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C-peptide of preproinsulin-like peptide 7: localization in the rat brain and activity in vitro

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Abstract

With the use of a rabbit polyclonal antiserum against a conserved region (54–118) of C-peptide of human preproinsulin-like peptide 7, referred to herein as C-INSL7, neurons expressing C-INSL7-immunoreactivity (irC-INSL7) were detected in the pontine nucleus incertus, the lateral or ventrolateral periaqueductal gray, dorsal raphe nuclei and dorsal substantia nigra. Immunoreactive fibers were present in numerous forebrain areas, with a high density in the septum, hypothalamus and thalamus. Pre-absorption of C-INSL7 antiserum with the peptide C-INSL7 (1 µg/ml), but not the insulin-like peptide 7 (INSL7; 1 µg/ml), also known as relaxin 3, abolished the immunoreactivity. Optical imaging with a voltage-sensitive dye DiSBAC₄(3) showed that C-INSL7 (100 nM) depolarized or hyperpolarized a small population of cultured rat hypothalamic neurons studied. Ratiometric imaging studies with calcium-sensitive dye fura-2 showed that C-INSL7 (10–1000 nM) produced a dose-dependent increase in cytosolic calcium concentrations [Ca²⁺]_i in cultured hypothalamic neurons with two distinct patterns: 1) a sustained elevation lasting for minutes; and 2) a fast, transitory rise followed by oscillations. In a Ca²⁺-free Hanks' solution, C-INSL7 again elicited two types of calcium transients: 1) a fast, transitory increase not followed by a plateau phase, and 2) a transitory rise followed by oscillations. INSL7 (100 nM) elicited a depolarization or hyperpolarization in a small population of hypothalamic neurons, and an increase of [Ca²⁺]_i with two patterns that were dissimilar from that of C-INSL7. [¹²⁵I]C-INSL7 bindings to rat brain membranes were inhibited by C-INSL7 in a dose-dependent manner; the K_d and B_{max} values were 17.7 ± 8.2 nM and 45.4 ± 20.5 fmol/mg protein. INSL7 did not inhibit [¹²⁵I]C-INSL7 binding to rat brain membranes, indicating that C-INSL7 and INSL7 bind to distinct binding sites. Collectively, our result raises the possibility that C-INSL7 acts as a signaling molecule independent from INSL7 in the rat central nervous system.

Keywords

calcium imaging; immunohistochemistry; nucleus incertus; hypothalamus

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The insulin superfamily is represented by several peptides with a signature motif of two peptides, the A and B chain, linked by a connecting peptide in the middle, the C-peptide. In addition to insulin, other family members include relaxin, insulin-like growth factor I (IGF-I) (Le Bouc et al., 1986) and II (IGF-II) (Bell et al., 1984), insulin-like 6 (INSL6) or Leydig cell insulin-like peptide or relaxin-like factor (RLF) (Adham et al., 1993), and insulin-like peptide 4 (INSL4) (Burkhardt et al., 1994) or early placenta insulin-like peptide (Chassin et al., 1995), insulin-like peptide 5 (INSL5) (Conklin et al., 1999), and insulin-like peptide 7 (INSL7) or relaxin 3 (Bathgate et al., 2002; Liu et al., 2003a, b).

Immunohistochemical or in situ hybridization studies show that INSL7, also known as relaxin 3, which is the most recently identified member of the insulin family, is expressed in neurons of the pontine nucleus incertus, lateral and ventrolateral periaqueductal gray, dorsal raphe nuclei and dorsal substantia nigra of the rat (Burazin et al., 2002; Liu et al., 2003b; Tanaka et al., 2005; Ma et al., 2007). Notwithstanding different antisera used in these studies, the distribution pattern of positively labeled neurons; i.e., concentration in the pontine nucleus incertus, is shown to be similar among these reports. Thus, an antiserum directed against a synthetic peptide equivalent to amino acid residues 85–101 of the relaxin 3 pro-peptide, which is a highly conserved region of the C-peptide, labeled a group of neurons with a distribution pattern (Ma et al., 2007) similar to that of neurons labeled with a monoclonal antibody raised against the N-terminal of the A-chain of relaxin 3 (Tanaka et al. 2005). More importantly, the C-peptide of relaxin 3 antiserum failed to recognize the mature A/B chain form of relaxin 3 (Ma et al., 2007), raising the possibility that the C-peptide of INSL7 is processed from the pro-hormone and functions as a signaling molecule independent of INSL7. To further identify a potential role of C-INSL7 as an independent signaling molecule, our first series of studies was to localize immunohistochemically C-INSL7 in the rat brain using an antiserum directed against a conserved fragment (54–118) of C-peptide of human INSL7.

INSL7 is biologically active. For example, in vitro studies show that INSL7 binds with a high affinity to the orphan G protein-coupled receptor GPR135 or GPR142 (Liu et al., 2003a, b). The peptide by intracerebroventricular administration or microinjection into hypothalamic sites increased food intake and body weight in rats (Hida et al., 2006; McGowan et al., 2007). There has been no report regarding a biological activity of C-INSL7. In the case of the C-peptide of insulin, a number of more recent studies show that the peptide is biologically active, contrary to the earlier notion that C-peptide of insulin is biologically inert (Hills and Brunskill, 2008). For example, C-peptide activates Na^+ , K^+ -ATPase of the rat kidney tubules (Ohtomo et al., 1996) and stimulates calcium influx in bovine aortic endothelial cells (Wallerath et al., 2003). Based on the findings of C-peptide of insulin, we hypothesized that C-INSL7 is biologically active. The second series of experiments were conducted to evaluate the biological activity of C-INSL7 by assessing its ability to mobilize calcium or alter membrane potential in dissociated cultured rat hypothalamic neurons using Ca^{2+} and optical imaging methodologies. Lastly, radiolabeled C-INSL7 was employed to determine whether or not C-INSL7 and INSL7 interact with the same or different binding site.

Experimental procedures

Adult male Sprague Dawley rats, 275–300 gm, were used in immunohistochemical and radiolabeled experiments (Ace Animals Inc., Boyertown, PA). Ca^{2+} or voltage measurements were conducted on dissociated hypothalamic neurons harvested from 1 to 3 days old rats, which were cultured for 5 days. Animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee.

Immunohistochemistry

Rats anesthetized with urethane (1.2 g/kg, IP) were intracardially perfused with 0.1 M phosphate buffered saline (PBS) followed by 4% paraformaldehyde/0.2% picric acid in PBS. Brains were removed, postfixed for 2 hr, and stored in 30% sucrose/PBS solution overnight. Tissues were processed for C-INSL7 immunoreactivity (irC-INSL7) by the avidin-biotin complex procedure or fluorescent method (Brailoiu et al., 2005; Dun et al., 2006). The brain was embedded in agar and coronal sections of 40 μ m were prepared with the use of a Vibratome. Tissues were first treated with 3% H₂O₂ to quench endogenous peroxidase, washed several times, blocked with 10% normal goat serum, and incubated in C-INSL7 antiserum (1:1,000 dilution), a rabbit polyclonal raised against a conserved region of the C-peptide of human prepro INSL7 (54–118) (⁵⁴SDI LAH EAMGDT FPDADADEDSLAGELEAMG SSEWLAL TKS PQAFYRGRPSW QGTPG VLR GSR¹¹⁸) (Phoenix Pharmaceuticals, Inc., Burlingame, CA). C-INSL7 antiserum exhibits 100% cross-reactivity with C-INSL7 peptide by ELISA, but not INSL7, human insulin-like peptide 3, C-peptide of human insulin-like peptide 3, C-peptide of human insulin (Phoenix Pharmaceuticals, Inc.). After thorough rinsing, sections were incubated in biotinylated anti-rabbit IgG (1:200 dilution, Vector Laboratories, Burlingame, CA) for 2 hr, and rinsed with PBS and incubated in avidin-biotin complex solution for 1.5 hr (1:100 dilution, Vector Laboratories). Following several washes in Tris-buffered saline, sections were developed in 0.05% diaminobenzidine/0.001% H₂O₂ solution and washed for at least 2 hr with Tris-buffered saline. Sections were mounted on slides with 0.25% gel alcohol, air-dried, dehydrated with absolute alcohol followed by xylene, and coverslipped with Permount. For fluorescent immunohistochemistry, sections were incubated with a secondary antiserum conjugated to fluorescein isothiocyanate, and examined under a confocal scanning laser microscope (Leica TCS SL) with excitation/emission wavelengths set to 488/520 nm.

For control experiments, brainstem sections were processed with C-INSL7 antiserum pre-absorbed with the human C-INSL7 (1 μ g/ml) or INSL7 (1 μ g/ml) overnight.

Neuronal Cell Culture

Cells were isolated from the hypothalamus of postnatal 1 to 3 days old rats by enzymatic digestion with 0.5 mg papain/100 mg tissue as described (Dun et al., 2006). Cells were plated at a density of 10³ cells/mm² in a Neurobasal-ATM medium, supplemented with 10% fetal calf serum, 2 mM glutamine, 100 units/ml penicillin and 100 μ g/ml streptomycin (Invitrogen, Carlsbad, CA), and maintained at 37°C in a humidified atmosphere with 5% CO₂. Glial cell growth was inhibited by the mitotic inhibitor cytosine β -arabino furanoside (1 μ M) (Sigma-Aldrich, St. Louis, MO). Neurons cultured for 5 days were then transferred to a medium without fetal serum 12 hr prior to Ca²⁺ or voltage measurements.

Cytosolic Ca²⁺ concentrations

Cytosolic Ca²⁺ measurements were performed as described previously (Brailoiu et al., 2006, 2008). Cells were incubated with 5 μ M fura-2 AM (Molecular Probes, Eugene, OR) in Hanks' balanced salt solution (HBSS) at room temperature for 45 min in the dark, washed three times with dye-free buffer, and then incubated for another 45 min to allow for complete de-esterification of the dye. Coverslips were subsequently mounted in a custom-designed bath on the stage of an Eclipse TE 2000-U Nikon inverted microscope equipped with a Photometrics CoolSnap HQ CCD camera (Roper Scientific, Tucson, AZ). Cells were routinely superfused with HBSS at a flow rate of 1 ml/min. Fura-2 fluorescence (emission = 510 nm), following alternate excitation at 340 nm and 380 nm, was acquired at a frequency of 0.3 Hz using a Lambda 10–2 filter shutter (Sutter Instrument Co). Images were acquired and analyzed off-line using a Metafluor software. The ratio of fluorescence signal (340 nm/380 nm) was converted to Ca²⁺ concentrations (Grynkiewicz et al., 1985). For Ca²⁺- free experiments, HBSS without Ca²⁺ and supplemented with 2.5 mM EGTA was used.

Optical imaging using DiSBAC₄(3)

Slow voltage sensitive fluorescent dyes, such as bis-oxonol (bis-[1,3-dibutylbarbituric acid] trimethineoxonol [DiSBAC₄(3)]), have been successfully used to assess relative changes in membrane potential of single neurons (Ebner and Chen, 1995; Kunkler et al., 2005; Brailoiu et al., 2008). Briefly, cells were incubated for 30 min in HBSS containing 0.5 mM DiSBAC₄(3). The fluorescence (excitation wavelength = 480 nm, emission wavelength = 540 nm) was continuously recorded at a rate of 10 points min⁻¹. Background values (windows of identical area placed beside the cells) were subtracted. The dye partition between the cell membrane and the cytosol is a function of membrane potentials. Depolarization of the membrane leads to a sequestration of the dye into cytosol and is associated with an increase in fluorescence intensity; whereas, the dye concentrates in the cell membrane during hyperpolarization, leading to a decrease of fluorescence intensity (Brauner et al., 1984).

Calibration of DiSBAC₄(3) Fluorescence

Calibration of DiSBAC₄(3) fluorescence was performed using the Na⁺-K⁺ ionophore gramicidin in Na⁺-free physiological solution (Brauner et al., 1984). The osmolarity was maintained constant by addition of N-methylglucamine. In the presence of gramicidin (1 μM), the Na⁺ concentration gradient is zero, and the membrane potential is approximately equal to K⁺ equilibrium potential which is determined by the Nernst equation. The intracellular K⁺ and Na⁺ concentrations were assumed to be 130 mM and 10 mM, respectively. The addition of gramicidin with various concentrations of K⁺ to the cultured neurons alters the cell membrane potential, thereby, altering fluorescence. Extracellular K⁺ concentrations used in this study were 17, 25 and 80 mM; consequently, membrane potentials varied between -56, -45 and -14 mV, as calculated by the Nernst equation. According to the calibration measurements, changes in DiSBAC₄(3) fluorescence intensity by 1.092 are equivalent to a change in membrane potential of 1 mV.

[¹²⁵I]C-INSL7 binding to rat brain membranes

[¹²⁵I]C-INSL7 (1839 Ci/mmol) and C-INSL7 were obtained from Phoenix Pharmaceuticals Inc. Preparation of rat brain membranes was performed according to a modification of our published procedures (Chen et al., 2004). [¹²⁵I]C-INSL7 binding assay was carried out similarly to that described (Chen et al., 2004). Rat brain membranes (~0.5 mg) were incubated with several concentrations of unlabeled C-INSL7 and INSL7 (from 1 to 1000 nM) at room temperature for 1 hr in binding buffer (50 mM Tris-HCl, 1 mM EDTA and 0.1 mM PMSF, pH 7.4) and then ~0.3 nM [¹²⁵I] C-INSL was added and incubated in a final volume of 0.5 ml for another 1 hr. Membrane bound and free radioligand were separated by centrifuging at 13,000 x g at 4°C for 25 min through 5% sucrose. Radioactivity associated with membranes was measured in a gamma counter and binding data were analyzed with the KELL (Radlig) program (Biosoft, Ferguson, MO).

Statistical analysis

In calcium measurement experiments, statistical significance between groups was tested with one-way ANOVA followed by Bonferroni test, p < 0.05 being considered significant.

Results

irC-INSL7 neurons and cell processes

Positively labeled neurons were clustered in the pontine nucleus incertus, which is situated bilaterally to the mid line of the brainstem (Fig. 1A). These cells measured 10–18 μm in diameter, and appeared multipolar with extensive branching. The number of irC-INSL7 neurons with a clearly defined nucleus in the nucleus incertus was counted. The average number

of irC-INSL7 neurons in the nucleus incertus was $1,290 \pm 314$ ($n=3$ rats). Additionally, a fewer number of irC-INSL7 neurons was scattered in the lateral periaqueductal gray (LPAG) and ventrolateral periaqueductal gray (VLPAG) (Fig. 1C & D), dorsal raphe nuclei and dorsal to the substantia nigra (not shown). Unlike the pontine nucleus incertus, irC-INSL7 neurons in these regions appeared to be bipolar, with few branching (Fig. 1C and D). Positively labeled neurons were not observed in any of the forebrain areas. Thus, the brainstem nuclei, particularly the pontine nucleus incertus, are the main source of irC-INSL7 neurons in the rat brain.

While irC-INSL7 cells were not observed in the forebrain, irC-INSL7 fibers were widely distributed to the cortex, septum, hypothalamus, thalamus, amygdala and hippocampus. At the rostral levels, irC-INSL7 fibers were seen in the lateral septum nucleus (intermediate and ventral, Fig. 2 A–B), medial septal nucleus (Fig. 2C), nucleus of vertical limb of the diagonal band (Fig. 2C), bed nucleus of stria terminalis (Fig. 2D). Varying intensities of irC-INSL7 fibers were present in several hypothalamic regions such as medial preoptic area (Fig. 2E), lateral preoptic area (Fig. 2F), paraventricular hypothalamic nucleus (Fig. 2G), supraoptic nucleus (Fig. 2H), ventromedial hypothalamic nucleus (Fig. 2J), dorsal hypothalamic area and dorsomedial hypothalamic nucleus (Fig. 2K) and tuber cinereum region (Fig. 2L). A particularly dense network of irC-INSL7 fibers were seen in the lateral hypothalamus area (Fig. 2I). In the thalamus, irC-INSL7 fibers were present in the lateral habenular nucleus (Fig. 2M), paraventricular thalamic nucleus, anterior (Fig. 2N), and sparse in medial habenular nucleus (Fig. 2M). irC-INSL7 fibers were also seen in the endopeduncular nucleus (Fig. 2O) and basolateral amygdaloid nucleus, anterior (Fig. 2P).

In control experiments, immunoreactivity was not detected in any of the pons sections processed with C-INSL7 antiserum pre-absorbed with the C-INSL7 peptide (1 $\mu\text{g/ml}$) overnight (Fig. 1B). Whereas, processing the pontine sections with C-INSL7 antiserum pre-absorbed with INSL7 peptide (1 $\mu\text{g/ml}$) did not significantly change the number and/or intensity of irC-INSL7 cells (not shown).

INSL7 and calcium responses

For comparative purposes, the effect of INSL7 (100 nM) on $[\text{Ca}^{2+}]_i$ was first examined. The mean basal $[\text{Ca}^{2+}]_i$ of rat cultured hypothalamic neurons was 86 ± 2.3 nM ($n=534$); which is in the range of cytosolic calcium concentrations (30–200 nM) reported in mammalian central neurons (Connor, 1986). Two types of INSL7-induced calcium responses could be discerned in cultured hypothalamic neurons. First, INSL7 elicited a fast, transitory increase of $[\text{Ca}^{2+}]_i$ in 17% of the hypothalamic neurons tested; the mean increase being 817 ± 6.3 nM ($n=27/158$). A representative experiment is shown in Fig. 3A. Second, INSL7 evoked a fast calcium transient followed by calcium oscillations in 5% of the hypothalamic neurons tested; the mean amplitude of the first spike was 1268 ± 39 nM ($n=8/158$). A representative recording is illustrated in Fig. 3B.

In a Ca^{2+} -free medium, INSL7 caused two patterns of $[\text{Ca}^{2+}]_i$ changes, similar in many respects to that produced in a normal calcium-containing medium. First, INSL7 produced a rapid and transitory increase in $[\text{Ca}^{2+}]_i$ by 307 ± 4.2 nM ($n=11/76$); a representative experiment is shown in Fig. 4A. Second, INSL7 induced $[\text{Ca}^{2+}]_i$ oscillations with a mean amplitude of the first spike 583 ± 7.4 nM ($n=5/76$); a representative response is illustrated in Fig. 4B.

Effect of C-INSL7 on cytosolic Ca^{2+}

Administration of C-INSL7 (100 nM) induced two types of calcium responses as well. In 36 of 276 (13%) neurons tested, C-INSL7 produced a fast and sustained increase in $[\text{Ca}^{2+}]_i$ with a mean amplitude of 405 ± 3.1 nM; an example is shown in Fig. 3C (black trace). C-INSL7 induced a fast, transitory peak followed by several smaller oscillations in 4% (11/276) of

neurons tested; the amplitude of the first spike was 763 ± 4.2 nM (Fig. 3D, black trace). Similar types of responses were observed when C-INSL7 was administered in the concentrations of 10 nM or 1000 nM. Administration of C-INSL7 (10 nM) induced a stable $[Ca^{2+}]_i$ elevation by 118 ± 2.4 nM ($n = 6$; Fig. 3C, green trace) or oscillations with the amplitude of the first spike averaging 137 ± 2.6 nM ($n = 6$; Fig. 3D, green trace). Administration of a higher concentration of C-INSL7 (1,000 nM) produced a fast and sustained increase in $[Ca^{2+}]_i$ with a mean amplitude of 731 ± 4.7 nM ($n = 8$; Fig. 3C, red trace) or $[Ca^{2+}]_i$ oscillations with the amplitude of the first spike of 1051 ± 9.8 nM ($n = 7$; Fig. 3D, red trace).

Pretreatment with ryanodine (5 μ M; 15 min) had no significant effect on C-INSL7 (100 nM)-induced fast and sustained raise in $[Ca^{2+}]_i$ ($\Delta [Ca^{2+}]_i = 391 \pm 3.6$ nM; $n = 7$) (Fig. 3E), while abolishing the $[Ca^{2+}]_i$ oscillations. Instead, in the presence of ryanodine, C-INSL7 produced a fast and transitory $[Ca^{2+}]_i$ increase by 427 ± 3.2 nM ($n = 9$; Fig. 3F). This response is significantly smaller ($P < 0.05$) as compared to the first $[Ca^{2+}]_i$ spike of the oscillatory response.

In a Ca^{2+} -free saline, C-INSL7 again produced two types of calcium responses. First, the peptide produced a single Ca^{2+} spike, not followed by a plateau phase, in 15 cells (11%); the mean increase in $[Ca^{2+}]_i$ was 231 ± 2.6 nM. A representative trace is shown in Fig. 4C. Second, C-INSL7 caused calcium oscillations in Ca^{2+} -free saline in 7 cells (5%); the amplitude of the first spike was 497 ± 3.6 nM (Fig. 4D).

C-INSL7 and membrane potential change

The mean resting membrane potential of cultured hypothalamic neurons was -53 ± 2.1 mV ($n=153$), as monitored by the voltage-sensitive dye DiSBAC₄(3). This value is close to the membrane potential measured from hypothalamic neurons, for example, pre-autonomic hypothalamic paraventricular neurons, by electrophysiological techniques (Stern, 2001). Superfusion of cultured hypothalamic neurons with C-INSL7 (100 nM) produced a membrane depolarization with a mean amplitude of 11 ± 1.6 mV ($n=7/153$), and a hyperpolarization having a mean amplitude of 7 ± 1.3 mV ($n=15/153$). A representative tracing of membrane depolarization or hyperpolarization is illustrated in Fig. 5A; the group data of membrane depolarizations or hyperpolarizations is shown in Fig. 5B.

INSL7 and membrane potential change

Similar to the membrane potential changes induced by C-INSL7, superfusion of INSL7 (100 nM) depolarized or hyperpolarized a small population of cultured hypothalamic neurons tested (not shown), with a mean depolarization of 9 ± 2.6 mV ($n=4$), and hyperpolarization of 5 ± 1.8 mV ($n=6$).

[¹²⁵I]C-INSL7 binding to rat brain membranes

C-INSL7 inhibited [¹²⁵I]C-INSL7 binding to rat brain membranes in a dose-dependent manner and inhibition reached the maximum at 5×10^{-7} M (Fig. 6). The non specific binding was defined as the binding in the presence of 1 μ M C-INSL7. The specific binding of [¹²⁵I]C-INSL7 represented 25–30% of total binding. The K_d and B_{max} values were determined to be 17.7 ± 8.2 nM ($n=3$) and 45.4 ± 20.5 fmol/mg protein ($n=3$), respectively. In contrast to C-INSL7, INSL7 did not inhibit [¹²⁵I]C-INSL7 binding to rat brain membranes (Fig. 6), indicating that C-INSL7 and INSL7 bind to distinct sites.

Discussion

Results from immunohistochemical, voltage and calcium imaging studies support the hypothesis that C-INSL7 is processed from the prepro-INSL7 and is biologically active in the rat central nervous system. First, the antiserum directed against a conserved fragment of C-

INSL7 labels specifically a population of neurons in the rat brainstem. Second, C-INSL7 depolarizes or hyperpolarizes cultured hypothalamic neurons and increases cytosolic calcium.

With respect to the immunohistochemical studies, irC-INSL7 is expressed mainly in neurons of the pontine nucleus incertus, with fewer cells located in the lateral or ventrolateral periaqueductal gray, dorsal raphe nuclei and substantia nigra. This pattern of distribution is similar to that reported in mammalian brains using immunohistochemistry and/or *in situ* hybridization (Bathgate et al., 2002; Burazin et al., 2002; Liu et al., 2003b; Tanaka et al., 2005; Ma et al., 2007). The antiserum used in our study is a rabbit polyclonal directed against the C-peptide of human INSL7 or prepro-INSL7 (54–128), and is different from those employed in earlier studies. In the previous two studies, a monoclonal antibody directed against the N-terminal peptide (Tanaka et al., 2005) or an affinity purified polyclonal against the pro-peptide (85–101) (Ma et al., 2007) was used. In our study, irC-INSL7 is abolished by pre-absorption of the antiserum with the peptide C-INSL7, but not INSL7, indicating the antiserum is specific for C-INSL7. Irrespective of the antiserum, neurons labeled by these three antibodies show a similar pattern of distribution to the pontine nuclei (Bathgate et al., 2002; Burazin et al., 2002; Liu et al., 2003b; Tanaka et al., 2005; Ma et al., 2007). Further, the average number of irC-INSL7 neurons observed in the pontine nucleus incertus is about 1,200, which is close to the 2,000 neurons noted in the same region by *in situ* hybridization (Tanaka et al., 2005). Because only labeled neurons with a clearly identified nucleus were counted in our study, the number may have been smaller as compared to the number of cells obtained in the *in situ* hybridization study. This number together with about 1,500 INSL7 neurons noted in other pontine nuclei, the total number of INSL7 neurons in the rat brain is estimated to be 3,500 (Tanaka et al., 2005). The findings that C-INSL7 and INSL7 neurons are uniquely and specifically distributed to the pontine nucleus incertus and that the number of C-INSL7 cells recorded here is close to the number of INSL7 cells reported by others (Tanaka et al., 2005) provide supportive evidence that irC-INSL7 and irINSL7 neurons belong to the same set of pontine medullary neurons.

Positively labeled neurons in the pontine nucleus incertus appear to be multipolar, with extensive branching. In contrast, irC-INSL7 neurons in other pontine nuclei, such as the lateral or ventrolateral periaqueductal gray, seem to be bipolar with few branching. It is not known whether or not these two types of morphologically dissimilar irC-INSL7 neurons may project to discrete forebrain areas and/or perform distinct physiological functions. A similar observation is made regarding the morphology of relaxin 3-immunoreactive cells by Tanaka et al. (2005). Double labeling experiments show that the majority of relaxin 3-expressing neurons in the pontine nucleus incertus contain glutamic acid decarboxylase-65, thus are γ -aminobutyric acid (GABA)-containing (Ma et al., 2007); many of these neurons in turn express corticotrophin-releasing factor (CRF) type 1 receptors (Tanaka et al., 2005). The observation that relaxin 3 mRNA in some of the nucleus incertus neurons is up-regulated in rats subjected to water-restraint stress (Tanaka et al., 2005), raises the possibility that relaxin 3 neurons of the nucleus incertus may be an integral component of the stress responding circuitry (Tanaka et al., 2005). In view of the widespread innervation of irC-INSL7 fibers to many forebrain regions including the medial septum, thalamus and hypothalamus, C-INSL7 is likely to subserve diverse physiological activities, including but not limited to stress response.

Calcium mobilization is a characteristic feature of activation of a G-protein coupled receptor (Kiselyov et al., 2003), and was used, in addition to membrane potential changes, as an assay of the biological activity of C-INSL7. The peptide increased cytosolic Ca^{2+} in cultured rat hypothalamic neurons with two distinct patterns: 1) fast rise followed by a plateau increase of $[\text{Ca}^{2+}]_i$, and 2) Ca^{2+} oscillations. These two patterns of response may correspond to openings of different calcium channels, resulting from the peptide interacting with two functionally distinct sites. In Ca^{2+} -free saline, C-INSL7 produced a short lasting rise in $[\text{Ca}^{2+}]_i$, instead of

a prolonged elevation noted in normal Ca^{2+} solution. This indicates that the calcium response in normal Ca^{2+} solution consists of two phases: 1) fast transitory phase, that persists in a Ca^{2+} -free saline, is probably related to Ca^{2+} release from internal stores; and 2) sustained component, that is eliminated by Ca^{2+} -free saline, is likely caused by Ca^{2+} influx from extracellular space. In some neurons, C-INSL7 induces Ca^{2+} oscillations, which are thought to be mediated by Ca^{2+} release from internal stores.

Calcium entry through plasmalemmal Ca^{2+} channels can activate ryanodine receptors through a calcium-induced calcium-release mechanism, which may in turn generate Ca^{2+} oscillations (Keizer and Levine, 1996). In some neurons, C-INSL7 induced Ca^{2+} oscillations were abolished by ryanodine. In contrast, ryanodine had no effects on C-INSL7-induced sustained $[\text{Ca}^{2+}]_i$ elevation. The observations that C-INSL7 elicits two types of calcium responses in cultured hypothalamic neurons and their differential sensitivity to ryanodine suggest the activation of two functionally distinct types of receptor such that one type of receptors is coupled to plasmalemmal and internal calcium stores and the other type may couple only to internal calcium stores.

For comparative purposes, the peptide INSL7 when administered to cultured hypothalamic neurons also elicited two types of calcium responses; i.e., fast calcium spike and calcium oscillations. A noticeable difference between the type 1 calcium responses induced by C-INSL7 and INSL7 is the transitory nature of INSL7-induced calcium spike, which is not significantly altered in a Ca^{2+} -free solution, suggesting that the receptor in question is coupled to internal calcium stores. As a corollary, INSL7 and C-INSL7 may interact with distinct receptors, which are coupled to different calcium stores.

Optical imaging using voltage sensor probes has been shown to be a reliable approach in monitoring membrane potential changes in neurons (Ebner and Chen, 1995; Kunkler et al., 2005; Chen et al., 2006). Here, optical imaging studies using a slowly responding, voltage-sensitive fluorescent dye DiSBAC₄(3) show that C-INSL7 or INSL7 depolarized or hyperpolarized a small percentage of cultured hypothalamic neurons tested. Thus, membrane potential changes as monitored by optical imaging techniques are consistent with the idea that C-INSL7 or INSL7 may interact with two functionally distinct receptors, leading to a membrane depolarization or hyperpolarization. The phenotype(s) of hypothalamic neurons sensitive to C-INSL7 or INSL7 is not known. The number of cultured hypothalamic neurons sensitive to either INSL7 or C-INSL7 was relatively small. The small number of cultured hypothalamic neurons sensitive to INSL7 or C-INSL7 may be related to the heterogeneity of dissociated rat hypothalamic neurons expressing an array of pharmacological receptors. In the process of random sampling, C-INSL7 receptor- or INSL7 receptor-expressing hypothalamic neurons may have been missed, contributing to a low yield of C-INSL7- and INSL7-responding hypothalamic neurons.

Earlier results propose INSL7 may bind to two different GPCR receptors GPCR135 and GPCR142, although the latter is not expressed natively in the rat brain (Liu et al., 2003a, b). Recombinant human relaxin 3 potently stimulated GTPγS binding and inhibited cAMP formation in cells over-expressing GPCR135 or GPCR145. Moreover, ¹²⁵I-relaxin 3 binds to COS-7 cells transiently expressing GPCR135 or GPCR142 with a K_d of 0.31 nM and 1.9 nM (Liu et al., 2003 a, b). It is not known the two types of membrane response induced by INSL7 or C-INSL7, i.e., depolarization and hyperpolarization, observed in our study may correspond to the activation of subtypes of receptor or to receptors coupled to different signaling pathways. As C-INSL7 and INSL7 elicit different patterns of cytosolic calcium elevation in hypothalamic neurons, the receptor(s) with which the two peptides interact may not be the same. The development of pharmacological antagonists specific for each type of responses will be necessary to identify the pharmacological nature of the receptor(s) in question. In the case of

insulin, the C-peptide of proinsulin appears to exert its biological effect via a G-protein coupled receptor that is distinct from that of insulin (Wahren et al., 2000).

Radiolabeled C-INSL7 binds to rat brain membranes with a K_d value of 17.7 ± 8.2 nM and a B_{max} value of 45.4 ± 20.5 fmol/mg protein. The K_d value is higher than those reported for most other neuropeptides to their cognate receptors, which generally is in the nM or sub-nM range. Several technical issues may contribute to the relatively high K_d value obtained here. For instance, a low density of receptors and thus the need to use a large amount of brain membranes are possible confounding factors. Second, the specific binding of [125 I]C-INSL7 represents only 25–30% of total binding. The high level of nonspecific binding is likely due to the hydrophobic nature of the peptide, as C-INSL7 is a large peptide (51 amino acids). The low specific binding may lead to potential errors in determination of binding affinity. Generally, a ligand with structure distinctly different from the radiolabeled ligand would be used to define nonspecific binding. Because there was no other ligand known to bind to this putative receptor/binding site, homologous competition experiments were used in determining K_d and B_{max} , which is not the optimal method. Since C-INSL7 is an agonist, the binding condition may not be optimal for detecting the high-affinity state. Consequently, the reported value may represent a combination of both high and low affinity states. In spite of the relatively high K_d value of [125 I]C-INSL7 for its binding sites, our result shows that INSL7 does not bind to the same site as the C-INSL7, supporting the contention that these two peptides may represent two independent signaling molecules.

Contrary to the long held opinion that the C-peptide of proinsulin is biologically inert, results from more recent studies support the idea that the cleaved product of proinsulin is biologically active (see Hills and Brunskill, 2008). Results from the present study provide the first evidence that the C-INSL7 is biologically active, extending the concept that the C-peptide of other members of the insulin family may also be biologically active.

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Abbreviations

[Ca ²⁺] _i	cytosolic calcium concentrations
C-INSL7	C-peptide of human preproinsulin-like peptide 7
DiSBAC ₄ (3)	bis-[1,3-dibutylbarbituric acid] trimethineoxonol
HBSS	Hanks' balanced salt solution
INSL7	insulin-like peptide 7
irC-INSL7	C-INSL7 immunoreactivity

References

- Adham IM, Burkhardt E, Benahmed M, Engel W. Cloning of a cDNA for a novel insulin-like peptide of the testicular Leydig cells. *J Biol Chem* 1993;268:26668–26672. [PubMed: 8253799]
- Bathgate RA, Samuel CS, Burazin TC, Layfield S, Claasz AA, Reytomas IG, Dawson NF, Zhao C, Bond C, Summers RJ, Parry LJ, Wade JD, Tregear GW. Human relaxin gene 3 (H3) and the equivalent mouse relaxin (M3) gene. Novel members of the relaxin peptide family. *J Biol Chem* 2002;277:1148–1157. [PubMed: 11689565]

- Bell GI, Merryweather JP, Sanchez-Pescador R, Stempien MM, Priestley L, Scott J, Rall LB. Sequence of a cDNA clone encoding human preproinsulin-like growth factor II. *Nature* 1984;310:775–777. [PubMed: 6382021]
- Brailoiu E, Churamani D, Pandey V, Brailoiu GC, Tuluc F, Patel S, Dun NJ. Messenger-specific role for nicotinic acid adenine dinucleotide phosphate in neuronal differentiation. *J Biol Chem* 2006;281:15923–15928. [PubMed: 16595650]
- Brailoiu GC, Brailoiu E, Chang JK, Dun NJ. Excitatory effects of human immunodeficiency virus 1 Tat on cultured rat cerebral cortical neurons. *Neuroscience* 2008;151:701–710. [PubMed: 18164555]
- Brailoiu GC, Dun SL, Yin DL, Yang J, Chang JK, Dun NJ. Insulin-like 6-immunoreactivity in the mouse brain and testis. *Brain Res* 2005;1040:187–190. [PubMed: 15804440]
- Brauner T, Hulser DF, Strasser RJ. Comparative measurements of membrane potentials with microelectrodes and voltage-sensitive dyes. *Biochim Biophys Acta* 1984;771:208–216.
- Burazin TC, Bathgate RA, Macris M, Layfield S, Gundlach AL, Tregear GW. Restricted, but abundant, expression of the novel rat gene-3 (R3) relaxin in the dorsal tegmental region of brain. *J Neurochem* 2002;82:1553–1557. [PubMed: 12354304]
- Burkhardt E, Adham IM, Brosig B, Gastmann A, Mattei MG, Engel W. Structural organization of the porcine and human genes coding for a Leydig cell-specific insulin-like peptide (LEY I-L) and chromosomal localization of the human gene (INSL3). *Genomics* 1994;20:13–19. [PubMed: 8020942]
- Chassin D, Laurent A, Janneau JL, Berger R, Bellet D. Cloning of a new member of the insulin gene superfamily (INSL4) expressed in human placenta. *Genomics* 1995;29:465–470. [PubMed: 8666396]
- Chen C, Li J, Bot G, Szabo I, Rogers TJ, Liu-Chen L-Y. Heterodimerization and cross-desensitization between the mu-opioid receptor and the chemokine CCR5 receptor. *Eur J Pharmacol* 2004;483:175–186. [PubMed: 14729105]
- Chen Y, Geisler WS, Seidemann E. Optimal decoding of correlated neural population responses in the primate visual cortex. *Nat Neurosci* 2006;9:1412–1420. [PubMed: 17057706]
- Conklin D, Lofton-Day CE, Haldeman BA, Ching A, Whitmore TE, Lok S, Jaspers S. Identification of INSL5, a new member of the insulin superfamily. *Genomics* 1999;60:50–56. [PubMed: 10458910]
- Connor JA. Digital imaging of free calcium changes and of spatial gradients in growing processes in single, mammalian central nervous system cells. *Proc Natl Acad Sci USA* 1986;83:6179–6183.
- Dun SL, Brailoiu E, Wang Y, Brailoiu GC, Liu-Chen LY, Yang J, Chang JK, Dun NJ. Insulin-like peptide 5: expression in the mouse brain and mobilization of calcium. *Endocrinol* 2006;147:3243–3248.
- Ebner TJ, Chen G. Use of voltage-sensitive dyes and optical recordings in the central nervous system. *Prog Neurobiol* 1995;46:463–506. [PubMed: 8532849]
- Gryniewicz G, Poenie M, Tsien RY. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* 1985;260:3440–3450. [PubMed: 3838314]
- Hida T, Takahashi E, Shikata K, Hirohashi T, Sawai T, Seiki T, Tanaka H, Kawai T, Ito O, Arai T, Yokoi A, Hirakawa T, Ogura H, Nagasu T, Miyamoto N, Kuromitsu J. Chronic intracerebroventricular administration of relaxin-3 increases body weight in rat. *J Recept Signal Transduct Res* 2006;26:147–158. [PubMed: 1677712]
- Hills CE, Brunskill NJ. Intracellular signalling of C-peptide. *Exper Diabetics Res* 2008;2008:635158.
- Keizer J, Levine L. Ryanodine receptor adaptation and Ca^{2+} -induced Ca^{2+} release-dependent Ca^{2+} oscillations. *Biophys J* 1996;71:3477–3487. [PubMed: 8968617]
- Kiselyov K, Shin DM, Muallem S. Signalling specificity in GPCR-dependent Ca^{2+} signalling. *Cell Signal* 2003;15:243–253. [PubMed: 12531423]
- Kunkler PE, Hulse RE, Schmitt MW, Nicholson C, Kraig RP. Optical current source density analysis in hippocampal organotypic culture shows that spreading depression occurs with uniquely reversing currents. *J Neurosci* 2005;25:3952–3961. [PubMed: 15829647]
- Le Bouc Y, Dreyer D, Jaeger F, Binoux M, Sondermeyer P. Complete characterization of the human IGF-I nucleotide sequence isolated from a newly constructed adult liver cDNA library. *FEBS Lett* 1986;196:108–112. [PubMed: 2935423]
- Liu C, Chen J, Sutton S, Roland B, Kuei C, Farmer N, Sillard R, Lovenberg TW. Identification of relaxin-3/INSL7 as a ligand for GPCR142. *J Biol Chem* 2003a;278:50765–50770. [PubMed: 14522967]

- Liu C, Eriste E, Sutton S, Chen J, Roland B, Kuei C, Farmer N, Jornvall H, Sillard R, Lovenberg TW. Identification of relaxin-3/INSL7 as an endogenous ligand for the orphan G-protein-coupled receptor GPCR135. *J Biol Chem* 2003b;278:50754–50764. [PubMed: 14522968]
- Ma S, Bonaventure P, Ferraro T, Shen P-J, Burazin TCD, Bathgate RAD, Liu C, Tregear GW, Sutton SW, Gundlach AL. Relaxin-3 in GABA projection neurons of nucleus incertus suggests widespread influence on forebrain circuits via G-protein-coupled receptor-135 in the rat. *Neurosci* 2007;144:165–190.
- McGowan BM, Stanley SA, White NE, Spangeus A, Patterson M, Thompson EL, Smith KL, Donovan J, Gardiner JV, Ghatei MA, Bloom SR. Hypothalamic mapping of orexigenic action and Fos-like immunoreactivity following relaxin-3 administration in male Wistar rats. *Am J Physiol Endocrinol Metab* 2007;292:E913–E919. [PubMed: 17132825]
- Ohtomo Y, Bergman T, Johansson BL, Jörnvall H, Wahren J. Differential effects of proinsulin C-peptide fragments on Na⁺, K⁺-ATPase activity of renal tubule segments. *Diabetologia* 1998;41:287–291. [PubMed: 9541168]
- Stern JE. Electrophysiological and morphological properties of pre-autonomic neurones in the rat hypothalamic paraventricular nucleus. *J Physiol* 2001;537:161–177. [PubMed: 11711570]
- Tanaka M, Lijima N, Miyamoto Y, Fukusumi S, Itoh Y, Ozawa H, Ibata I. Neurons expressing relaxin 3/INSL7 in the nucleus incertus respond to stress. *Eur J Neurosci* 2005;21:1659–1670. [PubMed: 15845093]
- Wahren J, Ekberg K, Johansson J, Henriksson M, Pramanik A, Johansson B-L, Rigler R, Jörnvall H. Role of C-peptide in human physiology. *Am J Physiol Endocrinol Metab* 2000;278:E759–E768. [PubMed: 10780930]
- Wallerath T, Li H, Gödtel-Ambrust U, Schwarz PM, Förstermann U. Stimulation of endothelial nitric oxide synthase by proinsulin C-peptide. *Nitric Oxide* 2003;9:95–102. [PubMed: 14623175]

Fig. 1.

Sections of pons of the rat labeled with C-INSL7 antiserum or C-INSL7 antiserum pre-absorbed with the C-INSL7 peptide (1 μ g/kg) overnight. A, irC-INSL7 neurons are distributed to the nucleus incertus pars compacta (Nlc) and nucleus incertus pars dissipata (Nld); they appear multipolar with extensive branching. B, a pontine section, at a level similar to that shown in panel A, labeled with C-INSL7 antiserum pre-absorbed with the peptide (1 μ g/ml) overnight; irC-INSL7 neurons are absent in the nucleus incertus. C and D, sections of pons where few irC-INSL7 neurons are noted in the lateral periaqueductal gray (LPAG) and ventrolateral periaqueductal gray (VLPAG); they appear bipolar, with few branching. Abbreviations: 4V, 4th ventricle; Aq, aqueduct; DTg, dorsal tegmental nucleus. Scale bar: A–D, 100 μ m.

Fig. 2.

Fluorescence images of rat forebrain sections labeled with C-INSL7 antiserum A–C, in the septum, a dense network of irC-INSL7 fibers is seen in the lateral septum nucleus, intermediate (LSI) and medial septum (MS); a moderate density of irC-INSL7 fibers is present in lateral septum nucleus, ventral (LSV), and nucleus of vertical limb of the diagonal band (VDB). D, irC-INSL7 fibers are present in the bed nucleus of stria terminalis, medial division, anterior (BSTMA) and ventral division (BSTV). E–L, varying intensities of irC-INSL7 fibers are present in several hypothalamic nuclei such as: medial preoptic area (MPA), lateral preoptic area (LPO), paraventricular hypothalamic nucleus (PVN), supraoptic nucleus (SO), lateral hypothalamic area (LH), ventromedial hypothalamic nucleus (VMH), periventricular hypothalamic nucleus (Pe), dorsal hypothalamic area (DA) and dorsomedial hypothalamic nucleus (DMD), and tuber cinereum (TC). M–N, irC-INSL7 fibers were present in lateral habenular nucleus (LHb), paraventricular thalamic nucleus, anterior (PVA) and scarce in the medial habenular nucleus (MHb). O–P, irC-INSL7 fibers in endopeduncular nucleus (EP) and basolateral amygdaloid nucleus, anterior (BLA). Abbreviations: aca, anterior commissure,

anterior; 3V, 3rd ventricle; D3V, dorsal 3rd ventricle; opt, optic tract; sox; supraoptic decussation. Scale bar, 50 μ m.

Fig. 3.

Elevation of cytosolic Ca^{2+} concentrations in cultured hypothalamic neurons by INSL7 and C-INSL7. A, INSL7 (100 nM) produced a fast and transitory increase in $[Ca^{2+}]_i$ in this neuron; the mean $[Ca^{2+}]_i$ increase was 817 ± 6.3 nM ($n=27$). B, A fast response followed by calcium oscillations was evoked by INSL7 (100 nM) in 8 neurons; the mean amplitude of the first spike was 1268 ± 39 nM. C, C-INSL7 (10, 100 and 1000 nM) produced a fast and sustained increase in $[Ca^{2+}]_i$ in a concentration-dependent manner: the mean $[Ca^{2+}]_i$ increase was 118 ± 2.4 nM ($n=6$) for 10 nM C-INSL7 (green trace), 405 ± 3.1 nM ($n=36$) for 100 nM (black trace) and 731 ± 4.7 nM ($n=8$) for 1000 nM C-INSL7 (red trace) respectively. D, C-INSL7 induced calcium oscillations also in a concentration-dependent manner: the mean amplitude of the first spike was 137 ± 2.6 nM ($n=6$) for 10 nM (green), 763 ± 4.2 nM ($n=11$) for 100 nM (black) and 1051 ± 9.8 nM ($n=7$) for 1000 nM, respectively (red). E, pretreatment with ryanodine (5 μ M; 15 min) had no significant effect on C-INSL7 (100 nM)-induced fast and sustained increase in $[Ca^{2+}]_i$ ($\Delta [Ca^{2+}]_i = 391 \pm 3.6$ nM; $n=7$). F, pretreatment with ryanodine abolished

$[Ca^{2+}]_i$ oscillation, C-INSL7 (100nM) caused a fast, transitory increase of $[Ca^{2+}]_i$ by 427 ± 3.2 nM ($n = 9$).

Fig. 4. Calcium responses produced by INSL7 and C-INSL7 in Ca^{2+} -free saline. A, INSL7 (100 nM) produced a rapid and transitory increase in $[Ca^{2+}]_i$ by 307 ± 4.2 nM (n=11). B, INSL7 induced $[Ca^{2+}]_i$ oscillations with a mean amplitude of the first spike of 583 ± 7.4 nM (n=5). C, In this neuron, C-INSL7 (100 nM) produced a fast and transitory increase in $[Ca^{2+}]_i$; the mean amplitude of $[Ca^{2+}]_i$ spike was 231 ± 2.6 nM (n=15). D, C-INSL7 induced $[Ca^{2+}]_i$ oscillations with a mean amplitude of the first spike of 497 ± 3.6 nM (n=7).

Fig. 5. Optical imaging of membrane potential changes by C-INSL7 in dissociated rat hypothalamic neurons. **A**, C-INSL7 depolarized (top trace) or hyperpolarized (bottom trace) hypothalamic neurons. **B**, the mean amplitude of depolarizations was $11 \pm 1.6\text{mV}$ ($n=7$) and the mean amplitude of hyperpolarizations was $7 \pm 1.3\text{mV}$ ($n=15$).

Figure 6.

C-INSL7 binding in rat brain membranes: $[^{125}\text{I}]$ C-INSL binding was performed in the presence or absence of various concentrations of unlabeled C-INSL7 and INSL7 as described in Experimental procedures. Data are expressed as mean $\pm$ s.e.m. of three independent experiments.