine* **27**, 913-922 (1999).
411 Petros, A., Bennett, D. & Vallance, P. Effect of nitric oxide synthase inhibitors on hypotension in patients with septic shock. *The Lancet* **338**, 1557-1558 (1991).
412 Lorente, J. A., Landín, L., de Pablo, R., Renes, E. & Liste, D. L-arginine pathway in the sepsis syndrome. *Critical care medicine* **21**, 1287-1295 (1993).
413 Bakker, J. *et al.* Administration of the nitric oxide synthase inhibitor NG-methyl-l-arginine hydrochloride (546C88) by intravenous infusion for up to 72 hours can promote the resolution of shock in patients with severe sepsis: Results of a randomized, double-blind, placebo-controlled multicenter study (study no. 144-002)*. *Critical care medicine* **32**, 1-12 (2004).
414 López, A. *et al.* Multiple-center, randomized, placebo-controlled, double-blind study of the nitric oxide synthase inhibitor 546C88: effect on survival in patients with septic shock. *Critical care medicine* **32**, 21-30 (2004).
415 Miyoshi, H., Nakaya, Y. & Moritoki, H. Nonednfothelial-derived nitric-oxide activates the ATP-sensitive K+ channel of vascular smooth-muscle cells *FEBS Lett.* **345**, 47-49, doi:10.1016/0014-5793(94)00417-x (1994).

416 Landry, D. & Oliver, J. The ATP-sensitive K+ channel mediates hypotension in endotoxemia and hypoxic lactic acidosis in dog. *Journal of Clinical Investigation* **89**, 2071 (1992).
417 Sorrentino, R. *et al.* Involvement of ATP-sensitive potassium channels in a model of a delayed vascular hyporeactivity induced by lipopolysaccharide in rats. *BPH British Journal of Pharmacology* **127**, 1447-1453 (1999).
418 Levy, B. *et al.* Vascular hyporesponsiveness to vasopressors in septic shock: from bench to bedside. *Intensive Care Med Intensive Care Medicine* **36**, 2019-2029 (2010).
419 Vanelli, G., Hussain, S. N. & Aguggini, G. Glibenclamide, a blocker of ATP-sensitive potassium channels, reverses endotoxin-induced hypotension in pig. *Experimental Physiology* **80**, 167-170 (1995).
420 Buckley, J. F., Singer, M. & Clapp, L. H. Role of KATP channels in sepsis. *Cardiovascular research* **72**, 220-230, doi:10.1016/j.cardiores.2006.07.011 (2006).
421 Warrillow, S., Egi, M. & Bellomo, R. Randomized, double-blind, placebo-controlled crossover pilot study of a potassium channel blocker in patients with septic shock. *Critical care medicine* **34**, 980-985 (2006).
422 Morelli, A. *et al.* Phenylephrine versus norepinephrine for initial hemodynamic support of patients with septic shock: a randomized, controlled trial. *Critical Care* **12**, R143 (2008).
423 Baker, C. & Wilmoth, F. Microvascular responses to E. coli endotoxin with altered adrenergic activity. *Circulatory shock* **12**, 165-176 (1983).
424 Silverman, H. J., Penaranda, R., Orens, J. B. & Lee, N. H. Impaired [beta]-adrenergic receptor stimulation of cyclic adenosine monophosphate in human septic shock: Association with myocardial hyporesponsiveness to catecholamines. *Critical care medicine* **21**, 31-39 (1993).
425 Meadows, D., Edwards, D. J., Wilkins, R. G. & Nightingale, P. Reversal of intractable septic shock with norepinephrine therapy. *Critical care medicine* **16**, 663-666 (1988).
426 Chernow, B. & Roth, B. L. Pharmacologic manipulation of the peripheral vasculature in shock: clinical and experimental approaches. *Circulatory shock* **18**, 141 (1986).
427 Landry, D. W. *et al.* Vasopressin pressor hypersensitivity in vasodilatory septic shock. *Critical care medicine* **25**, 1279-1282 (1997).
428 Landry, D. W. *et al.* Vasopressin deficiency contributes to the vasodilation of septic shock. *Circulation* **95**, 1122-1125 (1997).
429 Altura, B. Evidence that endogenous vasopressin plays a protective role in circulatory shock. Role for reticuloendothelial system using Brattleboro rats. *Experientia* **36**, 1080-1082 (1980).
430 Brackett, D. J., Schaefer, C. F. & Wilson, M. F. The role of vasopressin in the maintenance of cardiovascular function during early endotoxin shock. *Advances in shock research* **9**, 147-156 (1983).
431 Laycock, J., Penn, W., Shirley, D. & Walter, S. The role of vasopressin in blood pressure regulation immediately following acute haemorrhage in the rat. *The Journal of physiology* **296**, 267-275 (1979).
432 Valtin, H. & Schroeder, H. Familial hypothalamic diabetes insipidus in rats (Brattleboro strain). *American Journal of Physiology--Legacy Content* **206**, 425-430 (1964).

433 Graybiel, A. & Glendy, R. E. Circulatory effects following the intravenous administration of Pitressin in normal persons and in patients with hypertension and angina pectoris. *American Heart Journal* **21**, 481-489 (1941).
434 Wagner Jr, H. N. & Braunwald, E. The pressor effect of the antidiuretic principle of the posterior pituitary in orthostatic hypotension. *Journal of Clinical Investigation* **35**, 1412 (1956).
435 Aisenbrey, G. A., Handelman, W. A., Arnold, P., Manning, M. & Schrier, R. W. Vascular effects of arginine vasopressin during fluid deprivation in the rat. *Journal of Clinical Investigation* **67**, 961 (1981).
436 Errington, M. L. & Rocha e Silva, M., Jr. The secretion and clearance of vasopressin during the development of irreversible haemorrhagic shock. *J Physiol* **217 Suppl**, 43p-45p (1971).
437 Morales, D. *et al.* Reversal by vasopressin of intractable hypotension in the late phase of hemorrhagic shock. *Circulation* **100**, 226-229 (1999).
438 Argenziano, M. *et al.* Management of vasodilatory shock after cardiac surgery: Identification of predisposing factors and use of a novel pressor agent. *The Journal of Thoracic and Cardiovascular Surgery* **116**, 973-980, doi:http://dx.doi.org/10.1016/S0022-5223(98)70049-2 (1998).
439 Morales, D. L. *et al.* A double-blind randomized trial: prophylactic vasopressin reduces hypotension after cardiopulmonary bypass. *The Annals of thoracic surgery* **75**, 926-930 (2003).
440 Argenziano, M. *et al.* A prospective randomized trial of arginine vasopressin in the treatment of vasodilatory shock after left ventricular assist device placement. *Circulation* **96**, II-286-290 (1997).
441 Chen, J. M. *et al.* Vasopressin deficiency and pressor hypersensitivity in hemodynamically unstable organ donors. *Circulation* **100**, II-244-Ii-246 (1999).
442 Sharshar, T. *et al.* Circulating vasopressin levels in septic shock. *Critical care medicine* **31**, 1752-1758 (2003).
443 Zerbe, R. L., Feurstein, G. & Kopin, I. J. Effect of captopril on cardiovascular, sympathetic and vasopressin responses to hemorrhage. *European journal of pharmacology* **72**, 391-395 (1981).
444 Zerbe, R. L., Bayorh, M. A. & Feuerstein, G. Vasopressin: an essential pressor factor for blood pressure recovery following hemorrhage. *Peptides* **3**, 509-514 (1982).
445 Arnauld, E., Czernichow, P., Fumoux, F. & Vincent, J.-D. The effects of hypotension and hypovolaemia on the liberation of vasopressin during haemorrhage in the unanaesthetized monkey (Macaca mulatta). *Pflügers Archiv* **371**, 193-200 (1977).
446 Jochberger, S. *et al.* Serum vasopressin concentrations in critically ill patients. *Critical care medicine* **34**, 293-299 (2006).
447 Lin, I.-Y. *et al.* Low plasma vasopressin/norepinephrine ratio predicts septic shock. *The American journal of emergency medicine* **23**, 718-724 (2005).
448 Leclerc, F. *et al.* Admission plasma vasopressin levels in children with meningococcal septic shock. *Intensive care medicine* **29**, 1339-1344 (2003).
449 Wilson, M. *et al.* Elevated plasma vasopressin concentrations during endotoxin and E. coli shock. *Advances in shock research* **6**, 15-26 (1980).
450 Wilson, M. *et al.* Vasopressin release during sepsis and septic shock in baboons and dogs. *Surgery, gynecology & obstetrics* **153**, 869-872 (1981).

451 Brackett, D. *et al.* Evaluation of cardiac output, total peripheral vascular resistance, and plasma concentrations of vasopressin in the conscious, unrestrained rat during endotoxemia. *Circulatory shock* **17**, 273-284 (1984).
452 Dadoun, F. *et al.* Effect of endotoxin on the hypothalamic-pituitary-adrenal axis in sheep. *European Journal of Endocrinology* **138**, 193-197 (1998).
453 Giusti-Paiva, A., De Castro, M., Antunes-Rodrigues, J. & Carnio, E. C. Inducible nitric oxide synthase pathway in the central nervous system and vasopressin release during experimental septic shock*. *Critical care medicine* **30**, 1306-1310 (2002).
454 Oliveira-Pelegrin, G. R., Ravanelli, M. I. B., Branco, L. G. S. & Rocha, M. J. A. Thermoregulation and vasopressin secretion during polymicrobial sepsis. *Neuroimmunomodulation* **16**, 45-53 (2009).
455 Oliveira-Pelegrin, G. R., de Azevedo, S. V., Yao, S. T., Murphy, D. & Rocha, M. J. A. Central NOS inhibition differentially affects vasopressin gene expression in hypothalamic nuclei in septic rats. *Journal of neuroimmunology* **227**, 80-86 (2010).
456 Oliveira-Pelegrin, G. R., Aguila, F. A., Basso, P. J. & Rocha, M. J. A. Role of central NO-cGMP pathway in vasopressin and oxytocin gene expression during sepsis. *Peptides* **31**, 1847-1852 (2010).
457 Corrêa, P. B. F., Pancoto, J. A. T., de Oliveira-Pelegrin, G. R., Cárnio, E. C. & Rocha, M. J. A. Participation of iNOS-derived NO in hypothalamic activation and vasopressin release during polymicrobial sepsis. *Journal of neuroimmunology* **183**, 17-25 (2007).
458 Tsuneyoshi, I. *et al.* Hemodynamic and metabolic effects of low-dose vasopressin infusions in vasodilatory septic shock. *CRITICAL CARE MEDICINE-BALTIMORE-* **29**, 487-493 (2001).
459 Patel, B. M., Chittock, D. R., Russell, J. A. & Walley, K. R. Beneficial effects of short-term vasopressin infusion during severe septic shock. *The Journal of the American Society of Anesthesiologists* **96**, 576-582 (2002).
460 Ertmer, C. *et al.* Methylprednisolone reverses vasopressin hyporesponsiveness in ovine endotoxemia. *Shock* **27**, 281-288 (2007).
461 Pancoto, J. A. T., Corrêa, P. B. F., Oliveira-Pelegrin, G. R. & Rocha, M. J. A. Autonomic dysfunction in experimental sepsis induced by cecal ligation and puncture. *Autonomic Neuroscience* **138**, 57-63 (2008).
462 Malay, M. B., Ashton Jr, R. C., Landry, D. W. & Townsend, R. N. Low-dose vasopressin in the treatment of vasodilatory septic shock. *The Journal of trauma* **47**, 699-703; discussion 703-695 (1999).
463 Dünser, M. W. *et al.* Arginine Vasopressin in Advanced Vasodilatory Shock A Prospective, Randomized, Controlled Study. *Circulation* **107**, 2313-2319 (2003).
464 Luckner, G. *et al.* Arginine vasopressin in 316 patients with advanced vasodilatory shock. *Critical care medicine - Baltimore* **33**, 2659 (2005).
465 Russell, J. A. *et al.* Interaction of vasopressin infusion, corticosteroid treatment, and mortality of septic shock*. *Critical care medicine* **37**, 811-818 (2009).
466 Gordon, A. C. *et al.* The effects of vasopressin on acute kidney injury in septic shock. *Intensive care medicine* **36**, 83-91 (2010).
467 Russell, J. A. *et al.* Vasopressin compared with norepinephrine augments the decline of plasma cytokine levels in septic shock. *American journal of respiratory and critical care medicine* **188**, 356-364 (2013).

468 Lauzier, F., Lévy, B., Lamarre, P. & Lesur, O. Vasopressin or norepinephrine in early hyperdynamic septic shock: a randomized clinical trial. *Intensive care medicine* **32**, 1782-1789 (2006).
469 Critchley, H. D. & Harrison, N. A. Visceral influences on brain and behavior. *Neuron* **77**, 624-638 (2013).
470 Dantzer, R., O'Connor, J. C., Freund, G. G., Johnson, R. W. & Kelley, K. W. From inflammation to sickness and depression: when the immune system subjugates the brain. *Nature reviews neuroscience* **9**, 46-56 (2008).
471 Dubos, R. J. & Schaedler, R. W. The effect of bacterial endotoxins on the water intake and body weight of mice. *The Journal of experimental medicine* **113**, 921 (1961).
472 Holmes, J. E. & Miller, N. E. Effects of bacterial endotoxin on water intake, food intake, and body temperature in the albino rat. *The Journal of experimental medicine* **118**, 649-658 (1963).
473 Calapai, G., Squadrito, F., Massi, M., Caputi, A. & De Caro, G. Endotoxin inhibition of drinking behaviour in the rat. *Pharmacological research* **22**, 161-170 (1990).
474 Nava, F. & Carta, G. Repeated lipopolysaccharide administration produces tolerance to anorexia and fever but not to inhibition of thirst in rat. *International journal of immunopharmacology* **22**, 943-953 (2000).
475 Raghavendra, V., Agrewala, J. & Kulkarni, S. Role of centrally administered melatonin and inhibitors of COX and NOS in LPS-induced hyperthermia and adipsia. *Prostaglandins, Leukotrienes and Essential Fatty Acids (PLEFA)* **60**, 249-253 (1999).
476 Baert, K., De Boever, S., Duchateau, L. & De Backer, P. Sodium salicylate attenuates lipopolysaccharide (LPS)-induced adipsia, but not hypophagia, in broiler chickens. *British poultry science* **46**, 144-148 (2005).
477 Foca, A., Matera, G., Mastroeni, P. & Caputi, A. Evidence that prostaglandins within preoptic area (POA) may mediate the antidipsogenic effect of Escherichia coli endotoxin in the rat. *Circulatory shock* **17**, 137-145 (1984).
478 Calapai, G. *et al.* Mediation by nitric oxide formation in the preoptic area of endotoxin and tumour necrosis factor- induced inhibition of water intake in the rat. *British journal of pharmacology* **111**, 1328-1332 (1994).
479 Calapai, G. *et al.* Cloricromene antagonizes antidipsogenic effects induced by endotoxin, but not by TNFα, in the rat. *Life sciences* **51**, 2041-2048 (1992).
480 Siami, S. *et al.* Thirst perception and osmoregulation of vasopressin secretion are altered during recovery from septic shock. *PloS one* **8**, e80190 (2013).
481 Plata-Salamán, C. R., Oomura, Y. & Kai, Y. Tumor necrosis factor and interleukin-1β: suppression of food intake by direct action in the central nervous system. *Brain research* **448**, 106-114 (1988).
482 Luca, L. A. *et al.* Participation of α2- adrenoceptors in sodium appetite inhibition during sickness behaviour following administration of lipopolysaccharide. *The Journal of physiology* (2015).
483 Foca, A., Matera, G., Chisari, M., Mastroeni, P. & Caputi, A. Effects of different bacterial endotoxins on the drinking behaviour of the rat. *Giornale di batteriologia, virologia ed immunologia* **77**, 9-18 (1983).
484 Santiago, M. B., Vieira, A. A., Elias, L. L., Rodrigues, J. A. & Giusti-Paiva, A. Neurohypophyseal response to fluid resuscitation with hypertonic saline during septic shock in rats. *Exp Physiol* **98**, 556-563, doi:10.1113/expphysiol.2012.066241 (2013).

485 Grinevich, V., Ma, X. M., Jirikowski, G., Verbalis, J. & Aguilera, G. Lipopolysaccharide endotoxin potentiates the effect of osmotic stimulation on vasopressin synthesis and secretion in the rat hypothalamus. *Journal of neuroendocrinology* **15**, 141-149 (2003).
486 de Carvalho Borges, B. *et al.* Lesion of the anteroventral third ventricle (AV3V) reduces hypothalamic activation and hypophyseal hormone secretion induced by lipopolysaccharide in rats. *Brain Res* **1115**, 83-91, doi:10.1016/j.brainres.2006.07.087 (2006).
487 Siami, S. *et al.* Osmoregulation of vasopressin secretion is altered in the postacute phase of septic shock*. *Critical care medicine* **38**, 1962-1969 (2010).
488 Tazinafo, L. F., Felippotti, T. T. & da Rocha, M. J. A. Vasopressin secretion in sepsis-surviving animals following dehydration. *Critical Care* **17**, P103 (2013).
489 Granger, J. I., Ratti, P. L., Datta, S. C., Raymond, R. M. & Opp, M. R. Sepsis-induced morbidity in mice: effects on body temperature, body weight, cage activity, social behavior and cytokines in brain. *Psychoneuroendocrinology* **38**, 1047-1057, doi:10.1016/j.psyneuen.2012.10.010 (2013).
490 Hart, B. L. Biological basis of the behavior of sick animals. *Neuroscience & Biobehavioral Reviews* **12**, 123-137 (1988).
491 Maxime, V., Siami, S. & Annane, D. Metabolism modulators in sepsis: the abnormal pituitary response. *Critical care medicine* **35**, S596-S601 (2007).
492 Mazeraud, A. *et al.* Neuroanatomy and Physiology of Brain Dysfunction in Sepsis. *Clinics in chest medicine* **37**, 333-345 (2016).
493 Giusti-Paiva, A. & Brian Santiago, M. Neurohypophyseal dysfunction during septic shock. *Endocrine, Metabolic & Immune Disorders-Drug Targets (Formerly Current Drug Targets-Immune, Endocrine & Metabolic Disorders)* **10**, 247-251 (2010).
494 Annane, D. *et al.* Inappropriate sympathetic activation at onset of septic shock: a spectral analysis approach. *American journal of respiratory and critical care medicine* **160**, 458-465 (1999).
495 Munford, R. S. & Tracey, K. J. Is severe sepsis a neuroendocrine disease? *Molecular Medicine* **8**, 437 (2002).
496 Kelley, K. W. *et al.* Cytokine-induced sickness behavior. *Brain, behavior, and immunity* **17**, 112-118 (2003).
497 Wahab, F., Atika, B., Ravanelli Oliveira-Pelegrin, G. & Jose Alves Rocha, M. Recent advances in the understanding of sepsis-induced alterations in the neuroendocrine system. *Endocrine, Metabolic & Immune Disorders-Drug Targets (Formerly Current Drug Targets-Immune, Endocrine & Metabolic Disorders)* **13**, 335-347 (2013).
498 Bluthé, R.-M. *et al.* Lipopolysaccharide induces sickness behaviour in rats by a vagal mediated mechanism. *Comptes rendus de l'Academie des sciences. Serie III, Sciences de la vie* **317**, 499-503 (1994).
499 Watkins, L. *et al.* Neurocircuitry of illness-induced hyperalgesia. *Brain research* **639**, 283-299 (1994).
500 Banks, W. A. Blood-brain barrier transport of cytokines: a mechanism for neuropathology. *Current pharmaceutical design* **11**, 973-984 (2005).
501 Konsman, J. P., Vigues, S., Mackerlova, L., Bristow, A. & Blomqvist, A. Rat brain vascular distribution of interleukin- 1 type- 1 receptor immunoreactivity: Relationship to patterns of inducible cyclooxygenase expression by peripheral inflammatory stimuli. *Journal of Comparative Neurology* **472**, 113-129 (2004).

502 Schiltz, J. C. & Sawchenko, P. E. Distinct brain vascular cell types manifest inducible cyclooxygenase expression as a function of the strength and nature of immune insults. *The Journal of neuroscience* **22**, 5606-5618 (2002).
503 Cunningham, C., Wilcockson, D. C., Campion, S., Lunnon, K. & Perry, V. H. Central and systemic endotoxin challenges exacerbate the local inflammatory response and increase neuronal death during chronic neurodegeneration. *The Journal of Neuroscience* **25**, 9275-9284 (2005).
504 de Paiva, V. N. *et al.* Prostaglandins mediate depressive-like behaviour induced by endotoxin in mice. *Behavioural brain research* **215**, 146-151 (2010).
505 Yi, E. & Ulich, T. R. Endotoxin, interleukin-1, and tumor necrosis factor cause neutrophil-dependent microvascular leakage in postcapillary venules. *The American journal of pathology* **140**, 659 (1992).
506 Saper, C. B., Romanovsky, A. A. & Scammell, T. E. Neural circuitry engaged by prostaglandins during the sickness syndrome. *Nature neuroscience* **15**, 1088-1095 (2012).
507 Ott, D. *et al.* Neurons and glial cells of the rat organum vasculosum laminae terminalis directly respond to lipopolysaccharide and pyrogenic cytokines. *Brain research* **1363**, 93-106 (2010).
508 Kent, S., Bluthé, R.-M., Kelley, K. W. & Dantzer, R. Sickness behavior as a new target for drug development. *Trends in pharmacological sciences* **13**, 24-28 (1992).
509 Buckley, C. D., Gilroy, D. W., Serhan, C. N., Stockinger, B. & Tak, P. P. The resolution of inflammation. *Nat Rev Immunol* **13**, 59-66 (2013).
510 Roberts, N. J. Impact of temperature elevation on immunologic defenses. *Review of Infectious Diseases* **13**, 462-472 (1991).
511 Cunningham, E. T. & Sawchenko, P. E. Reflex control of magnocellular vasopressin and oxytocin secretion. *Trends in neurosciences* **14**, 406-411 (1991).
512 Sagar, S. M., Price, K. J., Kasting, N. W. & Sharp, F. R. Anatomic patterns of Fos immunostaining in rat brain following systemic endotoxin administration. *Brain research bulletin* **36**, 381-392 (1995).
513 Athayde, L. A., Oliveira-Pelegrin, G. R., Nomizo, A., Faccioli, L. & Rocha, M. A. Blocking central leukotrienes synthesis affects vasopressin release during sepsis. *Neuroscience* **160**, 829-836 (2009).
514 Ueta, Y. *et al.* Transgenic expression of enhanced green fluorescent protein enables direct visualization for physiological studies of vasopressin neurons and isolated nerve terminals of the rat. *Endocrinology* **146**, 406-413 (2005).
515 Stare, J. & Bourque, C. W. Measuring rat serum osmolality by freezing point osmometry. *Bio-protocol* **in press** (2016).
516 Abbadi, A., El-Khoury, J. M. & Wang, S. Stability of serum and plasma osmolality in common clinical laboratory storage conditions. *Clinical biochemistry* **47**, 686-687 (2014).
517 Curria, A., Pesta, E. G., Zampa, N., Rosenman, J. & Sacks, J. in *Scientific Poster, presented at the 2009 AACC Annual Meeting, Chicago, IL.*
518 Seifarth, C. C., Miertschischk, J., Hahn, E. G. & Hensen, J. Measurement of serum and plasma osmolality in healthy young humans–influence of time and storage conditions. *Clinical chemistry and laboratory medicine* **42**, 927-932 (2004).
519 Stachniak, T. J. *et al.* Osmoregulatory circuits in slices and en bloc preparations of rodent hypothalamus. *Isolated Central Nervous System Circuits*, 211-231 (2012).

520 Trudel, E. & Bourque, C. W. A rat brain slice preserving synaptic connections between neurons of the suprachiasmatic nucleus, organum vasculosum lamina terminalis and supraoptic nucleus. *Journal of neuroscience methods* **128**, 67-77 (2003).
521 Samson, W. Atrial natriuretic factor inhibits dehydration and hemorrhage-induced vasopressin release. *Neuroendocrinology* **40**, 277-279 (1985).
522 Yosten, G. L. & Samson, W. K. Pressor doses of vasopressin result in only transient elevations in plasma peptide levels. *Peptides* **33**, 342-345 (2012).
523 Keil, L. C., Rosella-Dampman, L. M., Emmert, S., Chee, O. & Summy-Long, J. Y. Enkephalin inhibition of angiotensin-stimulated release of oxytocin and vasopressin. *BRES Brain Research* **297**, 329-336 (1984).
524 Foca, A., Nicoletta, P., Matera, G., Mastroeni, P. & Caputi, A. Antidipsogenic effect of endotoxin in the rat. *Circulatory shock* **11**, 341-350 (1982).
525 Nava, F., Calapai, G., De Sarro, A. & Caputi, A. P. Interleukin-1 receptor antagonist does not reverse lipopolysaccharide-induced inhibition of water intake in rat. *European journal of pharmacology* **309**, 223-227 (1996).
526 Wang, K. & Evered, M. D. Endotoxin stimulates drinking in rats without changing dehydrational signals controlling thirst. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* **265**, R1043-R1051 (1993).
527 Wang, K., Waselenchuk, L. & Evered, M. D. Stimulation of drinking by bacterial endotoxins in the rat. *Physiology & behavior* **54**, 1005-1009 (1993).
528 Jones, C. W. & Pickering, B. Comparison of the effects of water deprivation and sodium chloride imbibition on the hormone content of the neurohypophysis of the rat. *The Journal of physiology* **203**, 449 (1969).
529 Kafa, I. M., Uysal, M., Bakirci, S. & Ayberk, K. M. Sepsis induces apoptotic cell death in different regions of the brain in a rat model of sepsis. *Acta neurobiologiae experimentalis* **70**, 246-260 (2009).
530 Dejager, L., Pinheiro, I., Dejonckheere, E. & Libert, C. Cecal ligation and puncture: the gold standard model for polymicrobial sepsis? *Trends in microbiology* **19**, 198-208 (2011).
531 Bourque, C. W., Ciura, S., Trudel, E., Stachniak, T. J. & Sharif- Naeini, R. Neurophysiological characterization of mammalian osmosensitive neurones. *Experimental physiology* **92**, 499-505 (2007).
532 Rivest, S. & Laflamme, N. Neuronal Activity and Neuropeptide Gene Transcription in the Brains of Immune- Challenged Rats. *Journal of neuroendocrinology* **7**, 501-525 (1995).
533 Xia, Y. & Krukoff, T. Cardiovascular responses to subseptic doses of endotoxin contribute to differential neuronal activation in rat brain. *Molecular brain research* **89**, 71-85 (2001).
534 du Plessis, I., Mitchell, D., Niesler, C. & Laburn, H. P. c-Fos immunoreactivity in selected brain regions of rats after heat exposure and pyrogen administration. *Brain research* **1120**, 124-130 (2006).
535 Zhang, Z. & Bourque, C. W. Osmometry in osmosensory neurons. *Nature Neurosci.* **6**, 1021-1022 (2003).
536 Voisin, D. L., Chakfe, Y. & Bourque, C. W. Coincident detection of CSF Na+ and osmotic pressure in osmoregulatory neurons of the supraoptic nucleus. *Neuron* **24**, 453-460 (1999).

537 Martins, T. F., Sorgi, C. A., Faccioli, L. H. & Rocha, M. J. A. Leukotriene synthesis inhibitor decreases vasopressin release in the early phase of sepsis. *Journal of neuroimmunology* **238**, 52-57 (2011).
538 Bourque, C. W. Activity-dependent modulation of nerve terminal excitation in a mammalian peptidergic system. *Trends in neurosciences* **14**, 28-30 (1991).
539 Sharif-Naeini, R., Ciura, S. & Bourque, C. W. TRPV1 gene required for thermosensory transduction and anticipatory secretion from vasopressin neurons during hyperthermia. *Neuron* **58**, 179-185 (2008).
540 Sudbury, J. R., Ciura, S., Sharif- Naeini, R. & Bourque, C. W. Osmotic and thermal control of magnocellular neurosecretory neurons–role of an N- terminal variant of trpv1. *European Journal of Neuroscience* **32**, 2022-2030 (2010).
541 Sudbury, J. R. & Bourque, C. W. Dynamic and Permissive Roles of TRPV1 and TRPV4 Channels for Thermosensation in Mouse Supraoptic Magnocellular Neurosecretory Neurons. *The Journal of Neuroscience* **33**, 17160-17165, doi:10.1523/jneurosci.1048-13.2013 (2013).
542 Cunningham, J. T. *et al.* Cardiovascular regulation of supraoptic vasopressin neurons. *Progress in brain research* **139**, 256-273 (2002).
543 Kuzmiski, J. B., Pittman, Q. J. & Bains, J. S. Metaplasticity of hypothalamic synapses following in vivo challenge. *Neuron* **62**, 839-849 (2009).
544 Sonneville, R. *et al.* Vasopressin synthesis by the magnocellular neurons is different in the supraoptic nucleus and in the paraventricular nucleus in human and experimental septic shock. *Brain Pathology* **20**, 613-622 (2010).
545 Bicknell, R. J. Optimizing release from peptide hormone secretory nerve terminals. *J. Exp. Biol.* **139**, 51-65 (1988).
546 Bourque, C. W. Activity-dependent modulation of nerve terminal excitation in a mammalian peptidergic system. *Trends Neurosci.* **14**, 28-30 (1991).
547 Bourque, C. W. Osmoregulation of vasopressin neurons: A synergy of intrinsic and synaptic processes. *Progress in brain research* **119**, 59-76 (1999).
548 Stare, J. *et al.* Effects of Peritoneal Sepsis on Rat Central Osmoregulatory Neurons Mediating Thirst and Vasopressin Release. *Journal of Neuroscience* **35**, 12188-12197, doi:10.1523/Jneurosci.5420-13.2015 (2015).
549 Kimura, T. *et al.* Effects of Acute Water Load, Hypertonic Saline Infusion, and Furosemide Administration on Atrial Natriuretic Peptide and Vasopressin Release in Humans*. *The Journal of Clinical Endocrinology & Metabolism* **62**, 1003-1010 (1986).
550 Bicknell, R. J. Optimizing release from peptide hormone secretory nerve terminals. *J Exp Biol* **139**, 51-65 (1988).
551 Stare, J. *et al.* Effects of Peritoneal Sepsis on Rat Central Osmoregulatory Neurons Mediating Thirst and Vasopressin Release. *The Journal of Neuroscience* **35**, 12188-12197, doi:10.1523/jneurosci.5420-13.2015 (2015).
552 Sendemir, E., Kafa, I., Schäfer, H. & Jirikowski, G. Altered oxytocinergic hypothalamus systems in sepsis. *Journal of chemical neuroanatomy* **52**, 44-48 (2013).
553 Ott, D. *et al.* Neurons and glial cells of the rat organum vasculosum laminae terminalis directly respond to lipopolysaccharide and pyrogenic cytokines. *Brain Res* **1363**, 93-106, doi:10.1016/j.brainres.2010.09.083 (2010).

554 Hosoi, T., Okuma, Y. & Nomura, Y. The mechanisms of immune-to-brain communication in inflammation as a drug target. *Current drug targets. Inflammation and allergy* **1**, 257-262 (2002).
555 Trudel, E. & Bourque, C. W. A rat brain slice preserving synaptic connections between neurons of the suprachiasmatic nucleus, organum vasculosum lamina terminalis and supraoptic nucleus. *J Neurosci Methods* **128**, 67-77 (2003).
556 Trudel, E. & Bourque, C. W. Central clock excites vasopressin neurons by waking osmosensory afferents during late sleep. *Nat Neurosci* **13**, 467-474, doi:nn.2503 [pii] 10.1038/nn.2503 (2010).
557 Stachniak, T. J., Trudel, E. & Bourque, C. W. Cell-specific retrograde signals mediate antiparallel effects of angiotensin II on osmoreceptor afferents to vasopressin and oxytocin neurons. *Cell reports* **8**, 355-362, doi:10.1016/j.celrep.2014.06.029 (2014).
558 Granger, J. I., Ratti, P.-L., Datta, S. C., Raymond, R. M. & Opp, M. R. Sepsis-induced morbidity in mice: Effects on body temperature, body weight, cage activity, social behavior and cytokines in brain. *Psychoneuroendocrinology* **38**, 1047-1057, doi:http://dx.doi.org/10.1016/j.psyneuen.2012.10.010 (2013).
559 Brooks, H., Osabutey, C., Moss, R., Andrews, P. & Davies, D. Caecal ligation and puncture in the rat mimics the pathophysiological changes in human sepsis and causes multi-organ dysfunction. *Metabolic brain disease* **22**, 353-373 (2007).
560 Abbott, S. B., Machado, N. L., Geerling, J. C. & Saper, C. B. Reciprocal Control of Drinking Behavior by Median Preoptic Neurons in Mice. *J Neurosci* **36**, 8228-8237, doi:10.1523/JNEUROSCI.1244-16.2016 (2016).
561 Gizowski, C., Zaelzer, C. & Bourque, C. W. Clock-driven vasopressin neurotransmission mediates anticipatory thirst prior to sleep. *Nature* **537**, 685-688, doi:10.1038/nature19756 (2016).
562 Kirkman, M. A., Albert, A. F., Ibrahim, A. & Doberenz, D. Hyponatremia and brain injury: historical and contemporary perspectives. *Neurocrit Care* **18**, 406-416, doi:10.1007/s12028-012-9805-y (2013).
563 Verbalis, J. G. Disorders of body water homeostasis. *Best Pract Res Clin Endocrinol Metab* **17**, 471-503, doi:S1521690X03000496 [pii] (2003).
564 Miller, M. Hyponatremia and arginine vasopressin dysregulation: mechanisms, clinical consequences, and management. *Journal of the American Geriatrics Society* **54**, 345-353 (2006).
565 Dreyfuss, D., Leviel, F., Paillard, M., Rahmani, J. & Coste, F. Acute infectious pneumonia is accompanied by a latent vasopressin-dependent impairment of renal water excretion. *Am Rev Respir Dis* **138**, 583-589 (1988).
566 Rosenow 3rd, E., Segar, W. & Zehr, J. in *Mayo Clinic Proceedings*. 169-174.
567 Shann, F. & Germer, S. Hyponatraemia associated with pneumonia or bacterial meningitis. *Archives of disease in childhood* **60**, 963-966 (1985).
568 Konsman, J., Kelley, K. & Dantzer, R. Temporal and spatial relationships between lipopolysaccharide-induced expression of fos, interleukin-1 β and inducible nitric oxide synthase in rat brain. *Neuroscience* **89**, 535-548 (1999).
569 Elmquist, J. K., Scammell, T. E., Jacobson, C. D. & Saper, C. B. Distribution of fos- like immunoreactivity in the rat brain following intravenous lipopolysaccharide administration. *Journal of Comparative Neurology* **371**, 85-103 (1996).

570 Lacroix, S. & Rivest, S. Functional circuitry in the brain of immune- challenged rats: Partial involvement of prostaglandins. *Journal of Comparative Neurology* **387**, 307-324 (1997).
571 Tkacs, N. C., Li, J. & Strack, A. M. Central amygdala Fos expression during hypotensive or febrile, nonhypotensive endotoxemia in conscious rats. *Journal of Comparative Neurology* **379**, 592-602 (1997).
572 Hare, A., Clarke, G. & Tolchard, S. Bacterial Lipopolysaccharide- Induced Changes in FOS Protein Expression in the Rat Brain: Correlation with Thermoregulatory Changes and Plasma Corticosterone. *Journal of neuroendocrinology* **7**, 791-799 (1995).
573 Quan, N., Stern, E. L., Whiteside, M. B. & Herkenham, M. Induction of pro-inflammatory cytokine mRNAs in the brain after peripheral injection of subseptic doses of lipopolysaccharide in the rat. *Journal of neuroimmunology* **93**, 72-80 (1999).
574 Gizowski, C., Zaelzer, C. & Bourque, C. Clock-driven vasopressin neurotransmission mediates anticipatory thirst prior to sleep. *Nature* **537**, 685-688 (2016).
575 Sharshar, T. *et al.* Apoptosis of neurons in cardiovascular autonomic centres triggered by inducible nitric oxide synthase after death from septic shock. *The Lancet* **362**, 1799-1805, doi:http://dx.doi.org/10.1016/S0140-6736(03)14899-4 (2003).
576 Noda, M. Hydromineral neuroendocrinology: mechanism of sensing sodium levels in the mammalian brain. *Experimental physiology* **92**, 513-522 (2007).
577 Chakfe, Y., Zhang, Z. & Bourque, C. W. IL-1β directly excites isolated rat supraoptic neurons via upregulation of the osmosensory cation current. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* **290**, R1183-R1190 (2006).
578 Ferri, C. C., Yuill, E. A. & Ferguson, A. V. Interleukin-1β depolarizes magnocellular neurons in the paraventricular nucleus of the hypothalamus through prostaglandin-mediated activation of a non selective cationic conductance. *Regulatory peptides* **129**, 63-71 (2005).
579 Penninger, J. M. & Crabtree, G. R. The actin cytoskeleton and lymphocyte activation. *Cell* **96**, 9-12 (1999).
580 Zeng, C. & Morrison, A. R. Disruption of the actin cytoskeleton regulates cytokine-induced iNOS expression. *American Journal of Physiology-Cell Physiology* **281**, C932-C940 (2001).
581 Stamatovic, S., Dimitrijevic, O., Keep, R. & Andjelkovic, A. in *Brain Edema XIII* 444-450 (Springer, 2006).
582 Koukouritaki, S. *et al.* TNF-alpha induces actin cytoskeleton reorganization in glomerular epithelial cells involving tyrosine phosphorylation of paxillin and focal adhesion kinase. *Molecular Medicine* **5**, 382 (1999).
583 Desmouliere, A. & Gabbiani, G. Modulation of fibroblastic cytoskeletal features during pathological situations: the role of extracellular matrix and cytokines. *Cell motility and the cytoskeleton* **29**, 195-203 (1994).
584 Lupp, C. *et al.* Differentiated control of deranged nitric oxide metabolism: a therapeutic option in sepsis? *Critical Care* **17**, 1 (2013).
585 Calapai, G. & Caputi, A. P. Nitric oxide and drinking behaviour. *Regulatory peptides* **66**, 117-121 (1996).
586 Calapai, G. *et al.* Evidence that nitric oxide modulates drinking behaviour. *Neuropharmacology* **31**, 761-764 (1992).

587 Liu, H., Terrell, M. L., Bui, V., Summy-Long, J. Y. & Kadekaro, M. Nitric oxide control of drinking, vasopressin and oxytocin release and blood pressure in dehydrated rats. *Physiology & behavior* **63**, 763-769 (1998).
588 Cao, L., Sun, X. & Shen, E. Nitric oxide stimulates both the basal and reflex release of vasopressin in anesthetized rats. *Neuroscience letters* **221**, 49-52 (1996).
589 Kozak, W. & Kozak, A. Genetic Models in Applied Physiology: Selected Contribution: Differential role of nitric oxide synthase isoforms in fever of different etiologies: studies using Nos gene-deficient mice. *J Appl Physiol* **94**, 2534-2544 (2003).
590 Steiner, A. A., Antunes-Rodrigues, J., McCann, S. M. & Branco, L. G. Antipyretic role of the NO-cGMP pathway in the anteroventral preoptic region of the rat brain. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* **282**, R584-R593 (2002).
591 Lin, J.-H. & Lin, M.-T. Inhibition of nitric oxide synthase or cyclo-oxygenase pathways in organum vasculosum laminae terminalis attenuates interleukin-1β fever in rabbits. *Neuroscience letters* **208**, 155-158 (1996).
592 Feleder, C., Perlik, V. & Blatteis, C. M. Preoptic nitric oxide attenuates endotoxic fever in guinea pigs by inhibiting the POA release of norepinephrine. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* **293**, R1144-R1151 (2007).
593 Amir, S., De Blasio, E. & English, A. M. NG-Monomethyl-L-arginine co-injection attenuates the thermogenic and hyperthermic effects of E2 prostaglandin microinjection into the anterior hypothalamic preoptic area in rats. *Brain research* **556**, 157-160 (1991).
594 Ataŏglu, H., Dŏgan, M. D., Mustafa, F. & Akarsu, E. S. Candida albicans and Saccharomyces cerevisiae cell wall mannans produce fever in rats: role of nitric oxide and cytokines. *Life sciences* **67**, 2247-2256 (2000).
595 Almeida, M. C., Trevisan, F. N., Barros, R. C., Carnio, E. C. & Branco, L. G. Tolerance to lipopolysaccharide is related to the nitric oxide pathway. *Neuroreport* **10**, 3061-3065 (1999).
596 Steiner, A. A. & Branco, L. G. Nitric oxide in the regulation of body temperature and fever. *Journal of Thermal Biology* **26**, 325-330 (2001).
597 Kadekaro, M. & Summy- Long, J. Y. Centrally produced nitric oxide and the regulation of body fluid and blood pressure homeostases. *Clinical and Experimental Pharmacology and Physiology* **27**, 450-459 (2000).
598 Liu, H., Terrell, M., Summy-Long, J. & Kadekaro, M. Drinking and blood pressure responses to central injection of L-NAME in conscious rats. *Physiology & behavior* **59**, 1137-1145 (1996).
599 Ribeiro, D. E. *et al.* Inhibition of nitric oxide synthase accentuates endotoxin-induced sickness behavior in mice. *Pharmacology Biochemistry and Behavior* **103**, 535-540 (2013).
600 Kadekaro, M. Nitric oxide modulation of the hypothalamo-neurohypophyseal system. *Brazilian Journal of Medical and Biological Research* **37**, 441-450 (2004).
601 Stern, J. E. & Ludwig, M. NO inhibits supraoptic oxytocin and vasopressin neurons via activation of GABAergic synaptic inputs. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology* **280**, R1815-R1822 (2001).

602 Jackson, M. B., Konnerth, A. & Augustine, G. J. Action potential broadening and frequency-dependent facilitation of calcium signals in pituitary nerve terminals. *Proceedings of the National Academy of Sciences* **88**, 380-384 (1991).
603 Ahern, G. P., Hsu, S. F. & Jackson, M. B. Direct actions of nitric oxide on rat neurohypophysial K+ channels. *The Journal of physiology* **520**, 165-176 (1999).
604 Krukoff, T. L. Central actions of nitric oxide in regulation of autonomic functions. *Brain research reviews* **30**, 52-65 (1999).
605 Calabrese, V. *et al.* Nitric oxide in the central nervous system: neuroprotection versus neurotoxicity. *Nat Rev Neurosci* **8**, 766-775 (2007).
606 Wong, M.-L. *et al.* Inducible nitric oxide synthase gene expression in the brain during systemic inflammation. *Nature medicine* **2**, 581-584 (1996).
607 Carnio, E. C. *et al.* Vasopressin release during endotoxaemic shock in mice lacking inducible nitric oxide synthase. *Pflügers Archiv* **450**, 390-394 (2005).
608 Giusti-Paiva, A., Elias, L. L. K. & Antunes-Rodrigues, J. Inhibitory effect of gaseous neuromodulators in vasopressin and oxytocin release induced by endotoxin in rats. *Neuroscience letters* **381**, 320-324 (2005).
609 Kojima, H. *et al.* Detection and imaging of nitric oxide with novel fluorescent indicators: diaminofluoresceins. *Analytical chemistry* **70**, 2446-2453 (1998).
610 Frenkel, S. R. *et al.* Effects of nitric oxide on chondrocyte migration, adhesion, and cytoskeletal assembly. *Arthritis & rheumatism* **39**, 1905-1912 (1996).
611 Clancy, R., Leszczynska, J., Amin, A., Levartovsky, D. & Abramson, S. B. Nitric oxide stimulates ADP ribosylation of actin in association with the inhibition of actin polymerization in human neutrophils. *Journal of leukocyte biology* **58**, 196-202 (1995).
612 Scheiermann, C., Kunisaki, Y. & Frenette, P. S. Circadian control of the immune system. *Nature Reviews Immunology* **13**, 190-198 (2013).
613 Cermakian, N., Westfall, S. & Kiessling, S. Circadian clocks and inflammation: reciprocal regulation and shared mediators. *Archivum immunologiae et therapiae experimentalis* **62**, 303-318 (2014).
614 Friese, R. S., Bruns, B. & Sinton, C. M. Sleep deprivation after septic insult increases mortality independent of age. *Journal of Trauma and Acute Care Surgery* **66**, 50-54 (2009).
615 Nygård, M., Lundkvist, G. B., Hill, R. H. & Kristensson, K. Rapid nitric oxide-dependent effects of tumor necrosis factor-α on suprachiasmatic nuclei neuronal activity. *Neuroreport* **20**, 213-217 (2009).
616 Kwak, Y. *et al.* Interferon-γ alters electrical activity and clock gene expression in suprachiasmatic nucleus neurons. *Journal of biological rhythms* **23**, 150-159 (2008).
617 O'Callaghan, E. K., Anderson, S. T., Moynagh, P. N. & Coogan, A. N. Long-lasting effects of sepsis on circadian rhythms in the mouse. *PloS one* **7**, e47087 (2012).

6.0 APPENDICES

6.1 Supplementary Data

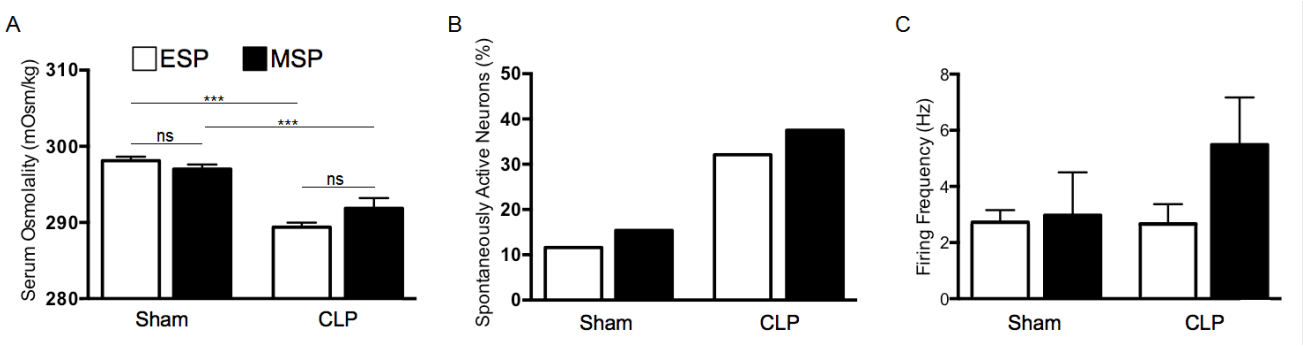

Figure 6.1.1 Preliminary data on circadian differences in serum osmolality and MNC firing behaviour. **A**, Serum osmolality was significantly higher (one-way ANOVA with Tukey's *post-hoc*, $F_{(3,88)} = 27.6$, $p < 0.0001$) in shams (ESP 298.1 ± 0.53 mOsm/kg, $n = 26$ rats; MSP 297.0 ± 0.60 mOsm/kg, $n = 21$ rats) compared to CLP rats (ESP 289.4 ± 6.1 mOsm/kg, $n = 24$ rats; MSP 291.9 ± 1.36 mOsm/kg, $n = 21$ rats), **B**, percent of spontaneously firing MNCs in shams (ESP 11.6%, $n = 5$ / 43 neurons, 3 rats; MSP 15.4%, $n = 2$ / 3 neurons, 1 rat) was significantly lower than in CLPs (ESP 32.1%, $n = 17$ / 53 neurons, 3 rats; MSP 37.5%, $n = 9$ / 24 neurons, 2 rats; χ^2, $p = 0.042$). **C,** MNC firing frequency was not statistically different (one-way ANOVA with Tukey's *post-hoc*, $F_{(2,26)} = 1.474$, $p = 0.2448$) between shams (ESP 2.73 ± 0.43 Hz, $n = 5$ neurons / 3 rats; MSP 2.98 ± 1.53 Hz, $n = 2$ neurons / 1 rat) and CLP rats (ESP 2.67 ± 0.70 Hz, $n = 16$ neurons / 3 rats; MSP 5.49 ± 1.7 Hz, $n = 7$ neurons / 2 rats). ESP, early sleep phase; MSP, late sleep phase; *** $p < 0.001$; ns, not significant.

6.2 Copyright Permissions

RE: Copyright Permission Request: FJ April 2015, 29(Suppl 1):968.17
JournalForms <JournalForms@faseb.org>
Mon 2016-12-05, 8:46 AMJerneja Stare <jerneja.stare@mail.mcgill.ca>;Mooneyhan, Cody <cmooneyhan@faseb.org>;M.R. Carey MRCarey@faseb.org

Permission is granted.

M.R. Carey
Journal Production Coordinator
FASEB Office of Publications
9650 Rockville Pike
Bethesda, Maryland 20814
Phone (301) 634-7801
E-mail mrcarey@faseb.org
Web www.FASEB.org

_____

From: Jerneja Stare [jerneja.stare@mail.mcgill.ca]
Sent: Friday, December 02, 2016 8:01 AM
To: JournalForms
Subject: Copyright Permission Request

1.
Jerneja Stare
2.
Abstract
3. Stare, J. et al. The Effects of Sepsis on Osmosensory Neurons Mediating Thirst. The FASEB Journal 29 (2015) http://www.fasebj.org/content/29/1_Supplement/968.17.short
4.
For republication in doctoral thesis.
5.
McGill University
http://www.mcgill.ca/physiology/home-page
6.
Any additional comments pertinent to the request.
- Request clarification that I may modify parts of the requested abstract for my thesis.

Thank you,

Jerneja

Jerneja Stare
Ph.D. Candidate, McGill University
Laboratory of Dr. C.W. Bourque
Centre for Research in Neuroscience
MGH-RI, Room R2-103
Montreal (QC) H3G 1A4
(514) 934-1934 x44605

bio-protocol

Bio-Protocol License to Publish

The following License must be signed and returned to Bio-protocol before a manuscript can be accepted for publication. If the copyright in the contribution is owned by an entity other than the author (such as the author's employer), the copyright owner or its authorized representative must co-sign this form.

This License to Publish ("**License**") is entered into as of 07/01 (mm/dd), 2016 (the "**Effective Date**") by and between Bio-protocol LLC ("**Bio-protocol**") and the person(s) identified as an author below (individually and collectively, "**Author**"). This License applies to the Author's protocol or article currently titled Measuring Rat Serum Osmolality by Freezing Point Osmometry and all associated supplemental materials, data, images, audio and/or video (collectively, the "**Article**"), which the Author is submitting for publication on Bio-protocol's website.

1. **License Grant.** Subject to the terms of this License, Author hereby grants to Bio-protocol the sole, exclusive, irrevocable, worldwide and royalty-free right to publish, reproduce, distribute, transmit, publicly display, store, translate, create derivative works from and otherwise use or exploit the Article in any form, manner, format or medium, whether known or hereafter developed, in any language, for the entire duration of any such right and any renewal or extension thereof and to permit and sublicense others through multiple tiers to do any or all of the foregoing.

2. **Reservation of Rights.**

a. Ownership. Author reserves ownership of the Article and all of Author's other proprietary rights with respect to the Article, including the copyright and any patent rights Author may have in any inventions described in the Article, subject to the rights granted to Bio-protocol in Section 1. Bio- protocol shall be the holder of title for the purpose of copyright registration.

b. Other Rights. Author retains the following non-exclusive rights to use the Article without further permission after the publication of the Article by Bio-protocol, provided that Author will reference (and, where applicable, require any third party to reference) the appropriate citation of the publication by Bio-protocol of the Article when exercising any of the rights retained by the Author in this subsection 2.b.:

i. Reprint the Article in print collections of Author's own writings;

ii. Reprint the Article in print format for inclusion in a thesis or dissertation that the Author writes for a degree granted by an educational institution;

iii. Present the Article orally;

iv. Reproduce the Article for use in courses that the Author is teaching (if the Author is employed by an academic institution, that institution may also reproduce the Article for course teaching at that institution);

v. Distribute photocopies of the Article to colleagues for non-commercial purposes only;

vi. Reuse figures or tables in the Article for future publications written by the Author; and

Author agrees to obtain prior consent from Bio-protocol for any uses not expressly authorized in this Section 2.

3. Enforcement. Author will promptly notify Bio-protocol if Author becomes aware of any known or suspected infringement of the copyright in the Article. Bio-protocol will be entitled to enforce the copyright against any third-party infringer. If Bio-protocol asserts or files any claim, suit, or action (a "**Claim**") against any such third-party infringer, Author will cooperate with Bio-protocol, at Bio- protocol's request, in the prosecution and settlement of such Claim, including joining as a party to such suit or action where reasonably required.

4. Warranties. Author represents and warrants that:

a. Author has all rights and licenses necessary to grant to Bio-protocol the rights in the Article specified in Section 1 above;

b. The Article has not been published anywhere else and Bio-protocol's use of the Article in compliance with the license granted in Section 1 will not infringe, misappropriate or violate a third party's intellectual property rights, rights of publicity or privacy, moral rights or any applicable law or regulation;

c. the Article is original, and that Author has made all reasonable efforts to ensure the accuracy of any factual information contained in the Article; and

d. Author's agreement to the terms and conditions of this License will not violate or conflict with any obligations owed by Author to a third party.

5. **Indemnification.** Author will indemnify and hold harmless Bio-protocol and its affiliates, employees, directors, officers, agents, customers and sublicensees against any and all claims, liabilities, damages, costs, and expenses directly or indirectly arising from or relating to any breach of or inaccuracy in any of representations or warranties made by Author under this License.

6. General. This License is governed by and construed in accordance with the laws of the State of California, excluding that body of law known as conflict of laws. Any legal action or proceeding arising under this License will be brought exclusively in the federal or state courts located in the Northern District of California and the parties hereby irrevocably consent to the personal jurisdiction and venue therein. Bio-protocol may freely assign or transfer this License, in whole or in part, by operation of law or otherwise. This License will bind and inure to the benefit of each party's successors and permitted assigns. If, for any reason, a court of competent jurisdiction finds any provision of this License invalid or unenforceable, that provision of the License will be enforced to the maximum extent permissible and the other provisions of this License will remain in full force and effect. The failure by either party to enforce any provision of this License will not constitute a waiver of future enforcement of that or any other provision. This License constitutes the complete and exclusive understanding and agreement between the parties regarding its subject matter and supersedes all prior or contemporaneous agreements or understandings, written or oral, relating to its subject matter. Any waiver, modification or amendment of any provision of this License will be effective only if in writing and signed by duly authorized representatives of both parties. This License may be executed in counterparts, each of which will be deemed an original, but all of which together will constitute one and the same instrument. In the event that Bio-protocol does not publish the Article in any medium, within two (2) years of the Effective Date, this License shall be null and void. Bio- protocol recognizes that an Article prepared under certain governmental grants or contracts may be subject to the government's prior non-exclusive rights to use the Article for non-commercial, governmental purposes to the extent provided in the contract or grant or other regulations.

If the Article was prepared jointly by multiple authors, please identify all co-authors below. If any co- authors or other copyright holders of the Article are not personally signing this License (each, a "**Non- Signing Author**"), the person designated below as the Corresponding Author represents and warrants that he or she (1) has informed all the Non-Signing Authors of the terms of this License, (2) has the authority to act as the agent for the Non-Signing Authors in relation to this License, and (3) is signing this License on behalf of all the Non-Signing Authors and also on his or her own behalf. The signatory must inform Bio-protocol of any changes in authorship.

bio-protocol

Corresponding Author
Name: Charles William Bourque Signature: _____ Date: July 4 2016
☑ Corresponding Author signing on behalf of all Non-Signing Authors.

Author
Name: _____ Signature: _____ Date: _____
(optional if the box for Corresponding Author is checked)

Author
Name: _____ Signature: _____ Date: _____
(optional if the box for Corresponding Author is checked)

Author
Name: _____ Signature: _____ Date: _____
(optional if the box for Corresponding Author is checked)

Author
Name: _____ Signature: _____ Date: _____
(optional if the box for Corresponding Author is checked)

The Journal of Neuroscience

J.Neurosci's Permissions Policy October 2, 2014

Starting in January of 2010, The Journal of Neuroscience adopted a License to Publish form to replace the previously used copyright form. The License to Publish form allows authors to retain the copyright to their article while granting the Society for Neuroscience a 6-month period to exclusively publish the article.

After six months, anyone may use the material for non-commercial purposes under the terms of the Creative Commons Attribution- Noncommercial-Share Alike 3.0 Unported License (http://creativecommons.org/licenses/by-nc-sa/3.0). This agreement allows data and text mining, use of figures in presentations, and posting the article online, as long as the original article is attributed.

Authors need NOT contact the journal to obtain rights for any non-commercial reuse their own material so long as authors provide attribution to the place of original publication and, for the first six months after publication, refrain from making the work publicly available. Authors are automatically granted permission to:

3. Reuse the article in print collections of their own writing.

4. Present a work orally in its entirety.

5. Use an article in a thesis and/or dissertation.

6. Reproduce an article for use in the author's courses.

7. Reuse a figure, photo and/or table in future non-commercial works. For any non-author uses of the work, please follow the "get permissions" link in the sidebar to the right of any full text article in J. Neurosci. This link will take you to Copyright Clearance Center, which processes these requests for J. Neurosci. If you are requesting permission to reprint with a nonprofit publisher, please email a detailed request directly to jnpermissions@sfn.org. In most cases, these requests are granted at no cost. Anyone requesting to reprint with a for-profit publisher, there will be a charge for each figure you reuse. We do not process these fees directly, but you can request permission online through the Copyright Clearance Center website. Please contact the J. Neurosci's Central office at jnpermissions@sfn.org with any questions.