Nesfatin-1 influences the excitability of neurons in the nucleus of the solitary tract and regulates cardiovascular function

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Submitted 20 May 2011; accepted in final form 21 March 2012

Mimee A, Smith PM, Ferguson AV. Nesfatin-1 influences the excitability of neurons in the nucleus of the solitary tract and regulates cardiovascular function. Am J Physiol Regul Integr Comp Physiol 302: R1297–R1304, 2012. First published March 21, 2012; doi:10.1152/ajpregu.00266.2011.—Nesfatin-1 has been identified as one of the most potent centrally acting anorexigenic peptides, and it has also been shown to play important roles in the control of cardiovascular function. In situ hybridization and immunohistochemical studies have revealed the expression of nesfatin-1 throughout the brain and, in particular, in the medullary autonomic gateway known as the nucleus of the solitary tract (NTS). The present study was thus undertaken to explore the cellular correlates and functional roles of nesfatin-1 actions in the medial NTS (mNTS). Using current-clamp electrophysiology recordings from mNTS neurons in slice preparation, we show that bath-applied nesfatin-1 directly influences the excitability of the majority of mNTS neurons by eliciting either depolarizing (42%, mean: 7.8 ± 0.8 mV) or hyperpolarizing (21%, mean: −8.2 ± 1.0 mV) responses. These responses were observed in all electrophysiologically defined cell types in the NTS and were site specific and concentration dependent. Furthermore, post hoc single cell reverse transcriptase polymerase reaction revealed a depolarizing action of nesfatin-1 on NPY and nucleobindin-2-expressing mNTS neurons. We have also correlated these actions of nesfatin-1 on neuronal membrane potential with physiological outcomes, using in vivo microinjection techniques to demonstrate that nesfatin-1 microinjected into the mNTS induces significant increases in both blood pressure (mean AUC (mean ± SEM) 201 ± 35 mmHg for the 300-μm coronal sections were cut using a vibratome (Leica, Nussloch, Germany). Slices were then incubated for 1 h before recording at 32°C in artificial cerebrospinal fluid (aCSF) made of (in mM) 125 NaCl, 2.5 KCl, 25 NaHCO₃, 2.5 CaCl₂, 1.25 MgCl₂, 1.0 NaH₂PO₄, 25 glucose, and 75 sucrose bubbled with 95% O₂-5% CO₂. A region of brain stem containing NTS was isolated, and microinjector (World Precision Instruments, Sarasota, FL) were pulled on a Sutter Instruments P97 flaming micropipette puller and filled with an intracellular solution made of (in mM) 125 potassium glutamate, 10 KCl, 0.1 CaCl₂, 5.5 EGTA, 10 HEPES, and 2 NaATP (pH 7.2 with KOH). When filled with the intracellular solution, electrodes had a resistance of 3–5 MΩ. After a high-resistance seal (>1 GΩ) was established, suction mode was applied to rupture the membrane and achieve whole cell configuration. Whole cell recordings were obtained with a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA) sampled at 10 kHz, filtered at 2.4 kHz using a Micro 1401 interface, and data were collected with Spike2 software for offline analysis (Cambridge Electronic Devices, Cambridge, UK). Neurons were rejected from further experimentation if they did not have action potentials with an amplitude greater than 60 mV or a stable baseline membrane potential. A liquid junction potential calculated to be ~15 mV has been subtracted from all membrane potentials. Before experimentation in current clamp, a series of current steps from −50 to −10 pA delivered
PCR products generated from the nested two reactions were run on 2.5% agarose gel containing ethidium bromide. With each reaction a positive and negative control was performed in which primers were run against cDNA made from whole NTS (positive control) and in a reaction which PCR grade water replaced the cDNA template (negative control). Experiments that did not pass either control were eliminated from the study.

**Microinjection.** Urethane-anesthetized (1.4 g/kg) male Sprague-Dawley rats (150–350 g) were fitted with a tracheal cannula (PE-205; Intramedic) to facilitate breathing and a femoral arterial catheter for the measurement of blood pressure (BP) and heart rate (HR). Animals were placed on a feedback-controlled heating blanket for the duration of the experiment to maintain body temperature at 37°C. The animal was then placed in a stereotaxic frame with its head positioned vertically (nose down). The dorsal surface of the medulla was exposed by a midline incision made at the level of the obex. A microinjection cannula (150 μm tip diameter; Rhodes Medical Instruments) was then positioned into the medial NTS (mNTS) and after a minimum 2 min stable baseline recording was obtained, 0.5 μl of 100 nM nesfatin-1 was microinjected into the region and the effects on BP and HR assessed.

At the conclusion of the experiment, animals were overdosed with anesthetic and perfused with 0.9% saline, followed by 10% Formalin, through the left ventricle of the heart. The brain was removed and placed in Formalin for at least 24 h. With the use of a vibratome, 50-μm coronal sections were cut through the region of NTS, mounted, and cresyl violet stained. The anatomical location of the microinjection site was verified at the light microscope level by an observer unaware of the experimental protocol or the data obtained.

**Analysis of blood pressure and heart rate data.** Animals were assigned to one of two groups (mNTS or non-NTS) according to the anatomical location of the microinjection sites. Animals with injection sites that were wholly confined to regions outside of the mNTS were classified as non-NTS sites. Injections that were on the border of mNTS and thus not wholly confined within mNTS were excluded from further analysis. Normalized BP and HR data (mean baseline BP and HR were calculated for 60 s before injection and subtracted from all data points before and after injection) were obtained for each animal 60 s before the time of microinjection (control period) until 500 s after microinjection. Area under the curve (AUC) (area between baseline and each blood pressure and heart rate response) was calculated for each animal for the 500-s time period immediately after the injection, and the mean AUC for BP and HR responses were then calculated. A Student’s t-test was used to determine whether BP and HR observed in response to nesfatin-1 were different.

**Table 1. Primers used in single cell RT-PCR experiments**

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GAPDH, glyceraldehyde 3-phosphate dehydrogenase; POMC, pro-opiomelanocortin; NPY, neuropeptide Y; GAD67, glutamate decarboxylase; NUCB2, nucleobindin-2; MC4R, melanocortin 4 receptor.
according to anatomical location of the microinjection site (mNTS vs. non-NTS). Vehicle (aCSF) control microinjections were not performed specifically for the current study as these experiments have been done previously in our laboratory and have been shown to be without effect on BP or HR (9).

Chemicals and drugs. All salts used to prepare slicing solution, aCSF and internal recording solution were obtained from Sigma Pharmaceuticals (Oakville, Ontario, Canada). Nesfatin-1 was obtained from Phoenix Pharmaceuticals (Belmont, CA). TTX citrate was obtained from Alomone Laboratories (Jerusalem, Israel).

RESULTS

Nesfatin-1 influences the excitability of mNTS neurons. Nesfatin-1 (10 nM) was bath applied to a total of 93 mNTS neurons during current-clamp recordings. The majority (63%) of neurons tested were responsive to nesfatin-1, with 42% (n = 39) exhibiting a slowly developing, long-lasting, and reversible depolarization (Fig. 1A). Another subset of neurons (21%, n = 20) showed temporally similar, reversible hyperpolarizations in response to 10 nM nesfatin-1 (Fig. 1B). The mean depolarization observed was 7.8 ± 0.8 mV, and the mean hyperpolarization was −8.2 ± 1.0 mV. The changes in membrane potential induced by nesfatin-1 corresponded with an altered firing frequency in the affected neurons. Cells that depolarized in response to nesfatin-1 showed significant increases in firing frequency from 0.12 to 1.1 Hz (paired t-test, P = 0.001), while those with a hyperpolarizing response were essentially silenced.
Nesfatin-1 effects are concentration dependent. Nesfatin-1 was next applied at varying concentrations to determine whether its effects on mNTS neurons are concentration dependent, as illustrated in Fig. 1D. When administered at a concentration of 100 pM ($n = 43$), nesfatin-1 influenced the same proportion of neurons and the observed effects were temporally similar to those observed at a concentration of 10 nM, though the amplitude of the response was reduced. Neurons showed both reversible depolarizing ($n = 17$, mean: 4.3 ± 0.5 mV) and hyperpolarizing ($n = 9$, mean: −3.5 ± 0.4 mV) responses to 100 pM nesfatin-1. A concentration of 10 pM nesfatin-1 failed to elicit any response ($n = 11$).

Nesfatin-1 exerts direct effects on mNTS neurons. We next investigated whether the depolarizing and hyperpolarizing effects of nesfatin-1 could occur in the presence of the voltage-gated sodium channel blocker TTX. A total of 12 mNTS neurons were examined in the presence of 1 μM TTX (Fig. 2, A and B) and 75% were responsive to nesfatin-1 in the presence of 1 μM TTX (Fig. 2, A and B), with 58% exhibiting a reversible depolarization ($n = 7$, mean: 7.6 ± 1.4 mV) and 17% a reversible hyperpolarization ($n = 2$, mean: −7.3 ± 3.4 mV). There was no difference in the magnitude of the depolarization ($P = 0.91$) or the hyperpolarization ($P = 0.79$) between TTX-treated and non-TTX-treated neurons. The proportion of responsive neurons in the presence of TTX was similar to that observed in its absence (Fig. 2C, $\chi^2 P = 0.50$), and there was no significant difference in the latency of effect to nesfatin-1 between TTX-treated and non-TTX-treated neurons (mean latency TTX-treated $= 139 \pm 24$ s, mean latency non-TTX-treated $= 132 \pm 8$ s, $P = 0.78$). These results thus suggest that nesfatin-1 influences the excitability of mNTS neurons via direct postsynaptic actions on these cells.

**Nesfatin-1 exerts similar effects on DE, PIR, and NON mNTS neurons.** The NTS contains three electrophysiologically defined cell types categorized based on their response to a large hyperpolarizing current pulse. Neurons showing a delayed return to baseline following the pulse due to a prominent A-type K$^+$ current are termed delayed excitation (DE) cells, those with a postinhibitory rebound (PIR) action potential due to a prominent low threshold Ca$^{2+}$ current are called PIR cells, and those with neither of these responses are NON cells (27). Since DE neurons have specifically been identified as exerting important control over autonomic processes (11, 24), we investigated whether nesfatin-1 would have selective effects on this specific cell type. Nesfatin-1 influenced the membrane potential of DE (70% depolarize, 15% hyperpolarize, 15% no response $= 13$, Fig. 3), PIR (52% depolarize, 14% hyperpolarize, 34% no response, $n = 21$), and NON cells (43% depolarize, 33% hyperpolarize, 24% no response, $n = 21$). Nesfatin-1 thus does not preferentially affect any particular cell type in the mNTS, suggesting that these effects cannot be...
attributed to the unique properties of a specific electrophysiological NTS cell type.

Nesfatin-1 depolarizes identified NPY and NUCB2 neurons and exerts heterogeneous effects on identified GAD67 and MC4R neurons. We next attempted to correlate electrophysiological actions of nesfatin-1 (10 nM) with the molecular phenotype of recorded neurons using post hoc single cell RT-PCR of the mRNA obtained from aspirated cytoplasm after completion of current-clamp recordings. These experiments were performed on a separate population of neurons as those used for the analyses based on electrophysiological fingerprint described above. There was no difference in the distribution of neurons that depolarized, did not respond, or hyperpolarized to 10 nM nesfatin-1 ($\chi^2 P = 0.43$) between these two sample populations nor in the amplitude of depolarizing responses (mean depolarization, 5.6 ± 0.6 mV, $P = 0.06$); however, the amplitude of hyperpolarizing responses was decreased in this population of neurons (mean hyperpolarization, −4.6 ± 0.4 mV, $P = 0.03$). Cytoplasm was obtained from a total of 56 neurons and was analyzed using primers directed at glyceraldehyde 3-phosphate dehydrogenase (GAPDH), pro-opiomelanocortin (POMC), neuropeptide Y (NPY), glutamate decarboxylase (GAD67, a GABAergic cell marker), nucleobindin-2 (NUCB2), and the melanocortin 4 receptor (MC4R). A total of 42 of 56 recorded neurons showed expression of the housekeeping gene GAPDH, indicating successful harvesting and amplification of mRNA, and we observed that most mNTS neurons expressed multiple genes of interest. We identified 11 NPY-expressing neurons, and all NPY neurons that were responsive to nesfatin-1 showed depolarizing responses ($n = 6$, Fig. 4, A and B). We found that all cells that expressed NUCB2 were depolarized by 10 nM nesfatin-1, suggesting a self-stimulatory action of this peptide on mNTS neurons ($n = 3$). Furthermore, 1 NUCB2 neuron coexpressed NPY, another NUCB2 neuron coexpressed GAD67, and a third NUCB2 neuron coexpressed both NPY and GAD67. Nesfatin-1 exerted heterogeneous effects on the 11 MC4R neurons we identified (depolarize = 6, hyperpolarize = 1) and on the 6 GAD67 neurons we identified (depolarize = 4, hyperpolarize = 2). Homogenous hyperpolarizing responses were not observed in any of the groups of identified neurons. We were unable to observe POMC expression in any of the 56 neurons examined.

Cardiovascular actions of nesfatin-1 in mNTS. We next examined the cardiovascular consequences of nesfatin-1 microinjection into the mNTS in a total of 25 urethane-anesthetized animals, of which 6 had microinjection sites histologically verified as within the anatomical boundaries of the mNTS, while 10 had sites outside of NTS (non-NTS). The remaining 9 animals had microinjection locations that could not be reliably classified as either NTS or non-NTS and thus were excluded from further analysis. Previous studies from our laboratory have confirmed that vehicle (aCSF) control microinjections into mNTS are without effect on BP or HR (9). Microinjection of 0.5 μl of 100 nM nesfatin-1 into mNTS caused a rapid, long-lasting increase in BP (mean AUC = 3354.1 ± 750.7 mmHg·s, $n = 6, P < 0.001$ compared with non-NTS sites), which was accompanied by an increase in HR (mean AUC = 164.8 ± 78.5 beats, $n = 6, P < 0.05$ compared with non-NTS sites) as illustrated in Fig. 5. These effects were shown to be site specific as microinjection into non-NTS sites was without effect on either BP (mean AUC = −638.3 ± 382.3 mmHg·s, $n = 10$) or HR (mean AUC = 17.8 ± 6.7 beats, $n = 10$).

DISCUSSION

The present study is the first to identify direct actions of the novel anorexigenic factor nesfatin-1 on the activity of electrophysiologically or neurochemically defined neuronal cell types in the NTS and to characterize potential physiological correlates of these effects on membrane potential to influence cardiovascular function in the whole animal. Our results reveal the mNTS may be a critical site through which nesfatin-1 exerts its effects on autonomic function.

To shed light on the mechanisms that underlie the satiety-promoting actions of nesfatin-1, we conducted in vitro whole cell patch-clamp experiments to examine the effect of nesfatin-1 on the excitability of neurons in the NTS, one of the most prominent central sites of nesfatin-1 expression (3, 5). Similarly to our findings in previous studies we performed in the paraventricular (PVN) and arcuate (ARC) nuclei of the hypothalamus (19, 20), nesfatin-1 exerted depolarizing or hyperpolarizing effects on different subsets of mNTS neurons tested. These opposing actions of the same peptide can potentially be attributed to the fact that nesfatin-1 may be affecting functionally separate populations of neurons that ultimately exert differential effects on cardiovascular regulation and energy homeostasis. An examination of the effects of nesfatin-1 on the three electrophysiologically defined populations of neurons in the NTS revealed this peptide both depolarizes and hyperpolarizes DE, PIR, and NON cells. Thus this categorization of neuronal populations cannot account for the differential effects of nesfatin-1. We therefore conducted studies to classify recorded neurons based on their peptide expression profile using post hoc single cell RT-PCR technology. Neurons in the NTS produce numerous peptides critically involved in the regulation of cardiovascular function and energy balance in addition to nesfatin-1, including the orexigenic NPY and anorexigenic POMC-derived melanocortins (13, 18). Indeed, our RT-PCR experiments now suggest potential coexpression of NUCB2...
and NPY in NTS neurons, as has been previously reported in the ARC (10). Furthermore, our RT-PCR studies revealed exclusively depolarizing actions of nesfatin-1 on NPY neurons in the mNTS, while our in vivo microinjections of nesfatin-1 into mNTS resulted in increases in BP and HR. Extensive studies conducted in the NTS have definitively established that microinjection of NPY into this medullary center causes decreases in BP and HR in the whole animal (1, 25). Furthermore, intracerebroventricular NPY injections and microinjections of NPY into other autonomic centers innervated by NTS NPY neurons, namely the PVN, also elicit bradycardic and depressor responses (21, 26). Thus stimulatory actions of nesfatin-1 on mNTS NPY neurons, involved either in local circuitry or projecting to higher brain centers, do not correlate with the ability of nesfatin-1 to increase HR and BP via the NTS. Our results therefore suggest that the medullary population of NPY neurons may not be responsible for the cardiovascular effects of nesfatin-1 observed in the mNTS. Future studies will thus be aimed at both identifying other neuronal populations that could mediate the nesfatin-1-induced effects on cardiovascular function (e.g., catecholaminergic neurons) and at elucidating the contribution of medullary NPY neurons to the cardiovascular and anorexigenic effects of this neuropeptide. Ultimately, the effects of nesfatin-1 on the cardiovascular system and on food intake are likely mediated via multiple populations of neurons in the NTS with different peptidergic profiles acting as part of a neural network involving other key autonomic and energy homeostatic centers such as the PVN, ARC, and lateral nucleus of the hypothalamus, which share either direct or indirect connections with the NTS. Thus we hypothesize that endogenous nesfatin-1 synthesized in the NTS can influence both incoming visceral signals on autonomic and energy status that are then relayed to higher brain centers, as well as inputs from hypothalamic regions, to ultimately modify cardiovascular parameters and feeding behaviour. Further studies aimed at elucidating the precise functional pathways of nesfatin-1 effects will be necessary to better understand its mechanism of action.

It is important to note that we were unable to detect the expression of POMC in any of the mNTS neurons we examined in the present study, and past experiments from our laboratory identified only 1 POMC-positive neuron from a population of 37 mNTS neurons (9). While this can partially be explained by the fact that the majority of POMC expressing cells in the NTS are located in the caudal commissural NTS (4) and our studies were focused on the medial NTS, it is also critical to mention that the NTS population of neurons expresses only 1/10 of the POMC mRNA expressed by neurons in the ARC (4). The lack of POMC-positive NTS neurons detected in our single cell RT-PCR studies provides important emphasis on how sparse the medullary POMC neuronal population is.

Fig. 4. Nesfatin-1 depolarizes NPY and nucleobindin-2 (NUCB2) mNTS neurons. A: current-clamp recording from a mNTS neuron in slice preparation showing a depolarizing response to 10 nM bath-applied nesfatin-1 (horizontal shaded bar). Note this neuron expresses multiple genes of interest as identified by post hoc single cell RT-PCR: glyceraldehyde 3-phosphate dehydrogenase (GAPDH), NPY, glutamate decarboxylase (GAD67), and NUCB2 (inset). B: scatter plot grouping recorded neurons according to molecular phenotype as identified by RT-PCR. Neurons that responded to 10 nM bath-applied nesfatin-1 and that expressed either NPY or NUCB2 exhibited only depolarizing responses. Neurons that expressed GAD67 or MC4R showed heterogeneous responses to nesfatin-1. For comparison, a scatter plot of all the nesfatin-1 responses exhibited by GAPDH-positive neurons is included.
We have also correlated the actions of nesfatin-1 on neuronal membrane potential with physiological outcomes, using in vivo microinjection techniques to demonstrate that nesfatin-1 microinjected into the NTS induces significant increases in both BP and HR in rats. These effects were site specific, as injections into sites outside of the NTS did not produce any changes in cardiovascular parameters. Our results are in accordance with previous studies demonstrating tachycardic and hypertensive effects of nesfatin-1 after intracerebroventricular injections into the lateral ventricle of rats (28). Thus it would appear nesfatin-1 is capable of exerting important effects on the cardiovascular system at both hypothalamic and medullary levels. Furthermore, Yosten and Samson (28) also showed the nesfatin-1-induced effects on cardiovascular parameters are sympathetically mediated, as they were blocked by a nonspecific α-adrenergic antagonist. We hypothesize the same may be true at the level of NTS, although we did not directly examine sympathetic contribution to the cardiovascular effects we observed. The NTS is a critically important site for the control of cardiovascular function, as it is the first central relay for baroreceptor afferents, and it receives projections from, and sends efferents to, multiple brain regions involved in the maintenance of cardiovascular homeostasis (14). Numerous peptides that exert both stimulatory and inhibitory effects on cardiovascular parameters are expressed and act at the level of the NTS to ultimately influence BP and HR in the whole animal. As holds true for nesfatin-1-induced effects on food intake, it has been shown that the hypothalamic nesfatin-1-mediated effects on cardiovascular function are abolished by pharmacological blockade of central melanocortin and oxytocin receptors (28, 29). Since both melanocortin and oxytocin receptors are expressed on neurons in the NTS (2, 12, 15), it is tempting to speculate that oxytocin and melanocortin responsive neurons, and potentially others as well, are acting together at the level of NTS to exert effects that are ultimately integrated to produce a tachycardic and vasopressor response. Future experiments will thus be aimed at evaluating the contribution of the melanocortin and oxytocin systems to nesfatin-1-induced cardiovascular effects in the medulla. Furthermore, the nesfatin-1 receptor remains unknown at the present time, and future identification of this receptor will allow us to further

Fig. 5. Nesfatin-1 microinjection into mNTS increases blood pressure (BP) and heart rate (HR). Normalized mean BP (A) and HR (C) traces showing the response to nesfatin-1 microinjection (black arrow) into mNTS (black trace, n = 6) or non-NTS sites (white trace, n = 10). Summary bar graphs show mean area under the curve for BP (B) and HR (D) in response to nesfatin-1 microinjection in mNTS (solid bar) or non-NTS sites (open bar). *P < 0.05, ***P < 0.001. E: schematic illustrating the anatomical location of individual microinjection sites classified either as mNTS (●) or non-NTS (■). The photomicrograph on the right shows a mNTS microinjection site (dashed circle). Note the blood vessel directly underneath the microinjection site. Scale bars indicate 200 μm. Numbers in left hand corner represent coordinates relative to Bregma. AP, area postrema.
examine which populations of neurons are influenced by nesfatin-1 to affect cardiovascular parameters.

**Perspectives and Significance**

Our electrophysiological, single cell RT-PCR, and in vivo studies of the effects of nesfatin-1 on neurons in the mNTS are the first to show this peptide exerts direct actions on the excitability of this neuronal population, and these effects ultimately result in increases in BP and HR in the whole animal. Furthermore, we demonstrate that nesfatin-1 stimulates mNTS NPY and NUCB2 neurons and provide additional evidence for a role of nesfatin-1 as a “self-stimulatory” peptide. Our results provide critical insight into the circuitry and physiology involved in the effects of nesfatin-1 and highlight the NTS as a key structure mediating these autonomic actions.

**ACKNOWLEDGMENTS**

We thank Christie DeVille for technical assistance.

**GRANTS**

This work was supported by funding from Natural Sciences and Engineering Research Council of Canada and Le Fonds Québécois de la Recherche sur la Nature et les Technologies to A. Mimii, a Canadian Institutes of Health Research Banting and Best studentship to P. M. Smith, and Heart and Stroke Foundation of Ontario to A. V. Ferguson.

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**

Author contributions: A.M., P.M.S., and A.V.F. conception and design of research; A.M. and P.M.S. performed experiments; A.M. and P.M.S. analyzed data; A.M. and P.M.S. interpreted results of experiments; A.M. and P.M.S. prepared figures; A.M. and P.M.S. drafted manuscript; A.M., P.M.S., and A.V.F. edited and revised manuscript; A.M., P.M.S., and A.V.F. approved final version of manuscript.

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