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

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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 medullary 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 transcription 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 = 3354.1 ± 750.7 mmHg·s, n = 6) and heart rate (mean AUC = 164.8 ± 78.5 beats, n = 6) in rats. Our results provide critical insight into the circuity and physiology involved in the profound effects of nesfatin-1 and highlight the NTS as a key structure mediating these autonomic actions.

EXPERIMENTAL PROCEDURES

Slice preparation. Unanesthetized male Sprague-Dawley rats aged 21–28 days (Charles River, Quebec, Canada) were decapitated and their brains briefly immersed in ice-cold carbogenated slicing solution made of (in mM) 87 NaCl, 2.5 KCl, 0.5 CaCl2, 7 MgCl2, 1.25 NaH2PO4, 25 glucose, and 75 sucrose bubbled with 95% O2-5% CO2. A region of brain stem containing NTS was isolated, and their brains briefly immersed in ice-cold carbogenated slicing solution 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 = 3354.1 ± 750.7 mmHg·s, n = 6) and heart rate (mean AUC = 164.8 ± 78.5 beats, n = 6) in rats. Our results provide critical insight into the circuity and physiology involved in the profound effects of nesfatin-1 and highlight the NTS as a key structure mediating these autonomic actions.

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Nesfatin-1 has been identified as one of the most potent centrally acting anorexigenic peptides (17). An 82 amino acid cleavage product of the nucleobindin-2 (NUCB2) protein, nesfatin-1 acutely and chronically reduces feeding in rodents when injected either intracerebroventricularly or peripherally, and these effects are dependent on melanocortin and oxytocin signaling (16, 17, 22, 29). Furthermore, treatment with nesfatin-1 neutralizing antibodies markedly increases food consumption and weight gain in animals, underscoring the critical physiological, tonic inhibitory control this peptide exerts over ingestive behaviors (17). In addition to its remarkable effects on food intake, nesfatin-1 has also been shown to play important roles in the control of cardiovascular function, water intake, blood glucose regulation, stress responses, and puberty onset (6, 8, 28, 29). In line with its numerous functions, comprehensive immunohistochemical studies have revealed a widespread distribution of nesfatin-1 throughout the brain. Notably, the nesfatin-1 peptide is strongly expressed in regions involved in the control of energy homeostasis and autonomic function in both hypothalamic and medullary centres (5), including the autonomic gateway known as the nucleus of the solitary tract (NTS). Furthermore, central or peripheral injections of nesfatin-1 result in c-Fos expression in NTS neurons (16, 22). However, despite the detailed knowledge of the central expression of nesfatin-1, to date there is minimal data describing the specific functional roles of nesfatin-1 in these autonomic nuclei, and what information is available has centered only on actions in the hypothalamus (16, 19, 20, 28, 29). The present study was thus undertaken to explore the cellular correlates and functional roles of nesfatin-1 actions in the medullary NTS using current-clamp electrophysiology, post hoc single cell reverse transcriptase polymerase reaction (RT-PCR), and microinjection techniques.

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at 5-s intervals in 10-pA increments were used to define neurons as postinhibitory rebound (PIR), delayed excitation (DE), or neither (NON) (27). Nesfatin-1 and tetrodotoxin (TTX) were applied to slices via bath perfusion. Response to nesfatin-1 was determined by comparing the membrane potential of the neuron before and after application of the peptide. A response was considered significant if the change in membrane potential after nesfatin-1 application was at least twice the amplitude of the standard deviation of the baseline membrane potential obtained during a 100-s period immediately before peptide application. Postapplication membrane potential averaged over a 100-s period of the recording showed a maximal effect. This method of analysis was employed as it provides a consistent, unbiased evaluation of effects on each individual neuron based on the variability of the baseline membrane potential of the neuron.

Single cell RT-PCR. After the completion of current-clamp recordings, single cell RT-PCR was performed to detect the expression of genes of interest in recorded NTS neurons. Cytoplasm was aspirated from the neuron using gentle suction, and the recording pipette was slowly withdrawn from the cell to form an outside-out patch. Neurons that did not successfully form outside-out patches were excluded from further analysis. The recording pipette was subsequently broken and its contents expelled into a 0.5-ml PCR tube containing the following to reverse transcribe the mRNA collected from the neuron: dithiothreitol (26 mM), dNTPs (10 mM), random hexamer primers (5 μM), MgCl₂ (5 mM), RNAse inhibitor (20 units), and Superscript II reverse transcriptase (100 units) (Invitrogen, Burlington, ON, Canada). An additional reaction mixture was made up without superscript II reverse transcriptase and served as a control for genomic contamination.

A multiplex PCR approach was then used to amplify the cDNA obtained from the reverse transcription. The first step was a multiplex reaction containing “outside” primers (0.2 μM, Table 1) for all the genes of interest, the cDNA from the single cell and the reagents provided in the Qiagen multiplex kit (Qiagen, Mississauga, ON, Canada) to a 100-μl volume. The reaction mixture then underwent the following temperature protocol: 15 min at 95°C, then 30 cycles at 94°C for 30 s, 60°C for 90 s, and 72°C for 90 s. The second step consisted of individual reactions for each gene of interest using “nested” primer sets (0.2 μM, Table 1), and the reagents provided in the Qiagen multiplex kit to a 50-μl volume. The reaction mixture then underwent 30 cycles of the temperature protocol described above. PCR products generated from the nested reaction were run on a 2% 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 between the two groups.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Outside</th>
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<td>tccgtttggatcgcctc</td>
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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.

Fig. 1. Nesfatin-1 influences the excitability of medial nucleus of solitary tract (mNTS) neurons. Current-clamp recordings from two mNTS neurons in slice preparation, showing depolarizing (A) and hyperpolarizing (B) responses to 10 nM bath-applied nesfatin-1 (horizontal shaded bar). In both neurons, a return to baseline membrane potential and firing frequency was seen after washout of nesfatin-1. C: scatter plot showing the range of responses elicited by bath application of 10 nM nesfatin-1. Solid bars represent mean response ± SE, while each single point represents the response of a single mNTS neuron. Mean depolarization: 7.8 ± 0.8 mV, mean hyperpolarization: −8.2 ± 1.0 mV; D: bar graph showing the mean responses elicited by bath application of 10 nM nesfatin-1 (solid), 100 pM nesfatin-1 (shaded), and 10 pM nesfatin-1 (open). Mean depolarization 10 nM: 7.8 ± 0.8 mV, n = 39, mean depolarization 100 nM: 4.3 ± 0.5 mV, n = 17, mean depolarization 10 pM: −3.5 ± 0.4 mV, n = 9. A concentration of 10 pM did not elicit a response in the neurons tested, n = 11.
by nesfatin-1 (decrease from 0.6 Hz to 0.002 Hz, \( P = 0.0007 \)). There was no correlation between baseline membrane potential, which ranged from \(-44\) to \(-78\) mV (mean: \(-62\) mV), and response to nesfatin-1. Finally, the effects of nesfatin-1 on the membrane potential of neurons were specific to the mNTS, as 10 nM bath-applied nesfatin-1 did not have an effect on neurons in the commissural NTS \((n = 10)\), a site without nesfatin-1 mRNA or protein expression \((5)\).

**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 \((n = 17, \text{mean: } 4.3 \pm 0.5 \text{ mV})\) and hyperpolarizing \((n = 9, \text{mean: } -3.5 \pm 0.4 \text{ mV})\) 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 were examined in the presence of 1 \(\mu\)M TTX (Fig. 2, A and B) and 75% were responsive to nesfatin-1 in the presence of 1 \(\mu\)M TTX (Fig. 2, A and B), with 58% exhibiting a reversible depolarization \((n = 7, \text{mean: } 7.6 \pm 1.4 \text{ mV})\) and 17% a reversible hyperpolarization \((n = 2, \text{mean: } -7.3 \pm 3.4 \text{ mV})\). 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 \((\text{Fig. 2C, } \chi^2 P = 0.50)\), and there was no significant difference in the latency of effect to nesfatin-1 between TTX-treated and non-TTX-treated neurons \((\text{mean latency TTX-treated } = 139 \pm 24 \text{ s, mean latency non-TTX-treated } = 132 \pm 8 \text{ s, } P = 0.78)\). 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 \((70\% \text{ depolarize, } 15\% \text{ hyperpolarize, } 15\% \text{ no response, } n = 13)\), PIR \((52\% \text{ depolarize, } 14\% \text{ hyperpolarize, } 34\% \text{ no response, } n = 21)\), and NON cells \((43\% \text{ depolarize, } 33\% \text{ hyperpolarize, } 24\% \text{ no response, } n = 21)\). 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 (χ² 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 effects of nesfatin-1. For comparison, a scatter plot of all the nesfatin-1 responses exhibited by GAPDH-positive neurons is included.

![Fig. 4. Nesfatin-1 depolarizes NPY and nucleobindin-2 (NUCB2) mNTS neurons.](image)

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.

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.
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 \(\alpha\)-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). \(* P < 0.05, \*** P < 0.001.\)

### Graphs

- **A:** Normalized mean BP traces showing the response to nesfatin-1 microinjection (black arrow) into mNTS (black trace, \(n=6\)) or non-NTS sites (white trace, \(n=10\)).
  - **B:** Summary bar graph showing mean area under the curve for BP in response to nesfatin-1 microinjection in mNTS (solid bar) or non-NTS sites (open bar).
  - **C:** Normalized mean HR traces showing the response to nesfatin-1 microinjection (black arrow) into mNTS (black trace, \(n=6\)) or non-NTS sites (white trace, \(n=10\)).
  - **D:** Summary bar graph showing mean area under the curve for HR in response to nesfatin-1 microinjection in mNTS (solid bar) or non-NTS sites (open bar).

### Diagrams

- **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.

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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. performed experiments; 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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