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ORIGINAL ARTICLE

Nesfatin-1 Influences the Excitability of Paraventricular Nucleus Neurones

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Nesfatin-1 is a newly-discovered satiety peptide found in several nuclei of the hypothalamus, including the paraventricular nucleus. To begin to understand the physiological mechanisms underlying these satiety-inducing actions, we examined the effects of nesfatin-1 on the excitability of neurones in the paraventricular nucleus. Whole-cell current-clamp recordings from rat paraventricular nucleus neurones showed nesfatin-1 to have either hyperpolarising or depolarising effects on the majority of neurones tested. Both types of response were observed in neurones irrespective of classification based on electrophysiological fingerprint (magnocellular, neuroendocrine or pre-autonomic) or molecular phenotype (vasopressin, oxytocin, corticotrophin-releasing hormone, thyrotrophin-releasing hormone or vesicular glutamate transporter), determined using single cell reverse transcription-poylmerase chain reaction. Consequently, we provide the first evidence that this peptide, which is produced in the paraventricular nucleus, has effects on the membrane potential of a large proportion of different subpopulations of neurones located in this nucleus, and therefore identify nesfatin-1 as a potentially important regulator of paraventricular nucleus output.

Key words: hypothalamus, satiety, single cell RT-PCR, patch clamp, brain slice.

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Recent attempts to find novel molecules involved with regulating appetite and energy balance have identified nesfatin-1 as a potent inhibitor of feeding activity (1). This peptide, arising from cleavage of the calcium-binding protein nucleobindin2, is expressed in many hypothalamic nuclei, including the paraventricular (PVN), arcuate, lateral hypothalamic and supraoptic nuclei (1). Significantly, in starved rats, nesfatin-1 levels decreased only in the PVN, a nucleus with a well known association with the control of feeding behaviour and metabolism (2, 3). These data identify the PVN as a location where signals associated with the depletion of energy reserves are received, resulting in the inhibition of the synthesis of this satiety signal (1). Although the PVN has been identified as a source for nesfatin-1, to date, no information is available regarding the roles of nesfatin-1 in controlling the excitability of the neurones in this vital hypothalamic autonomic control centre. Therefore, using whole-cell patch-clamp recordings from PVN neurones in rat brain slices, we performed experiments examining the effect of nesfatin-1 on the membrane potential and excitability of PVN neurones.

Materials and methods

Slice preparation

All animal procedures conformed to the standards of the Canadian Council on Animal Care and were approved by the Queen's University Animal Care Committee. Male Sprague-Dawley rats (Charles River, Quebec, Canada), postnatal days 21–27 (approximately 50–100 g), were used in the preparation of hypothalamic slices. Rats were quickly decapitated and the brain dissected out and placed into ice cold carbogenated slicing solution, consisting of (in mM): 87 NaCl, 2.5 KCl, 25 $NaHCO_3$, 0.5 $CaCl_2$, 7 $MgCl_2$, 1.25 NaH_2PO_4, 25 glucose and 75 sucrose. A tissue block containing the hypothalamus was obtained and 300 μm coronal slices cut using a vibratome. Slices were then stored in a water bath at 32 °C for at least 1 h, before recordings commenced, in carbogenated artificial cerebrospinal fluid (ACSF) composed of (in mM): 126 NaCl, 2.5 KCl, 26 $NaHCO_3$, 2 $CaCl_2$, 2 $MgCl_2$ 1.25 NaH_2PO_4 and 10 glucose.

Electrophysiology

Slices were placed in a chamber that was continuously perfused at approximately 2 ml/min with carbogenated ACSF heated to between 28 and 32 °C.

Neurones were visualised using an infrared differential interference contrast system on an upright microscope (Nikon, Tokyo, Japan). Whole cell current and voltage clamp recordings were made using a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA, USA) and sampled using a Micro1401 interface and Spike2 software (Cambridge Electronic Design, Cambridge, UK) for offline analysis. Digitisation rate was 5 kHz and signals were filtered at 10 kHz. Electrodes were filled with an intracellular solution that was prepared under RNase-free conditions and contained (in mM): 125 potassium gluconate, 2 $MgCl_2$, 5.5 ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′–tetraacetic acid, 10 KCl, 0.1 $CaCl_2$, 2 NaATP and 10 Hepes (pH 7.2–7.3 with KOH). When filled with this solution, electrodes had resistances of 3–7 MΩ. Neurones that did not have overshooting action potentials with amplitudes over 60 mV, or that did not possess a stable baseline, were excluded from further experiments. Nesfatin-1 was applied to the cell via the bath perfusion. Neurones were said to have responded to nesfatin-1 if there was a change in membrane potential that was at least twice the amplitude of the SD of the mean baseline membrane potential. This value was obtained by taking the mean ± SD of all the points during the 50-s control period immediately prior to peptide application. Response amplitudes were quantified by subtracting the mean baseline membrane potential from the peak membrane potential, averaged over a 50-s segment of the recording showing the maximal effect. To establish electrophysiological fingerprints for neurones in the PVN, neurones were maintained at a potential of approximately −75 mV and a series of current steps from −60 to +40 pA in 10 pA increments were administered to the neurone and the presence of a low threshold spike or prominent A current determined. Alternatively, neurones were given a series of biphasic current pulse, with the first pulse bringing the membrane potential to approximately −100 mV. This was immediately followed by a current pulse to bring the neurones to a less hyperpolarised potential and allow for the detection of low threshold spikes or an A current (4, 5). Input resistance measurements were obtained by delivering a series of hyperpolarising current pulses in 10-pA increments and obtaining the slope from the resulting voltage/current plot.

Single cell reverse transcription-polymerase chain reaction (PCR)

Following the completion of an electrophysiological experiment, gentle suction was applied to the electrode interior and, under visual control, the cytoplasmic contents were collected. The electrode was then carefully withdrawn from the cell until the electrode detached, forming an outside-out patch. The electrode tip was subsequently broken and its contents aspirated into a 0.5-ml microcentrifuge tube to which the following were added to set up the reverse transcriptase reaction (approximate final concentration): dithiothreitol (26 mM), dNTPs (3 mM), random heximer primers (3 μM), $MgCl_2$ (4 mM), RNase inhibitor (20 U) and superscript II reverse transcriptase (100 U) (all from Invitrogen, Burlington, Ontario, Canada). The reverse transcriptase reaction ran overnight at 37 °C after which the cDNA was stored at −80 °C until the start of the multiplex reaction.

A multiplex PCR approach was employed to amplify the cDNA obtained from the reverse transcriptase reaction using primers specific for certain neuropeptides and transmitters (Table 1). The first step was a multiplex reaction containing primers (outside) for all the genes of interest along with cDNA from the single cell. The second reaction was a nested PCR reaction using a single set of primers (nested) for each gene of interest. The initial multiplex reaction was performed in a 100 μl volume using the reagents provided with the Qiagen Multiplex kit (Qiagen, Mississauga, Ontario, Canada) and 0.2 μM of each primer. Each reaction was denatured at 95 °C for 15 min then cycled 20 times through a temperature protocol consisting of 30 s at 94 °C, 90 s at 60 °C and 90 s at 72 °C. The final product in early experiments was diluted 1 : 1000 and used as the template for the next round of PCR. In later experiments the first round product was used undiluted as template for the second round of PCR. The second round of PCR consisted of individual 50 μl reactions for each of the genes of interest. Reactions were performed again using the reagents contained in the Qiagen Multiplex kit and 0.2 μM of each primer. The reaction mixture was cycled 35 times through the same temperature protocol as indicated above, afterwards the PCR products were run on a 2% (w/v) agarose gel containing ethidium bromide and sequenced to confirm their identity (Robarts Institute, London, Ontario, Canada).

Chemicals and drugs

Salts used in the preparation of the intracellular solution and normal and high sucrose ACSF were obtained from Sigma (Oakville, Ontario, Canada). Nesfatin-1 was obtained as a gift from Phoenix Pharmaceuticals (Belmont, CA, USA), tetrodotoxin (TTX) citrate was obtained from Alomone Laboratories (Jerusalem, Israel).

Table 1. Inside and Outside Primer Pairs Used in Multiplex Polymerase Chain Reaction.

Primer	Outside	Inside
GAPDH	Sense: 5′-GATGGTGAAGGTCGGTGTG-3′	5′-TACCAGGGCTGCCTTCTCT-3′
	Antisense: 5′-GGGCTAAGCAGTTGGTGGT-3′	5′-CTCGTGGTTCACACCCATC-3′
Vasopressin	Sense: 5′-CCTCACCTCTGCCTGCTACTT-3′	5′-CCAGAACTGCCCAAGAGG-3′
	Antisense: 5′-GCTTCCGCAAGGCTTCTG-3′	5′-GCTTCCGCAAGGCTTCTG-3′
Oxytocin	Sense: 5′-CTGCCCCAGTCTCGCTTG-3′	5′-CTGCCCCAGTCTCGCTTG-3′
	Antisense: 5′-CCTCCGCTTCCGCAAGGCTTC-3′	5′-GCGAGGGCAGGTAGTTCTCC-3′
TRH	Sense: 5′-AGAGGGGAGACTTGGGAGAA-3′	5′-ATTCATGGGCAGATGAGGAG-3′
	Antisense: 5′-CTTTGCTTCACCAGGGTCTC-3′	5′-GGCGTTTCTCAGGCATTAAG-3′
CRH	Sense: 5′-GGGGAAAGGCAAAGAAAAGG-3′	5′-GGAGAAGAGAAAGGAGAAGAG-3′
	Antisense: 5′-GACAGAGCCACCAGCAGCAT-3′	5′-GGACACCAGCAGCCGCAG-3′
VGluT2	Sense: 5′-AGGTTGGCTACCACCTCCTT-3′	5′-CCCGCAAAGCATCCAACCA-3′
	Antisense: 5′-TGAGAGTAGCCAACAACCAGA-3′	5′-CCTGCAGAAGTTTGCAACAA-3′
Genomic	Sense: 5′-GCCTGCATTCATCTTCATCTG-3′	5′-GCCTGCATTCATCTTCATCTG-3′
	Antisense: 5′-AAAGGTGGAACTCGCCCGTTT-3′	5′-TGTGTCGGTTGTCGTGGTTC-3′

TRH, Thyrotrophin-releasing hormone; CRH, corticotrophin-releasing hormone; VGuT2, vesicular glutamate transporter.

Results

Nesfatin-1 influences the excitability of the majority of PVN neurones

Nesfatin-1 (10 nM) was administered by bath perfusion onto PVN neurones during current clamp recordings (baseline membrane potentials ranged between −56 and −70 mV). In the vast majority of neurones tested (70.6% of 85 neurones), nesfatin-1 application resulted in a change in membrane potential that met or exceeded our criteria of twice the SD of the baseline noise; 43.5% of neurones tested hyperpolarised by a mean of 7.5 ± 0.7 mV (n = 37, Fig. 1A,C), whereas a further 27% of neurones tested depolarised by a mean of 5.4 ± 0.8 mV (n = 23, Fig. 1B,C). There was no significant correlation between the baseline membrane potential prior to nesfatin-1 application and whether cells were depolarised or hyperpolarised. Both the hyperpolarising and depolarising effects of nesfatin-1 are likely direct actions on PVN neurones because they were still observed when TTX (1 μM) was present in the ACSF to isolate the neurones from action potential-evoked synaptic influences (Fig. 1D). In a subset of neurones, the effect of nesfatin-1 on input resistance was monitored and no consistent effects were observed in either neurones that depolarised (4/8 decreased, and 4/8 increased input resistance), or those that hyperpolarised (4/9 decreased, 5/9 increased input resistance) in response to nesfatin-1. Therefore it is likely that multiple ionic mechanisms are responsible for the depolarising and hyperpolarising responses elicited by nesfatin-1 in PVN neurones.

Nesfatin-1 has similar effects on neurones irrespective of electrophysiological identity

PVN neurones can be subdivided into three electrophysiologically distinct categories which are an indication of the output of each

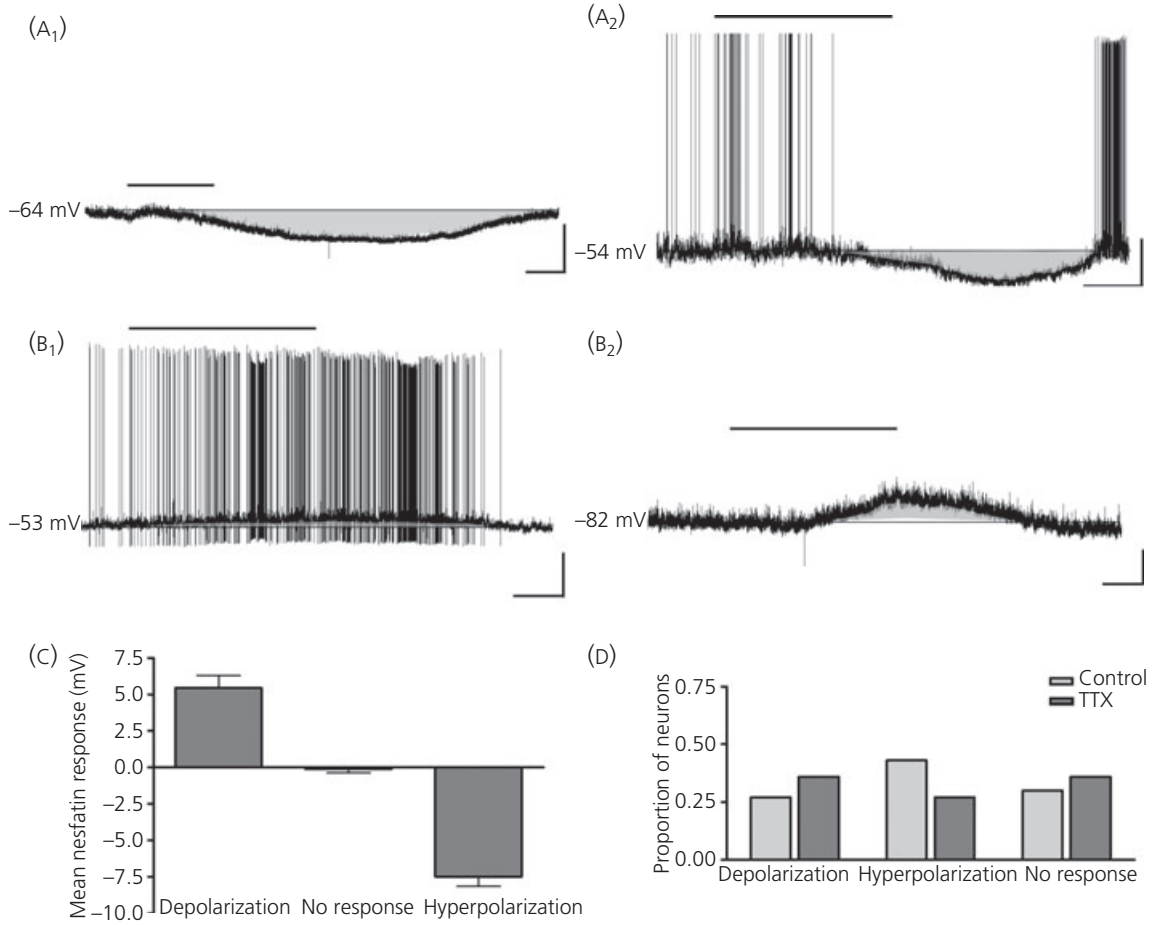

Fig. 1. Nesfatin-1 effects on the membrane potential of neurones in the paraventricular nucleus (PVN). ($A_{1,2}$) Nesfatin-1 (10 nM) induced hyperpolarisation that recovered to near baseline values following removal of nesfatin-1 from the bath. Scale: 25 and 20 mV/100 s. ($B_{1,2}$) In other neurones depolarisation was seen that recovered to near baseline values upon removal of nesfatin-1 from the bath. Scale: 25 and 5 mV/50 s. (C) Summary histogram illustrating the mean change in membrane potential in neurones that depolarised, hyperpolarised or did not respond. (D) Histogram showing the proportion of neurones that depolarised, hyperpolarised or showed no effect under control conditions and where action potential evoked release of neurotransmitter was inhibited by preincubation with the sodium channel blocker tetrodotoxin (TTX) (1 μM). Bars over traces indicate the time of application of nesfatin-1. Thin lines show the position of the baseline membrane potential whose value is noted to the left of each trace.

neuronal cell type. Magnocellular neurones possess a dominant A current and release the neuropeptides vasopressin and oxytocin into the circulation at the posterior pituitary. By contrast, preautonomic parvocellular (low-threshold spike; LTS) possessing a T-type calcium current-dependent low threshold spike and project to the medulla and spinal cord. Finally neuroendocrine cells show neither of these characteristics (4, 5) and send axonal projections to the median eminence where they release neuropeptides into the hypothalamic portal circulation, which in turn control pituitary hormone secretion. Neurones possessing the same electrophysiological fingerprint (e.g. LTS) were seen to both depolarise (Fig. 2A_1) and hyperpolarise (Fig. 2A_2) following exposure to nesfatin-1. Overall, a similar proportion of neurones hyperpolarised and depolarised in response to nesfatin-1 within each of these three groups of neurones (Fig. 2B) (χ^2, P = 0.3728). Likewise, there was no statistically significant correlation between cell size (estimated using cell capacitance measurements), and responsiveness (hyperpolarisation versus depolarisation) of PVN neurones tested (one-way ANOVA: P > 0.05).

Nesfatin-1 has similar effects on identified oxytocin/vasopressin/corticotrophin-releasing hormone (CRH)/thyrotrophin-releasing hormone (TRH) neurones

We also identified neurones based on their molecular phenotype, using single cell RT-PCR of the mRNA contained in the cytoplasm collected from neurones upon completion of the recording. Similar to groupings based on electrophysiological criteria, for all neuropeptide phenotypes we investigated (oxytocin/vasopressin/CRH/TRH), both hyperpolarising and depolarising effects of nesfatin-1 were observed (Fig. 3$A_{1,2}$). Indeed, for the three neuropeptide phenotypes that we encountered most frequently: oxytocin, vasopressin and TRH, there was no significant difference in the frequency of occurrence between depolarising, hyperpolarising and nonresponses (Fig. 3B).

Discussion

These data showing that nesfatin-1 influences the excitability of the majority of PVN neurones have identified this peptide as a potentially important regulator of PVN neuronal activity and suggest potentially important physiological roles within this hypothalamic autonomic control centre. Furthermore, the finding that PVN neurones respond to nesfatin-1, and neurones in the PVN produce this peptide, suggests potentially important interactions between different subsets of PVN neurones involved in the control of multiple autonomic outputs. The promiscuous activity of nesfatin-1 across a range of different neuronal types, including cells with both putative neuroendocrine and central nervous system release sites, indicate that the actions of nesfatin-1 are complex and likely influence feeding through integrated roles in controlling neuroendocrine (oxytocin/vasopressin/CRH/TRH) and autonomic (medullary/spinal) output pathways originating in the PVN (6–8). CRH and TRH from neuroendocrine cells are both released during satiated states, and at the median eminence, initiating a chain of events that leads to, respectively, glucocorticoid release and increased catabolism and

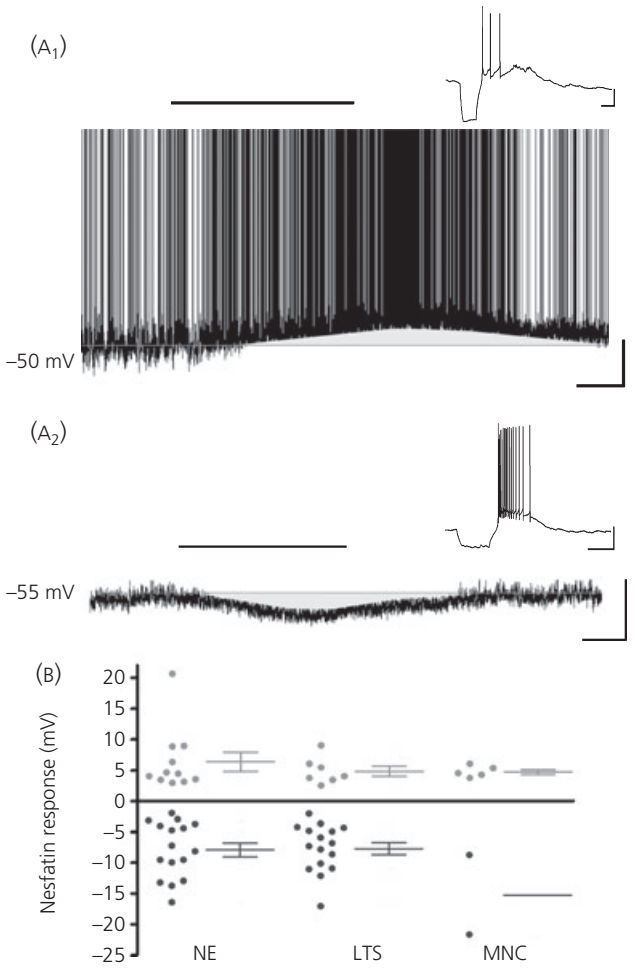

Fig. 2. Nesfatin-1 has similar effects on the membrane potential of paraventricular nucleus (PVN) neurones irrespective of electrophysiological fingerprint. (A_1) Nesfatin-1 induced depolarisation of a PVN neurone possessing a low-threshold spike (LTS)-like electrophysiological profile (see inset). Scale: 10 mV/50 s. (A_2). Nesfatin-1 induced hyperpolarisation of a second LTS-like neurone. Scale: 20 mV/50 s and 20 mV/400 ms for the inset. (B) Scatter plot of depolarising (light) and hyperpolarising (dark) responses to 10 nM nesfatin-1 for neurones with electrophysiological profiles characteristic of neuroendocrine (NE), preautonomic (LTS) and magnocellular (MNC) neurones. In addition, 11 of 41 NE, ten of 33 LTS and five of 12 MNC neurones did not respond to nesfatin-1. These are omitted from the figure for reasons of clarity. To the right of each scatter plot is the mean ± SE for the depolarising and hyperpolarising responses seen. Bars over traces indicate the time of application of nesfatin-1, whereas thin lines indicate the baseline membrane potential, whose value is noted to the left of each trace.

incorporation of fuels into lipids and thyroid hormone release and increased overall metabolism (9, 10). In addition, oxytocin neurones projecting to the nucleus tractus solitarius are excited during satiated states to promote inhibition of feeding (6). The collective excitation of these neurones by nesfatin-1 would aid in coordinating the actions of these separate peptide systems in the satiated animal. Intriguingly, the finding that both hyperpolarising and depolarising actions were routinely observed also suggests that nesfatin-1 actions may not be limited to the control of feeding because these output neurones of PVN are also well recognised for their roles in

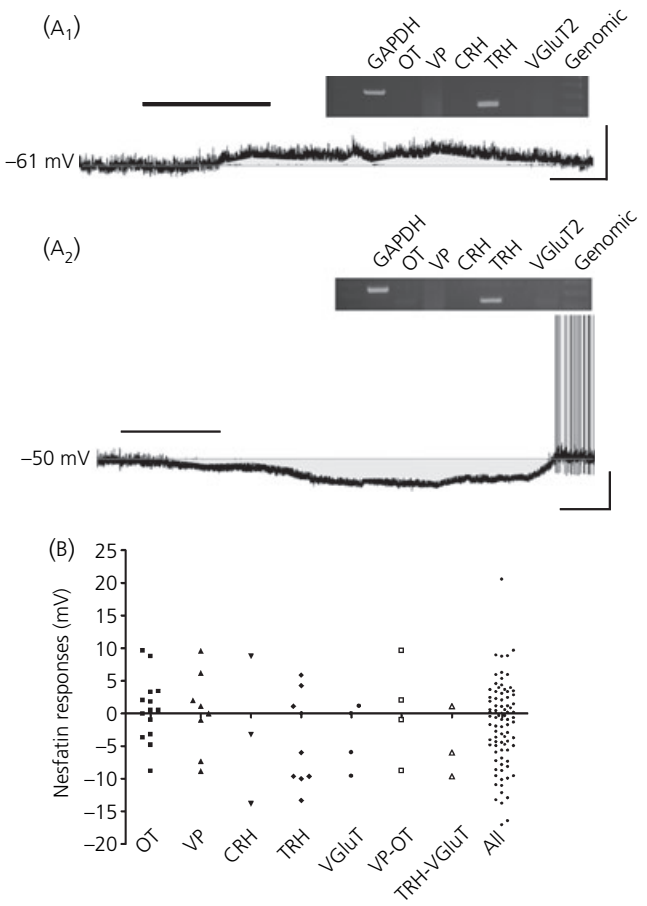

Fig. 3. Nesfatin-1 has similar effects on the membrane potential of neurones with different molecular phenotypes. (A_1) Nesfatin-1 induced depolarisation of a TRH-positive neurone, based on post-hoc reverse transcription-polymerase chain reaction identification (inset). Scale: 20 mV/100 s. (A_2) Hyperpolarisation seen in a second TRH neurone. Scale: 20 mV/100 s. (B) Scatter plots showing the distribution of responses to nesfatin-1 seen for all neuronal phenotypes. For comparison, a scatter plot of all the nesfatin-1 responses obtained is included. Bars over traces indicate the time of application of nesfatin-1, whereas thin lines indicate the baseline membrane potential, whose value is noted to the left of each trace. OT, oxytocin; VP, vasopressin; CRH, corticotrophin–releasing hormone; TRH, thyrotrophin–releasing hormone; VGluT, vesicular glutamate transporter.

regulating fluid balance, the cardiovascular system and the physiological response to stress (11, 12).

Our observations that nesfatin-1 induced either hyperpolarisations or depolarisations in nearly all of the subtypes of PVN neurones we examined, based on either electrophysiological or molecular phenotyping, are also of particular interest. They suggest that rather than exerting homogenous influences on functionally specific groups of PVN neurones, release of nesfatin-1 in the PVN may be highly localised to specific synapses, allowing for discrete state dependent effects on cells involved in the neuronal circuits controlling feeding, satiety and other potential autonomic outputs originating from this nucleus. Specificity of actions may also arise due to the co-expression of other receptors on nesfatin-1 sensitive neurones, such as melanocortin 4-receptor. This receptor was found to be associated with nesfatin-1 induced satiety and can be shown to be located on a subset of PVN neurones, including TRH and CRH neurones where it regulates the expression of these two peptides (1, 13, 14). Alternatively, it is possible that multiple nesfatin-1 receptors exist that are separately responsible for hyperpolarising or depolarising responses.

It has recently been shown that nesfatin-1 application to dissociated hypothalamic neurones elevated intracellular calcium concentrations using a pertussis toxin-dependent and L-type calcium channel mediated mechanism of action (15). This is consistent with our finding that many PVN neurones were directly depolarised by nesfatin-1, possibly via the activation of a g-protein coupled receptor. Thus, although we still do not know all the details regarding the cellular mediators of nesfatin-1 actions on PVN neurones (receptors, ion channels, signal transduction pathways), our data clearly demonstrate that this newly-discovered peptide may exert at least a part of its physiological actions on the control of food intake as a direct result of its role in controlling the excitability of neurones in the PVN, the hypothalamic nucleus where nesfatin-1 synthesis has been shown to be reduced during starvation (1).

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