Cortistatin radioligand binding in wild-type and somatostatin receptor-deficient mouse brain

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Abstract

Cortistatin-14 (CST-14) is a recently discovered member of the somatostatin family of neuropeptides. It shares 11 of its 14 amino acids with somatostatin-14 (SRIF-14). In the present study, binding sites for cortistatin-14 in the mouse brain were examined and compared to those for somatostatin using iodinated cortistatin-14 and iodinated somatostatin-14. By in vitro receptor autoradiography, high densities of cortistatin-14 and somatostatin-14 specific binding sites were detected in the cortex, hippocampal formation, basolateral amygdala and medial habenula. Unlabeled 100 nM cortistatin-14 inhibited iodinated somatostatin-14 binding in the hippocampus, but not in the cortex or amygdaloid nuclei. In somatostatin receptor subtype-2 knock-out (KO) mice, autoradiographic iodinated somatostatin-14 binding was observed in the hippocampus and habenula but was removed in the cortex and amygdaloid nuclei, specific iodinated cortistatin-14 binding sites were found in the hippocampus, habenula and throughout the cortex. We conclude that the somatostatin receptor subtype-2 is responsible for somatostatin binding in cortical and amygdaloid regions and that cortistatin predominantly interacts with the same receptors as somatostatin.

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1. Introduction

Cortistatin-14 (CST-14) is a recently discovered neuropeptide relative of somatostatin (somatotropin-release inhibiting factor, SRIF), named after its mainly cortical expression and ability to depress cortical activity [8]. The cortistatin precursor protein gene has now been cloned from mouse, rat and human sources [10,12] and encodes a 112-amino acid peptide, which is processed to produce two physiologically relevant neuropeptides, CST-28 and CST-14. CST-14 (rodent: P[CKNFFWKTFSSC]K) shares 11 of its 14 amino acids with somatostatin-14 (SRIF-14) (rodent: AG[CKNFFWKTFTSC]) including those residues thought to be responsible for SRIF-14 binding to its receptors [31], and the cysteine pair which likely renders the peptide cyclic. However, differences in cDNA sequences and chromosomal location clearly indicate that cortistatin and somatostatin are the products of separate genes (reviewed in Ref. [30]).

The five cloned SRIF receptors (sst1–5) belong to the G-protein coupled receptor family. Binding features (high or low affinity for octreotide or seglitide, respectively) distinguish two classes of receptors: SRIF1 including sst2, sst3 and sst5 and SRIF2 including sst1 and sst4 [6]. CST-14 binds to and activates the five sst1–5 receptors with affinities comparable to SRIF-14 itself [8,12,29]. SRIF...
receptors are widely distributed in the brain and periphery, with distinct patterns of expression \[17,21\]. In the rat, sst1 mRNA is abundant throughout the neuraxis, whereas sst2 is predominantly expressed in the cortex. sst5 is moderately expressed throughout the brain, sst4 is the least abundant somatostatin receptor and is found mainly in the hippocampus, and sst3 is preferentially expressed in the hippocampal formation and cerebellum (reviewed in Ref. \[21\]). The similarity of CST-14 and SRIF-14 structures and pharmacological properties might suggest that the in vivo role of CST-14 is similar to that of SRIF-14. However, this appears to not be the case as: (i) CST-14 levels are not upregulated in the somatostatin knock-out (KO) mouse \[34\]; (ii) preprocortistatin and preprosomatostatin genes respond to different signals \[4\]; (iii) the peptide precursors are expressed in partially overlapping but distinct populations of neurons \[9\]; and (iv) CST-14 exerts many markedly different physiological effects compared to SRIF (reviewed in Ref. \[30\]). Thus, cortistatin does not appear to be a somatostatin back up, but a neuropeptide with distinct functions.

We used in situ receptor autoradiography to identify areas of the brain containing receptors for CST-14 in comparison with SRIF-14. Iodinated CST-14 and SRIF-14 were used to map brain regions containing binding sites for these ligands. Cross competition studies with SRIF-14 or CST-14 were performed to examine the binding properties of CST-14 to SRIF receptors. Tissue specific radioligand-binding in WT and SRIF receptor subtype-2 knockout mice was also performed to characterize CST-14 and SRIF-14 binding sites in the absence of SRIF receptors.

2. Experimental procedures

2.1. Animals

Adult C57BL/6J mice were maintained at 22 °C on a 12:12-h light–dark cycle with free access to food and water. Mice were sacrificed by decapitation under halothane anesthesia in accordance with the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978). Brains were removed and rapidly frozen in mounting medium (Tissue-Tek O.C.T. compound) under dry ice. Brains were either sectioned directly or stored in air tight containers at –80 °C. sst2 knock-out mice (backcrossed for at least five generations into C57BL/6J background) were obtained from Merck, NJ, USA \[35\].

2.2. Autoradiography procedures

Brain coronal and sagittal sections of 12 μm thickness were cut on a cryostat (Leica) at –17 °C and thaw mounted onto superfrost plus glass slides (Fisher). Sections were either used directly or stored at –80 °C. For binding reactions, sections were brought up to room temperature and preincubated for 15 min in 50 mM Tris–HCl buffer, pH 7.6 containing 0.25 M sucrose and 1% BSA. The sections were then incubated for 45 min at room temperature in the same medium to which MgCl₂ (5 mM), phenylmethylsulfonylfluoride (PMSF; 10 μM), bacitracin (50 μg/ml), leupeptin (50 μg/ml), the iodinated ligand and competitor peptide if used were added. For each experimental condition, a minimum of 3 animals were tested and autoradiography was performed on 50 sections from each brain; 30 sections for total binding and 20 sections for binding competed with excess unlabelled ligand. The iodinated ligands used in this study were 125I-[Tyr⁹]SRIF-14 (NEN Life Science; specific activity 2200 Ci/mmol) and custom synthesized 125I-[Tyr¹⁰]CST-14 (Advanced Chemtech; specific activity 2100 Ci/mmol).

Radiolabeled ligands were used at 0.2 nM final concentration. Binding reaction mixtures were applied to the thaw mounted sections in a volume of 120 μl. Competitor ligands included unlabeled SRIF-14 and CST-14. After incubation, the sections were rinsed twice in ice cold supplemented Tris buffer (5 min/bath) and then rinsed in ice cold H₂O. The sections were air dried and exposed for 24–72 h to autoradiographic film (BioMax, MR) depending on signal strength. Autoradiographic images were photographed using a DC120 digital camera (Kodak) or scanned using a ScanWit 2720S film scanner (Acer). Brain structures were identified by comparison with the mouse brain atlas of Franklin and Paxinos \[11\]. Optical density on the autoradiographic films was measured using a computerized image analysis system (Biocom, Les Ulis, France).

2.3. Competition radioligand-binding studies

Membranes were prepared from cortex or hippocampus dissected from sacrificed halothane anesthetized mice. Dissected tissues were suspended in 4 °C homogenization buffer: 50 mM Tris–HCl pH 7.6, 5 mM MgCl₂ and PMSF 50 μg/ml. Membranes were Polytron disrupted and centrifuged at 2000×g for 10 min at 4 °C. The membrane-containing supernatant was centrifuged at 4 °C for 30 min at 40,000 g. The membrane pellet was resuspended in 4 ml homogenization buffer and recentrifuged as before, four times to wash the membranes. Protein concentration was determined by the BioRad Protein Assay Kit with BSA as a standard, and membrane containing solutions were normalized to the same protein concentration for each tissue. Binding reactions were carried out in a final volume of 200 μl in 96-well plates (Costar). 125I-[Tyr¹⁰]CST-14 and 125I-[Tyr⁹]SRIF-14 were used as radioligands at 0.2 nM final concentration. Membranes were incubated with either radioligand and the appropriate concentration of competing CST-14, for 1 h at room temperature. Binding reactions were terminated.
by rapid vacuum filtration, using a Tomtec Harvester 96 (Mach III m), over GF/C filters pre-soaked in 0.5% polyethylenimine for 1 h. The filters were washed three times with ice cold 50 mM Tris–HCl buffer and individual filter units counted for membrane bound radioactivity in a gamma counter. Data were plotted and curves fit using Graphpad Prism version 3.02 (Graphpad Software) assuming the binding was due to one or more binding sites, in order to determine IC50 values.

3. Results

3.1. Cortistatin-14 and somatostatin-14 autoradiographic binding

The most intense 125I-[Tyr10]CST-14 radioligand binding in the mouse brain was observed in the basolateral amygdala and the deep layers (V–VI) of the cerebral cortex (Fig. 1A). Other structures showing significant levels of

![Fig. 1. Regional distribution of [125I]-[Tyr10]CST-14 binding in the adult mouse brain. The overall regional distribution of radiolabeling by 125I-[Tyr10]CST-14 (0.2 nM) in coronal and sagittal 12 μm thick sections of the adult mouse brain. (A) Total 125I-[Tyr10]CST-14 (H) and non-specific binding (C) defined by addition of 1 μM CST are shown. Sections are representative of typical patterns of ligand binding taken from 50 brain sections (30 hot, 20 cold) from each of at least 3 animals. Dark regions are regions of high radioligand-binding. Strong 125I-[Tyr10]CST-14 labeling is found in the hippocampal formation (Hipp), deep layers of the cortex (Ctx) and amygdaloid formation (Amy). (B) Distribution of 125I-[Tyr7]SRIF-14 in adult mouse brain sections. Note that the overall pattern is indistinguishable from the CST-14 binding pattern.](image-url)
$^{125}$I-[Tyr$^{10}$]CST-14 binding include the hippocampal formation and the medial nucleus of the habenula. Serial coronal sections and sagittal sections show that the CST-14 binding sites are located in the deep layers in all cortical areas with the frontal cortex showing the most intense labeling. Excess unlabeled CST-14 (1 μM) was able to fully compete the radiolabeling of the structures above. $^{125}$I-[Tyr$^{10}$]CST-14 binding was not observed in the cerebellum or other hindbrain areas.

The pattern of receptor binding for $^{125}$I-[Tyr$^{0}$]SRIF-14 and $^{125}$I-[Tyr$^{10}$]CST-14 was similar in most areas (Fig. 1). $^{125}$I-[Tyr$^{0}$]SRIF-14 labeled the deep layers of the cerebral cortex, the hippocampal formation, basolateral amygdala and medial habenula, consistent with previous reports using $^{125}$I-[Tyr$^{0}$]SRIF-14 [18]. Highest receptor densities were also observed in the deep layers of the frontal cortex and the basolateral amygdala. $^{125}$I-[Tyr$^{0}$]SRIF-14 binding was fully competed by excess unlabeled SRIF-14 (1 μM). The $^{125}$I-[Tyr$^{0}$]SRIF-14 binding signal to noise ratio was higher than that of the $^{125}$I-[Tyr$^{10}$]CST-14 signal, which may be due to differences in peptide synthesis and iodination.

3.2. Peptide competition studies

In order to determine whether the similar pattern of CST-14 and SRIF-14 binding sites is an overlapping distribution to discrete populations of receptors, or due to both neuro-peptides interacting with the same binding sites, cross-competition experiments were performed (Fig. 2). Detectable $^{125}$I-[Tyr$^{0}$]SRIF-14 binding in mouse brain sections was effectively competed by co-incubation with either 1 μM CST-14 or 1 μM SRIF-14. However, 100 nM CST-14 was not able to compete $^{125}$I-[Tyr$^{0}$]SRIF-14 binding in the hippocampus, but not in the cortex, habenula and amygdaloid nuclei.

Fig. 3. Graphs of the displacement of $^{125}$I-[Tyr$^{0}$]SRIF-14 by CST-14 and SRIF-14 in different brain regions. In the hippocampal formation, both competing ligands showed similar displacement curves competing for high affinity binding sites (top). However, in the cerebral cortex and amygdala (bottom), CST-14 showed much lower affinity compared with SRIF.
to compete $^{125}\text{I}-\text{[Tyr}^0\text{]}\text{SRIF-14}$ binding in the cerebral cortex or basolateral amygdala whereas 100 nM SRIF-14 completely removed $^{125}\text{I}-\text{[Tyr}^0\text{]}\text{SRIF-14}$ binding in all brain regions. 100 nM CST-14 was able to remove $^{125}\text{I}-\text{[Tyr}^0\text{]}\text{SRIF-14}$ binding from the hippocampal formation (Fig 3). Quantification of the displacement of iodinated somatostatin by somatostatin and cortistatin also revealed qualitative regional differences (Table 1). SRIF-14 exhibits similar apparent affinities for its own binding sites in the hippocampus, amygdala and cerebral cortex with Hill numbers below one probably as a result of the presence of several SRIF-14 receptors in these brains areas recognized by the radioactive ligand (Table 1). Indeed, when CST-14 was used as the competing ligand, the binding profile was significantly different in the cortex and the amygdala compare to the hippocampus. Thus, CST-14 affinities for SRIF-14 binding sites in the cerebral cortex and amygdala are decreased and associated with Hill numbers higher to one because of the use of a concentration range apart from the IC$_{50}$ (Fig. 3 and Table 1). In contrast, $^{125}\text{I}-\text{[Tyr}^0\text{]}\text{SRIF-14}$ displacement curves exhibit similar shapes and binding parameters for both CST-14 and SRIF-14 in the hippocampus (Fig. 3 and Table 1).

### 3.3. Studies in mice deficient for somatostatin receptor subtypes 2

In order to examine which SRIF receptor subtypes CST-14 and SRIF-14 bind to in the various brain regions, autoradiography was performed with both ligands on sst2 KO mice. $^{125}\text{I}-\text{[Tyr}^0\text{]}\text{SRIF-14}$ binding was almost entirely removed from brain sections of SST2 KO mice (Fig. 4). There was no detectable SRIF-14 binding in the cerebral cortex of these animals except in the frontal cortex, which displayed weak $^{125}\text{I}-\text{[Tyr}^0\text{]}\text{SRIF-14}$ binding. SRIF-14 binding was also removed from the basolateral amygdala. Specific SRIF-14 binding sites were not removed in the hippocampus and the medial habenula, where strong binding was still observed. These results indicate that the number of SRIF-14 sites is decreased in the cortex and

### Table 1

Quantification of autoradiographic binding of $^{125}\text{I}$-SRIF-14 displaced by SRIF-14 or CST-14 on sections of WT mice

<table>
<thead>
<tr>
<th>Iodinated label</th>
<th>Brain region</th>
<th>Competitor ligand</th>
<th>Log IC$_{50}$±S.E.M.</th>
<th>Hill number</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{125}\text{I}$-SRIF-14</td>
<td>Hippocampus</td>
<td>SRIF-14</td>
<td>7.42 ± 0.26</td>
<td>0.77</td>
</tr>
<tr>
<td>$^{125}\text{I}$-SRIF-14</td>
<td>Hippocampus</td>
<td>CST-14</td>
<td>7.89 ± 0.71</td>
<td>0.56</td>
</tr>
<tr>
<td>$^{125}\text{I}$-SRIF-14</td>
<td>Cortex</td>
<td>SRIF-14</td>
<td>8.37 ± 0.89</td>
<td>0.57</td>
</tr>
<tr>
<td>$^{125}\text{I}$-SRIF-14</td>
<td>Cortex</td>
<td>CST-14</td>
<td>6.71 ± 0.12</td>
<td>&gt;1</td>
</tr>
<tr>
<td>$^{125}\text{I}$-SRIF-14</td>
<td>Amygdala</td>
<td>SRIF-14</td>
<td>8.05 ± 0.53</td>
<td>0.63</td>
</tr>
<tr>
<td>$^{125}\text{I}$-SRIF-14</td>
<td>Amygdala</td>
<td>CST-14</td>
<td>6.70 ± 0.23</td>
<td>&gt;1</td>
</tr>
</tbody>
</table>

Each data point represents $n=3$ replicates. IC$_{50}$ and Hill numbers are the results of the analysis of a sigmoidal dose response curve with a variable slope.

Fig. 4. $^{125}\text{I}$-[Tyr$^0$]SRIF-14 and $^{125}\text{I}$-[Tyr$^{10}$]CST-14 binding in SST2 knock-out mice. Total $^{125}\text{I}$-[Tyr$^0$]SRIF-14 and non-specific (NS) binding defined by addition of 1 mM SRIF-14 are shown. SST2 deficient mice exhibit diminished $^{125}\text{I}$-[Tyr$^0$]SRIF-14 binding sites throughout the cortex and amygdala, whereas binding in the hippocampus and habenula is not reduced compared with wild-type sections (see Fig 2). SST2 deficient animals exhibit diminished $^{125}\text{I}$-[Tyr$^{10}$]CST-14 binding sites in the amygdala, but not in the hippocampus and habenula compared with autoradiographs from wild-type mice (see Fig 1). Note specific $^{125}\text{I}$-[Tyr$^{10}$]CST-14 binding is found in superficial layers throughout the cortex of SST2 KO mice.
Each data point represents n>3 replicates.

4. Discussion

The pattern of radioligand binding observed in this study for SRIF-14 is in agreement with previous studies on the distribution of SRIF-14 binding sites using autoradiography [15,16,18]. This is the first study to report the distribution and radioligand-binding properties of CST-14 binding sites in the brain. The overall distribution of CST-14 binding sites in the WT mouse brain was not distinguishable from that of SRIF-14, with the majority of binding sites in the deep layers of the cortex, the hippocampal formation, basolateral amygdala and medial habenula. In addition, the CST-14 binding was effectively competed by SRIF-14, indicating that CST-14 interacts with receptors which exhibit high affinity for SRIF-14.

Cross-competition experiments with increasing concentrations of CST-14 showed that the affinities of CST-14 to receptors expressed in various brain regions may not be uniform. In the cortex and amygdala, iodinated SRIF-14 binding was not competed by up to 100 nM CST-14 whereas significantly lower concentrations of unlabeled SRIF-14 did effectively compete the iodinated SRIF-14 binding. Although not directly comparable due to the different experimental paradigms used, the difference in autoradiographic binding in the cortex and amygdala contrasts with the observed similarity of CST-14 and SRIF-14 binding affinities to the five known SRIF receptors by membrane binding in vitro and with our results from brain membranes in wild-type mice [5,12,21,28,29].

Mechanisms may exist which modulate the affinities of SRIF receptors compared to the isolated receptors when expressed in vitro. This may account for some of the physiological and behavioral differences observed on CST-14 and SRIF-14 injections in rodent brains (reviewed in...
Ref. [30]). Differences in the apparent affinities on brain tissue may be the result of the co-expression of various SRIF receptor subtypes in the same population of neurons. Indeed, heterodimerization of SRIF-receptor subtypes has been shown to modify receptor-binding affinities for SRIF receptor subtypes; for example heterodimerization of sst2A and sst3 results in disruption of sst3 receptor function [22]. Even though sst3 has been localized by some authors in neuronal cilia [13], other studies have placed this receptor in the plasma membrane and it may thus interact with other GPCRs of the somatostatin family.

The distribution of SRIF-14 binding sites in the brain of sst2 KO mice was recently reported [33] with observations in agreement to those presented in this study. The removal of detectable autoradiographic $^{125}$I-SRIF-14 labeling in all brain regions apart from the hippocampus and habenula by knock-out of sst2, indicates that under the binding conditions used in these experiments sst2 accounts for the majority of high affinity SRIF-14 binding in the brain. The observation of CST-14 binding but not SRIF-14 binding in the cortex of sst2 KO animals may be due to the binding conditions used favoring one ligand over the other, or biological reasons that may include the existence of binding sites with higher affinity for CST-14 than for SRIF-14 which do not involve sst2.

It is unlikely that the CST-14 binding sites in the cortex are due to upregulation of other somatostatin receptors, since all detectable $^{125}$I-SRIF binding is lost in this area, and there is no evidence of upregulation of other receptors in these mice [14]. It is also of interest to note that the areas in which CST-14-specific binding in sst2 deficient mice is observed includes the brain regions where the neuropeptide CST-14 is predominantly expressed, namely the neocortex and hippocampus [8–10,30].

Membrane radioligand-binding data showed that both SRIF-14 and CST-14 bind with indistinguishable apparent affinities in the cortex and hippocampus of sst2 KO mice. Although it may be interpreted that other somatostatin receptors are upregulated in the sst2 KO mice that could account for cortistatin binding in the cerebral cortex, recent data has shown little, if any compensation by other SRIF receptors in these mutant mice [14]. However, since labeled CST-14 displays higher background than SRIF-14, we cannot rule out that the differences in autoradiographic binding to sst2 KO mice are due to chemical instability of the ligands. It should be noted, however, that chemical modifications of CST14, including dTrp$^7$ CST14, do not result in prolonged or more potent in vivo activity [5]. Also, the difference in binding to sst2 KO sections between CST14 and SRIF 14 could be due to exposure of binding sites to ligand in the two different experimental procedures, as has been described for the two main populations of SRIF binding sites. Indeed, SRIF1 binding sites, which correspond to sst2, sst3 and sst5 are detected under binding conditions different from SRIF2 sites (corresponding to sst1 and sst4) [18].

Biological evidence for a cortistatin-specific receptor has been increasing since the discovery of this peptide [8]. CST-14, like SRIF-14, has been shown to be capable of activating all five of the known SRIF receptors in in vitro pharmacological assays and to stimulate the M-current in neurons [21]. However, it has not yet been shown that CST-14’s interaction with the SRIF receptors reflects in vivo events. A significant body of evidence points towards the in vivo effects of CST-14 being mediated by receptors distinct from the classically defined SRIF receptors. This evidence includes: (i) ICV injection of CST-14 causes hypomotility in rats [8], whereas injection of SRIF-14 causes hypermotility [1,32]; (ii) SRIF-14 application to the hippocampus and cortex increases cortical excitability, whereas this is depressed by CST-14 application [8]; (iii) i.c.v. injection of CST-14 increases slow wave sleep without effecting rapid eye movement (REM) sleep [8,12], whereas SRIF-14 injection increases REM sleep with no effect on slow wave sleep [2,3,7]; and (iv) CST-14 is able to augment the amplitude of the h current in hippocampal neurons which is unresponsive to SRIF-14 [27]. Indeed, the recent cloning of a human G-protein coupled receptor, called MrgX2 [23], that selectively binds CST-17 with nanomolar affinity and does not bind to SRIF, strongly suggests that part of the effects of cortistatin are independent of somatostatin receptors. It is intriguing, however, that no homologues of the MrgX2 receptor exist in rodents. It is noteworthy that the new receptor MrgX2 is expressed in the hippocampal formation in humans. We can speculate that the MrgX2 orthologue(s) in rodents may be expressed in the cerebral cortex and dentate gyrus. These data may suggest that, although CST-14 predominantly binds to the same populations of receptors as SRIF-14, there may be other receptors in the cerebral cortex which mediate cortistatin’s biological effects. Alternatively, the differential effects of cortistatin in rodents may be mediated through SRIF receptors which could be modified by mechanisms including dimerization [22,24,25], receptor activity modifying proteins (RAMPs) [19], or differential receptor internalization [26].

In this study, we show that CST-14 interacts with somatostatin receptors in the rodent brain. The major brain areas of CST-14 binding are the neocortex, hippocampal formation, habenula and amygdala. CST-14 and SRIF-14 have almost indistinguishable apparent affinities for their binding sites in the brain. Preliminary data from displacement with cold ligands and autoradiography in SST2 KO knock-out mice suggest the possibility of binding sites that appear to show selectivity for CST-14, although further characterization studies are required to confirm if this binding represents a new class of receptors. Recent cloning of human CST-17 selective receptors suggests that cortical specific CST-14 receptors may exist in rodents. Further molecular characterization of cortistatin receptors and the pharmacology of cortistatin will shed light into the neurobiology of this new peptidergic system.
Acknowledgements

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References