Nesfatin-1 evokes Ca2+ signaling in isolated vagal afferent neurons via Ca2+ influx through N-type channels

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Abstract

Nesfatin-1, processed from nucleobindin 2, is an anorexigenic peptide expressed in the brain and several peripheral tissues including the stomach and pancreas. Peripheral, as well as intracerebroventricular, administration of nesfatin-1 suppresses feeding behavior, though underlying mechanisms are unknown. In this study, we examined effects of nesfatin-1 on cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in the neurons isolated from the vagal afferent nodose ganglion of mice. Nesfatin-1 at 10^{-10} to 10^{-8} M increased $[Ca^{2+}]_i$ in the isolated neurons in a concentration-dependent manner, and at 10^{-8} M it increased $[Ca^{2+}]_i$ in 33 out of 263 (12.5%) neurons. These responses were inhibited under Ca^{2+}-free conditions and by N-type Ca^{2+} channel blocker, ω-conotoxin GVIA. All the nesfatin-1-responsive neurons also exhibited $[Ca^{2+}]_i$ responses to capsaicin and cholecystokinin-8. These results provide direct evidence that nesfatin-1 activates vagal afferent neurons by stimulating Ca^{2+} influx through N-type channels, demonstrating the machinery through which peripheral nesfatin-1 can convey signals to the brain.

Keywords

Nesfatin-1, nodose ganglion, cytosolic Ca^{2+}, feeding, N-type Ca^{2+} channel, cholecystokinin

Abbreviations

arcuate nucleus (ARC), capsaicin (CAP), cholecystokinin (CCK), cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$), dorsal motor nucleus of the vagus (DMV), HEPES-buffered Krebs-Ringer bicarbonate buffer (HKRB), hypothalamic paraventricular nucleus (PVN), intracerebroventricular (icv), lateral hypothalamic area (LHA), minimal essential medium (MEM), nesfatin-1 (Nesf-1), nucleobindin 2 (NUCB2), nucleus of the solitary tract (NTS), supraoptic nucleus (SON)

Introduction

Nesfatin-1 is a recently discovered anorexigenic peptide encoded in the precursor protein, nucleobindin 2 (NUCB2) [1]. Nesfatin-1/NUCB2 is localized in the hypothalamic paraventricular nucleus (PVN), arcuate nucleus (ARC), supraoptic nucleus (SON) and lateral hypothalamic area (LHA) [1], and in the brainstem nucleus of the solitary tract (NTS) and dorsal motor nucleus of the vagus (DMV) [1, 2], which are the feeding-related areas. Intracerebroventricular (icv) administration of nesfatin-1 suppresses food intake, whereas icv administration of nesfatin-1 antibody increases food intake [1]. Interestingly, nesfatin-1 also inhibits food intake in Zucker fatty rats whose leptin receptor is mutated [1], indicating that the anorexigenic effect of nesfatin-1 is leptin-independent.

Recently, nesfatin-1 immunoreactivity was also found in the peripheral

tissues including stomach [3] and pancreatic islets [4]. Peripheral administration of nesfatin-1 reduces food intake in normal and leptin-resistant mice such as db/db and high-fat diet-induced obese mice [5]. Since nesfatin-1 can cross blood-brain barrier, nesfatin-1 injected peripherally might directly act on the neurons in the feeding-controlling centers to reduce food intake. On the other hand, there is the possibility that the anorexia induced by peripheral nesfatin-1 is via the afferent nervous system. In fact, peripheral administration of nesfatin-1 induces expression of c-Fos in the NTS, which receives projections of vagal afferent nerves [5].

In this study, to investigate the effect of nesfatin-1 on the vagal afferent nerve, we monitored the activity of neurons isolated from the mouse nodose ganglion by measuring cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) and investigated direct effects of nesfatin-1. Cholecystokinin (CCK)-8 is the well established peripheral peptide that acts on the vagal afferent nerve to produce anorexia [6]. Capsaicin (CAP) is also known to activate vagal afferent nerves [7]. Therefore, we examined whether the nodose ganglion neuron that responds to nesfatin-1 is distinct from or overlapping with the CCK-8- or CAP-responsive neuron.

Materials and methods

Materials

Mouse nesfatin-1 was synthesized as previously reported [5]. CCK-8 (sulfated form) was purchased from Peptide Institute, Inc. (Osaka, Japan). CAP

was obtained from Sigma (MO, USA)

Animals

Male ICR mice (Japan SLC, Shizuoka, Japan) aged 6 to 13 weeks were housed for at least 1 week under conditions of controlled temperature (23 ± 1°C), humidity (55% ± 5%), and lighting (light on at 07:30 and off at 19:30). Food and water were available ad libitum. Procedures of animal experiments were approved by the Animal Care and Use Committee of the Jichi Medical University.

Preparation of nodose ganglion neurons from mice

Single neurons were isolated from mouse nodose gangalia, according to the methods reported by Simasko et al. with minor modifications [8]. Briefly, male ICR mice were anesthetized with a-chloralose and urethane (0.1 g/kg and 1 g/kg, i.p., respectively), and nodose ganglia were excised. These ganglia were rapidly cut into 4 or 5 pieces in ice-cold HEPES-buffered Krebs-Ringer bicarbonate buffer (HKRB) composed of 4.7 mM KCl, 1.2 mM KH_2PO_4, 129 mM NaCl, 5 mM $KaHCO_3$, 1.2 mM $MgSO_4$, 1.8 mM $CaCl_2$, and 10 mM HEPES with pH adjusted at 7.4 using NaOH supplemented with 5.6 mM glucose, The tissue pieces were dissociated by incubation for 20 minutes at 37 °C in the HKRB containing 0.7 mg/ml collagenase Ia (Sigma), 0.7 mg/ml dispase II (Roche, Basel, Swiss), 15 μg/ml DNase II type IV (Sigma), and 0.75 mg/ml bovin serum albumin. Cells were gently triturated using fire-polished pasteur pipettes and centrifugated 2

times in Eagle's minimal essential medium (MEM) containing 5.6 mM glucose supplemented with 10% feral bovine serum, 100 µg/ml streptomycin, and 100 units/ml penicillin. Cells were resuspended in MEM and plated onto coverslips coated with poly-L-lysin. Cells were cultured in MEM for 18-36 h.

Measurements of $[Ca^{2+}]_i$

Measurements of $[Ca^{2+}]_i$ in primary cultured nodose ganglion neurons were carried out according to the procedures reported previously [9]. Briefly, following incubating with 2 µM fura-2 AM (DOJINDO, Kumamoto, Japan) for 30 min at 37 °C, the cells were mounted in a chamber and superfused with HKRB at 1.3 ml/min at 30 °C. Fluorescence images at 510 nm due to excitation at 340 and 380 nm were detected every 10 s with an intensified charge-coupled device camera, and ratio images were produced by an Aquacosmos ver. 2.5 (Hamamatsu Photonics, Shizuoka, Japan). When $[Ca^{2+}]_i$ changed within 5 min after addition of agents and their amplitudes were at least twice larger than the fluctuations of the baseline, they were considered responses. Only the cells that responded to 55 mM KCl were analyzed.

Data analysis

Statistical analysis was performed by one-way ANOVA and Tukey's multiple comparison tests for multiple groups, and by unpaired t-test for two groups. All data were shown as means ± SEM. $P<0.05$ was considered significant.

Results

Nesfatin-1 increased $[Ca^{2+}]i$ in mouse nodose ganlion neurons

To evaluate directly effect of nesfatin-1 on vagal afferent nerve, we measured $[Ca^{2+}]_i$ changes in fura-2 loaded nodose ganlion neurons before and after treatment of nesfatin-1. Administration of nesfatin-1 at 10^{-8} M for 15 min increased $[Ca^{2+}]_i$ in nodose ganglion neurons as shown in Fig. 1A. The $[Ca^{2+}]_i$ returned to the basal level after washing out nesfatin-1. To confirm concentration-dependency, as shown in Fig. 1B nesfatin-1 at 10^{-10}, 10^{-9} and 10^{-8} M were sequentially applied to nodose ganglion neurons with treatment and washing periods for 4 and 8 min, respectively. Nesfatin-1 at 10^{-10}, 10^{-9} and 10^{-8} M raised $[Ca^{2+}]_i$ in 10 of 119 (8.4%), 15 of 119 (12.6%) and 18 of 119 (15.1%) neurons examined, respectively (Fig. 1C). Thus, incidence of nesfatin-1-responsive neurons increased in a concentration-dependent manner. Moreover, in these 18 nesfatin-1-responsive neurons, amplitudes of $[Ca^{2+}]_i$ responses to nesfatin-1 also increased in a concentration-dependent manner (Fig. 1D).

Inhibition of nesfatin-1-induced $[Ca^{2+}]_i$ increases under Ca^{2+}-free condition and by N-type Ca^{2+} channel blocker

Involvement of extracellular Ca^{2+} in nesfatin-1-induced $[Ca^{2+}]_i$ increases in nodose ganlion neurons was examined. In a Ca^{2+}-free condition made with no

added Ca^{2+} and 0.1 mM EGTA, nesfatin-1 at 10^{-8} M failed to increase $[Ca^{2+}]_i$ in all the cells examined. After bringing Ca^{2+} back to HKRB that often elevated $[Ca^{2+}]_i$ in a transient manner, nesfatin-1 elicited $[Ca^{2+}]_i$ increases in 14 of 100 neurons (14.0%) (Figs. 2A and 2C).

The effect of ω-conotoxin GIVA, a blocker of voltage-gated N-type Ca^{2+} channels, on nesfatin-1-induced $[Ca^{2+}]_i$ responses was examined with the protocol shown in Fig. 2B. In the presence of ω-conotoxin GVIA (0.5 μM), administration of 10^{-8} M nesfatin-1 induced either no change or small increase in $[Ca^{2+}]_i$, and after washing out the agents, second stimulation with nesfatin-1 (10^{-8} M) raised robust increases in $[Ca^{2+}]_i$ (Fig. 2B). Amplitude of $[Ca^{2+}]_i$ responses to 10^{-8} M nesfatin-1 was significantly smaller in the presence than the absence of ω-conotoxin GVIA (0.5 μM) (Fig. 2C). Furthermore, ω-conotoxin GIVA at 0.1 μM also attenuated the nesfatin-1-induced $[Ca^{2+}]_i$ increase, though to a lesser extent than at 0.5 μM (Fig. 2C), showing a dose-dependent blocking effect.

Relationship between nesfatin-1-responsive neurons and CCK-8- or CAP-responsive neurons

We examined whether nesfatin-1-responsive neurons are distinct from or overlap with CCK-8- or CAP-responsive neurons in the nodose ganglion. Nodose ganglion neurons were exposed sequentially to nesfatin-1 (10^{-8} M), CCK-8 (10^{-8} M), CAP (10^{-7} M), and KCl (55 mM) (Fig. 3A). Nesfatin-1, CCK-8,

and CAP evoked $[Ca^{2+}]_i$ increases in 33 (12.5%), 132 (50.2%), and 164 (62.4%) of 263 neurons that responded to KCl, respectively (Fig. 3B). Furthermore, all of 33 neurons that responded to nesfatin-1 with $[Ca^{2+}]_i$ increases also responded to both CCK-8 and CAP (Fig. 3C).

Discussion

This study demonstrated that nesfatin-1 directly interacts with nodose ganglion neuron and increases $[Ca^{2+}]_i$ by stimulating Ca^{2+} influx primarily through voltage-gated N-type Ca^{2+} channels, and that the nesfatin-1-responsive neurons occur in a portion of CAP- and CCK-8-responsive neurons.

The $[Ca^{2+}]_i$ responses to nesfatin-1 were markedly inhibited by ω-conotoxin GIVA, an N-type Ca^{2+} channel blocker. It has been reported that 10^{-7} M nesfatin-1-induced $[Ca^{2+}]_i$ increases in the hypothalamic single neurons were linked to L-type and P/Q-type Ca^{2+} channels [2]. These results by us and others suggest that the mechanisms by which nesfatin-1 increases $[Ca^{2+}]_i$ are different between the central neurons and the nodose ganglion neurons. The dominant role of the N-type Ca^{2+} channel in the nesfatin-1-induced $[Ca^{2+}]_i$ increases in the nodose ganglion neurons is apparently in accordance with the documents that this channel type is implicated in the regulation of the autonomic nerve activity [7].

In this study, the concentrations at which nesfatin-1 increased $[Ca^{2+}]_i$ in mouse nodose ganglion neurons were 10^{-10} M to 10^{-8} M. It was reported that serum concentration of nesfatin-1 in the humans fasted overnight is around 0.2

ng/ml (0.2×10^{-10} M). In our preliminary study, the plasma concentration of nesfatin-1 before breakfast in healthy humans is in a range of 1 to 10 ng/ml (10^{-10} to 10^{-9} M) (unpublished data). On the other hand, expressions of NUCB2 in the PVN [1] and gastric mucosa [3] are lower under fasting than ad libitum conditions, suggesting that the nesfatin-1 level in the circulation may be elevated by food intake. Furthermore, the nesfatin-1 level in the vicinity of nesfatin-1-producing tissues such as stomach and pancreas could be higher than that in the circulation. Collectively, it is suggested that, the effective concentration of nesfatin-1 to activate nodose ganglion neurons, 10^{-10} M, observed in this study, could be achieved at the site of vagal afferent fibers under *in situ* physiological concentrations and that nodose ganglion neurons are possibly activated by endogenous nesfatin-1.

The middle-segment of nesfatin-1 composed of 30 amino acids, M30, is as potent as nesfatin-1 in inducing anorexia, while the former- and post-segments were ineffective on food intake [5]. Both M30 and nesfatin-1 inhibit food intake when administered peripherally as well as centrally [1, 5]. However, in mice pretreated with CAP, a procedure to deplete CAP-sensitive afferent nerves, peripheral administration of M30 fails to show anorexigenic action [10]. These results have suggested that induction of anorexia by nesfatin-1 and its active segment M30 is, at least in part, due to activation of CAP-sensitive afferent nerves. The present study demonstrated that nesfatin-1 directly activates CAP-responsive nodose ganglia neurons of the vagal afferent.

CCK is one of the anorexigenic peptides secreted from enteroendocrine I cells after meal intake. It has been demonstrated that CCK exerts its anorexigenic effect via acting on the vagal afferent nerves [11]. We found that CCK-8 increases $[Ca^{2+}]_i$ in 50.2% of mouse nodose ganglion neurons, confirming the previous document in rat nodose ganglion neurons [12]. Moreover, in the present study, nesfatin-1 increased $[Ca^{2+}]_i$ exclusively in the neurons that responded to CCK-8. Our data indicate that the nesfatin-1-responsive neurons occupy a portion of CCK-8-responsive neurons, and suggest that nesfatin-1 and CCK-8 share the same target neurons of nodose ganglia that could be linked to inhibition of feeding. Conversely, it has been reported that peripheral administration of CCK stimulates c-Fos expression in nesfatin-1-containing neurons in the hypothalamic PVN and brain stem NTS [13]. These data by us and others, taken together,, raise the possibility that peripheral and central nesfatin-1 could have a strong connection with the neurons that respond to peripheral CCK with respect to the regulation of food intake.

Conclusions

This study revealed that nesfatin-1 induces Ca^{2+} signaling in the vagal nodose ganglion neurons, and that the nesfatin-1-responsive neurons occur in the CAP- and CCK-8-responsive neurons. The results suggest that nesfatin-1 activates the nodose ganglion neurons that are implicated in feeding. Our study, by revealing a key cellular process, substantiated the hypothesis that peripheral

nesfatin-1 signals to the brain via interacting with the afferent vagus nerves [5, 10]. This interaction could underlie the anorexigenic action of peripherally-produced nesfatin-1 under physiological conditions and serve as the therapeutic sensor of exogenous nesfatin-1 for treating patients with hyperphagic obesity.

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Figure legends

Figure 1. Nesfatin-1 increases $[Ca^{2+}]_i$ in mouse nodose ganglion neurons.

A: Administration of 10^{-8} M nesfatin-1 (Nesf-1) induced a continuous increase in $[Ca^{2+}]_i$ in a single nodose ganglion neuron that subsequently responded to 55 mM KCl. **B**: Repetitive administration of increasing concentrations of Nesf-1 at 10^{-10}, 10^{-9} and 10^{-8} M induced repeated $[Ca^{2+}]_i$ increases in a concentration-dependent manner in a single nodose ganglion neuron that responded to CAP and 55 mM KCl. In (A) and (B), $[Ca^{2+}]_i$ is expressed by fura-2 ratio and bars above the tracings indicate the periods of administration of agents specified. The data are representative of 22 neurons in (A) and 18 neurons in (B). **C and D**: Concentration-dependent effects of Nesf-1 to increase $[Ca^{2+}]_i$ in single nodose ganglion neurons examined by the protocol indicated in (B). Incidence of $[Ca^{2+}]_i$ responses expressed by the percentage of neurons that responded to Nesf-1 (C), and amplitude of $[Ca^{2+}]_i$ responses to Nesf-1 in Nesf-1-responsive neurons (D) are shown. In C, numbers above each point indicate the number of neurons that responded to Nesf-1 over that responded to 55 mM KCl. In (D), values are normalized to those with 10^{-8} M Nesf-1 in each neuron and then averaged and expressed by means ± SEM (n = 18). *$p < 0.01$ by ANOVA.

Figure 2. Nesfatin-1-induced $[Ca^{2+}]_i$ increases are inhibited under Ca^{2+}-free conditions and by a blocker of N-type Ca^{2+} channels.

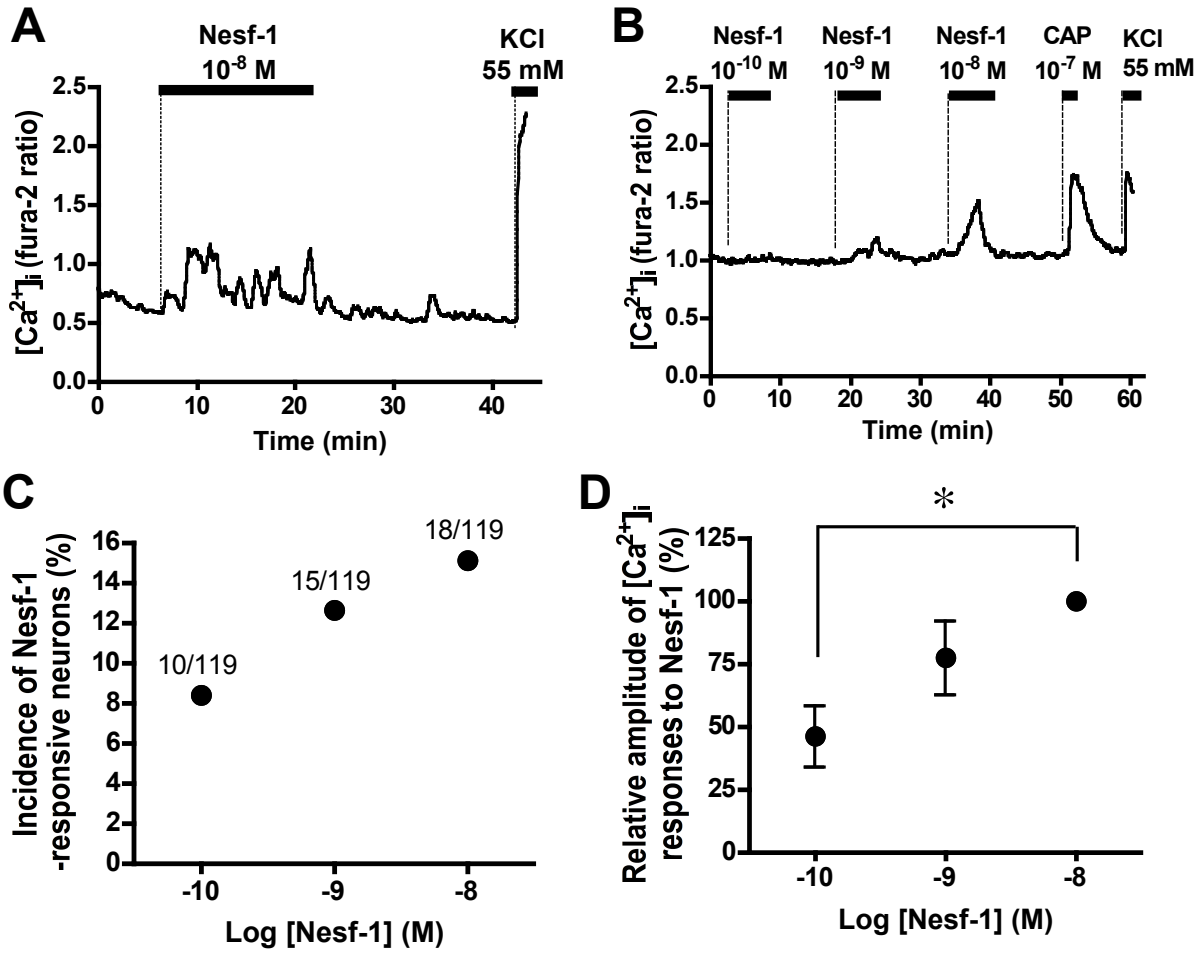
A: Nesfatin-1 (Nesf-1) at 10^{-8} M failed to increase $[Ca^{2+}]_i$ under Ca^{2+}-free

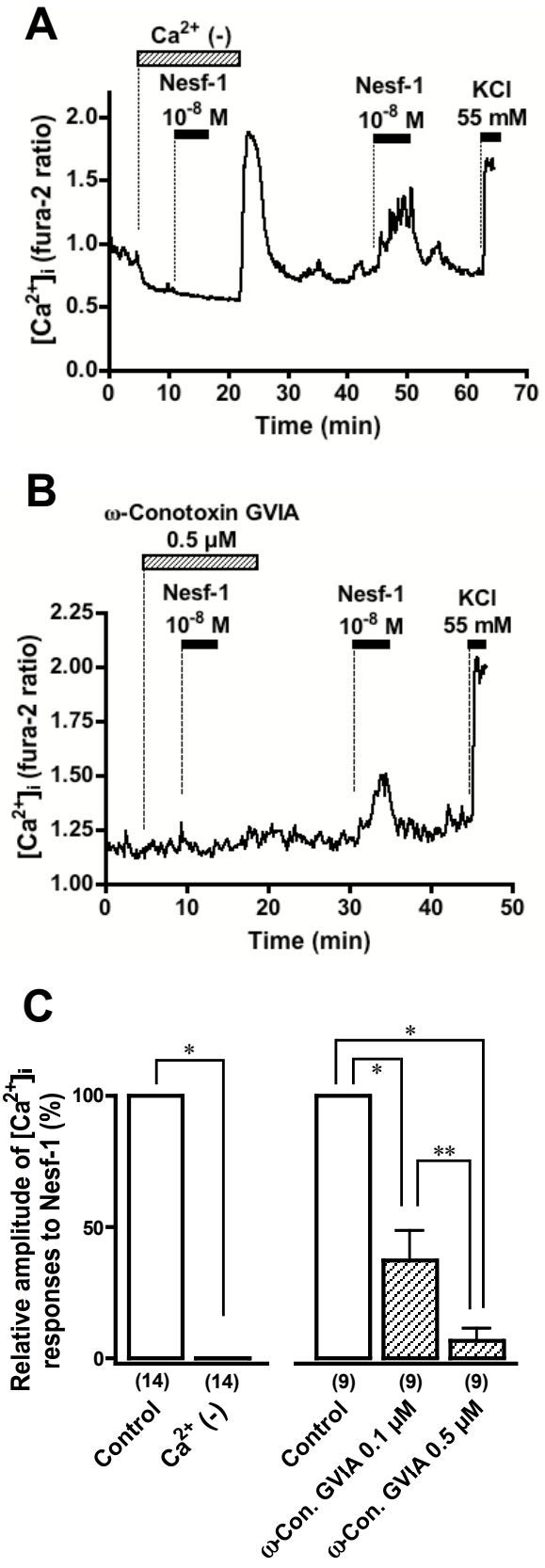
conditions made with 0.1 mM EGTA and no added Ca^{2+} in a single nodose ganglion neuron. Following the perfusate was switched to the control Ca^{2+}-containing HKRB that elevated $[Ca^{2+}]_i$ in a transient manner, Nesf-1 elicited an increase in $[Ca^{2+}]_i$. The neuron also responded to 55 mM KCl. **B**: Nesf-1 at 10^{-8} M failed to increase $[Ca^{2+}]_i$ in the presence of an N-type Ca^{2+} channel blocker ω-conotoxin GVIA (0.5 μM), but elicited an increase in $[Ca^{2+}]_i$ after washing out the drug in a single nodose ganglion neuron that responded to 55 mM KCl. The data are representative of 14 neurons in (A) and 9 in (B). **C**: The amplitude of $[Ca^{2+}]_i$ responses to 10^{-8} M Nesf-1 under control conditions, Ca^{2+}-free conditions and those with ω-conotoxin. The amplitude of the response under test conditions is normalized to that to the second Nesf-1 administration under control conditions and expressed by percentage in each neuron, and then averaged for the neurons examined and expressed by means ± SEM. *$p < 0.01$ by unpaired t-test for Ca^{2+}-free experiments. *$p < 0.01$ and **$p < 0.05$ by ANOVA for ω-conotoxin experiments.

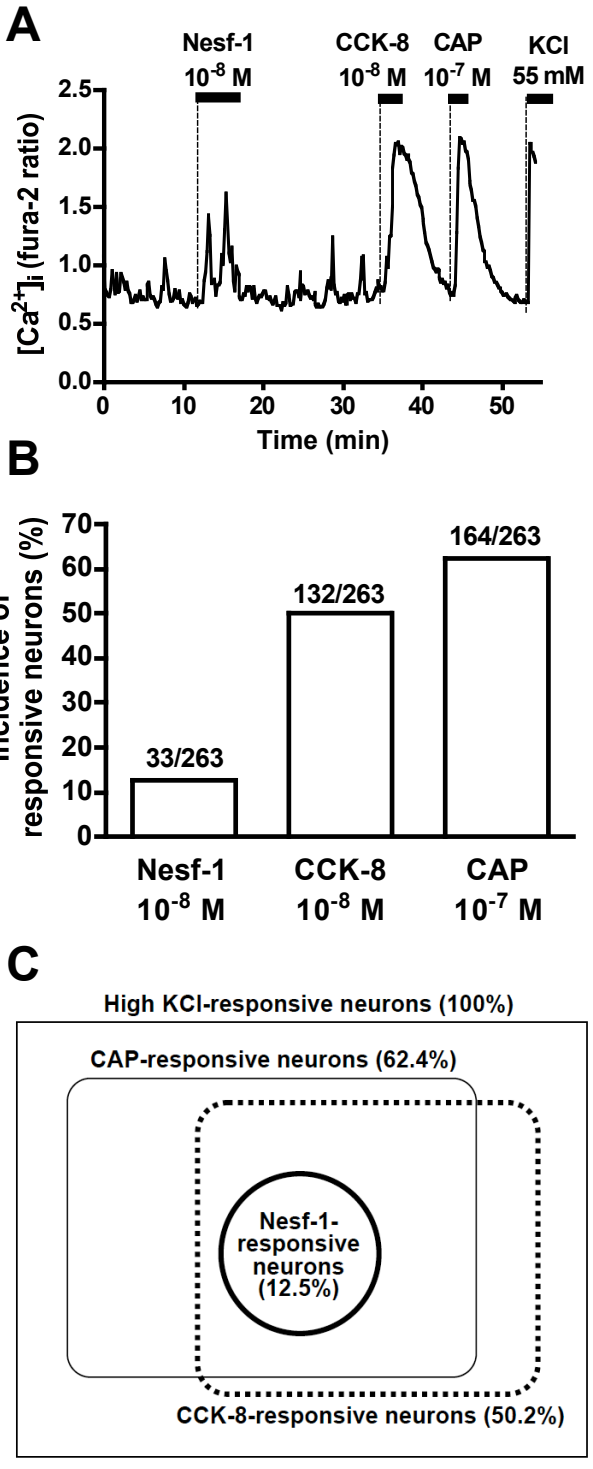
Figure 3. Incidence and distribution of nesfatin-1-, CCK-8- and CAP-responsive nodose ganglion neurons.

A: A representative $[Ca^{2+}]_i$ recording of the nodose ganglion neurons that responded to nesfatin-1 (Nesf-1), CCK-8 and CAP (n = 33). **B**: Incidence of $[Ca^{2+}]_i$ responses to Nesf-1, CCK-8 and CAP in the neurons that responded to 55 mM KCl, expressed by percentage. **C**: Overlap of the Nesf-1-, CCK-8- and

CAP-responsive nodose ganglion neurons. Incidences of specific subpopulations are; 16.0% for CAP-responsive, CCK-8- and Nesf-1-unresponsive neurons, 3.8% for CCK-8-responsive, CAP- and Nesf-1-unresponsive neurons, 33.8% for CAP- and CCK-8-responsive Nesf-1-unresponsive neurons, and 33.8% for neurons responsive to none of these agents.


A
Nesf-1
10-8 M
KCl
55 mM
[Ca2+]i (fura-2 ratio)
Time (min)
B
Nesf-1
10-10 M
Nesf-1
10-9 M
Nesf-1
10-8 M
CAP
10-7 M
KCl
55 mM
[Ca2+]i (fura-2 ratio)
Time (min)
C
Incidence of Nesf-1
-responsive neurons (%)
10/119
15/119
18/119
Log [Nesf-1] (M)
D
Relative amplitude of [Ca2+]i
responses to Nesf-1 (%)
*
Log [Nesf-1] (M)


A
Ca2+ (-)
Nesf-1
10-8 M
Nesf-1
10-8 M
KCl
55 mM
[Ca2+]i (fura-2 ratio)
Time (min)
B
ω-Conotoxin GVIA
0.5 μM
Nesf-1
10-8 M
Nesf-1
10-8 M
KCl
55 mM
[Ca2+]i (fura-2 ratio)
Time (min)
C
Relative amplitude of [Ca2+]i responses to Nesf-1 (%)
(14)
(14)
(9)
(9)
(9)
Control
Ca2+ (-)
Control
ω-Con. GVIA 0.1 μM
ω-Con. GVIA 0.5 μM


A
Nesf-1
10-8 M
CCK-8
10-8 M
CAP
10-7 M
KCl
55 mM
[Ca2+]i (fura-2 ratio)
2.5
2.0
1.5
1.0
0.5
0.0
0
10
20
30
40
50
Time (min)
B
Incidence of responsive neurons (%)
70
60
50
40
30
20
10
0
33/263
132/263
164/263
Nesf-1
10-8 M
CCK-8
10-8 M
CAP
10-7 M
C
High KCl-responsive neurons (100%)
CAP-responsive neurons (62.4%)
Nesf-1-responsive neurons (12.5%)
CCK-8-responsive neurons (50.2%)