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Full-length review

Cortistatin: a member of the somatostatin neuropeptide family with distinct physiological functions

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Abstract

Cortistatin is a recently discovered neuropeptide relative of somatostatin named after its predominantly cortical expression and ability to depress cortical activity. Cortistatin-14 shares 11 of the 14 amino acids of somatostatin-14 yet their nucleotide sequences and chromosomal localization clearly indicate they are products of separate genes. Now cloned from human, mouse and rat sources, cortistatin is known to bind all five cloned somatostatin receptors and share many pharmacological and functional properties with somatostatin including the depression of neuronal activity. However, cortistatin also has many properties distinct from somatostatin including induction of slow-wave sleep, apparently by antagonism of the excitatory effects of acetylcholine on the cortex, reduction of locomotor activity, and activation of cation selective currents not responsive to somatostatin. Expression of mRNA encoding cortistatin follows a circadian rhythm and is upregulated on deprivation of sleep, suggesting cortistatin is a sleep modulatory factor. This review summarizes recent advances in our understanding of the neurobiology of cortistatin, examines the similarities and differences between cortistatin and somatostatin, and asks the question: does cortistatin bind to a cortistatin-specific receptor?

Theme: Neorotransmitters, modulators, transporters, and receptors

Topic: Peptides: anatomy and physiology

Keywords: G protein coupled receptor; CST; Memory; Sleep; LTP; h-current

Contents

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1. Cloning, chromosomal mapping and structure of the cortistatin gene

The cDNA encoding cortistatin was isolated through characterization of regional specific rat brain mRNAs using directional tag PCR subtractive hybridization by de Lecea et al. in 1996 [19]. The sequence indicated the clone to be a novel 112 amino acid protein with a striking homology to somatostatin at its distal C-terminal end. The C-terminal 14 predicted amino acids, which are preceded by a pair of lysines, potentially forming a site for proteolytic cleavage, share 11 residue identities with somatostatin-14. Furthermore, the residues common with somatostatin include the FWKT tetramer that is critical for somatostatin binding to its receptors [82] (Fig. 1), and two homologously placed cysteine residues which are likely to cause the predicted peptide to be cyclic. The new protein was named preprocortistatin in recognition of its predominantly cortical expression and its neuronal depressant properties (see below). Despite the obvious similarities with somatostatin the new gene was concluded to be distinct from that encoding somatostatin as: (1) the alignment of the cortistatin and somatostatin 14mer peptides is shifted by one residue, with the alignment of cortistatin beginning at residue 2 of somatostatin and the terminal lysine residue of cortistatin extending one residue beyond the C-terminal cysteine of somatostatin; (2) the cDNA sequences are not homologous except for the regions encoding the 14mers; and (3) the mouse gene for cortistatin (*cort*) was mapped to chromosome 4 whereas somatostatin is located on chromosome 16 [21]. The region of the mouse chromosome 4 where *cort* maps is syntenic to human 1p36. Interestingly a human neuroblastoma susceptibility gene has recently been mapped to this region [1].

Cortistatin has thus far been cloned from mouse, rat and human tissues [19,26]. Initially cloned from rat, the 112 amino acid predicted preprocortistatin protein is comparable to the 116 residues of preprosomatostatin. The preprocortistatin gene product begins with a 27-amino acid apparent secretion-signal sequence which contains six CTG (leucine) trinucleotide repeats. Interestingly when this region is expanded in other genes it has been implicated in neurological diseases such as myotonic dystrophy [8]. Preprocortistatin also contains a number of predicted dibasic amino-acids (KR, RK, KK, RR) which constitute possible sites of action for prohormone convertases (see below). With the predicted signal sequence removed the remaining procortistatin protein could be further cleaved to produce cortistatin-29 (rCST29), rCST14, rCST13, rCST17, rCST31 and additional peptide products produced by partial cleavage (Fig. 2). CST29 and CST14 are analogous to somatostatin-28 (SST28) and SST14. rCST14 may also be processed by carboxypeptidase enzymes which would cleave its C-terminal lysine residue. The preprocortistatin gene also encodes two consensus polyadenylation signals located at its 3′ end.

Cloning of the mouse preprocortistatin gene reveals an 86% nucleotide sequence identity between mouse and rat genes (after incorporation of two gaps), with complete identity in the region corresponding to rCST14 and the adjacent lysine doublet [21]. The mouse gene sequence indicates that the translated protein is likely to contain 108 amino, acids compared to 112 for rat. Alignment of these predicted protein sequences shows that rat and mouse gene products are 82% identical (after incorporation of two gaps). Interestingly, upstream of the processing site of mCST14 several points of divergence in DNA and predicted protein sequence are found, including the deletion of two pairs of dibasic residues which may act as sites for proteolytic cleavage. Thus cleavage of mouse preprocortistatin may result in mCST14, mCST44 or mCST58 (by partial digestion) protein products. This would suggest that mCST14 is the only physiologically relevant peptide processed from the mouse cortistatin precursor protein.

The human cortistatin peptide was cloned by PCR based methods [21] and also identified by screening a human expressed sequence tag (EST) database [26]. The human preprocortistatin nucleotide sequence showed a lower degree of identity to the rat sequence (71%) [21]. Human preprocortistatin is predicted to have 114 residues and begin with a 29-amino acid hydrophobic secretory signal

Fig. 1. Alignment of predicted amino acid sequences from rat, mouse and human cortistatin-29 and somatostatin-28. Consensus residues between the known cortistatin sequences are shown (CST consensus), as are residues in common between all cortistatin and somatostatin sequences (All consensus). The basic residues thought to be substrates for prohormone convertase enzymes are underlined. The conserved cysteine residues which are likely to render the peptides cyclic are also highlighted.

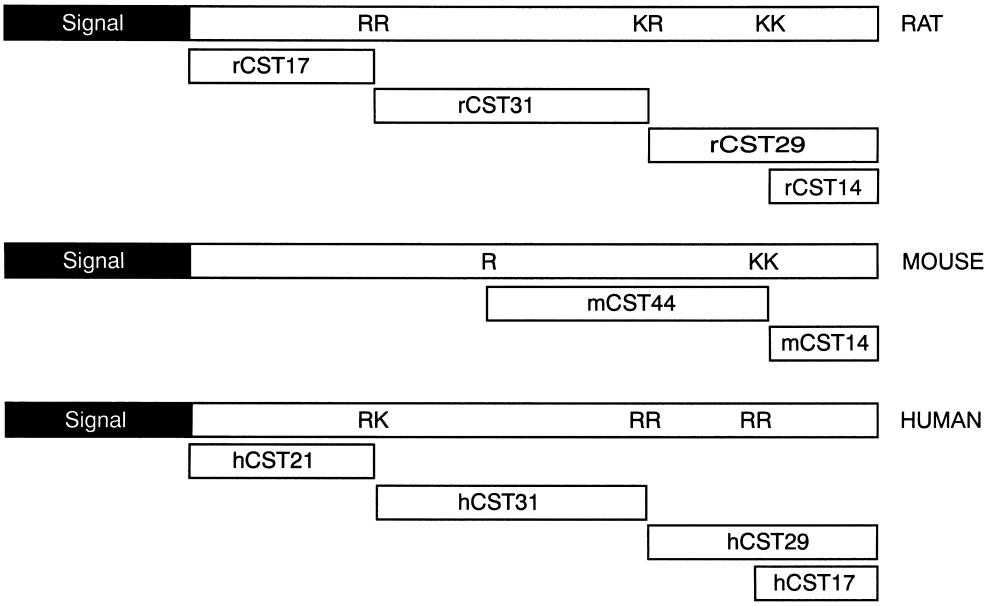

Fig. 2. Predicted proteolytic products generated from the processing of rat, mouse and human preprocortistatin peptides with prohormone convertase enzymes (schematic, not to scale).

sequence. Within this putative signal sequence are four CTG triplet (encoding leucine) repeats which compare to six in the rat and three in the mouse, suggesting that this region is not stable in length. Overall, human and mouse predicted preprocortistatin amino acid sequences are 55% identical [26]. However, rCST14 shares 13 of its 14 residues with the aligned sequence from the human, although the two lysines immediately N-terminal of the rCST14 peptide are not represented in the human precursor protein. Instead, a pair of arginine residues, three amino acid positions N-terminal of the predicted rCST14 cleavage site, are present in the deduced human preprocortistatin amino acid sequence. These residues suggest the human equivalent of rCST14 is 17 residues in length (hCST17; Fig. 3). Additional predicted cleavage sites suggest the possibility of producing hCST21, hCST31, hCST29, and hCST17 peptides (and others by partial cleavage).

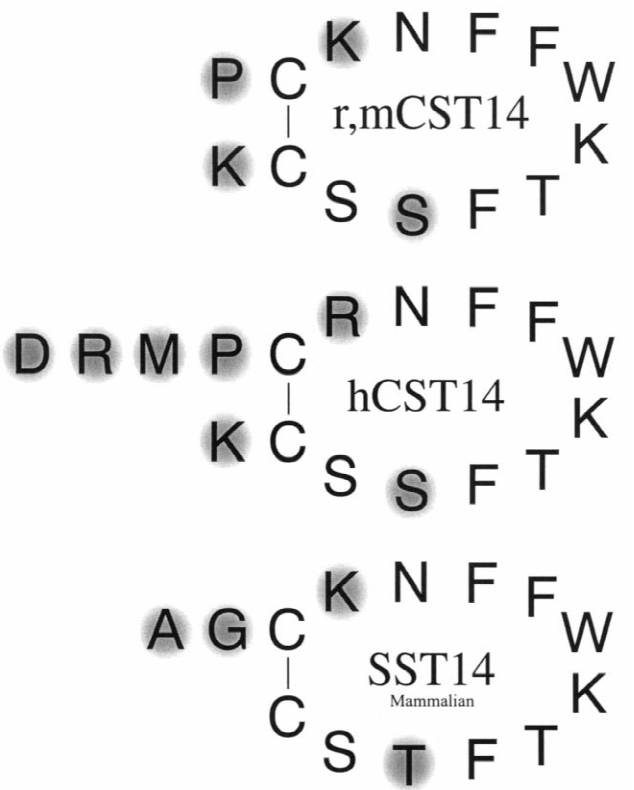

Fig. 3. Predicted secondary structures of rodent and human cortistatin-14 and 17, and somatostatin-14.

Preprocortistatin mRNA is detected by Northern blot analysis as a single band of approximately 600 nucleotides from rat brain tissues [19,21] and as an approximately 1 Kb band in human tissues [26]. In a recent study the mouse preprocortistatin gene was cloned and its nucleotide sequence determined [9]. The transcriptional region of the mouse preprocortistatin gene was shown to include two exons of 146 and 419 base pairs, with an intervening intron of 1065 nucleotides, as also found in the somatostatin gene [3,29,30]. The similarity of somatostatin and cortistatin gene structures suggests the occurrence of an early gene duplication event.

2. Cortistatin regulatory elements

Analysis of the regulatory elements for the preprocortistatin gene, recently determined by Calbet et al. [9], indicates that the regulatory elements of preprocortistatin and preprosomatostatin genes share few similarities (<30% using the BESTFIT program). The preprocortis-

tatin transcriptional regulatory region lacks a TATA box or Initiator element and is GC rich in the proximal region. The promoter region contains a GATA motif at the −107 position which raises the possibility of basal transcriptional regulation of preprocortistatin [2]. An E-box is present at position −25 which may bind USF, a regulatory element capable of binding TFIID, and hence recruit transcriptional machinery to the DNA without a TATA box. Additionally, E-box binding has been shown to decrease with administration of kainate in hippocampal neurons which is of relevance in light of the proposed neuroprotective role of cortistatin [7]. E-boxes have also been postulated to be involved in circadian regulation of certain genes [23]. Regulation of cortistatin by such mechanisms may underlie the proposed role of cortistatin as a sleep modulatory neuropeptide. Other potential regulatory elements of significance include several GATA, AP-1 and NFkB consensus elements. NFkB has been shown to be an important transcription factor in response to stimuli which affect neurons, such as kainate administration [37,62,64].

Within the intronic sequence of the preprocortistatin gene are two MEF2 sites. MEF2 is a DNA binding protein usually associated with development of the myogenic phenotype but is also temporally and spatially regulated during brain development [51,54,55]. MEF2C is highly expressed in the cortex and hippocampus along with preprocortistatin (see below) suggesting a potential role for this factor in regulating cortistatin expression. Following the transcriptional start site, as determined by primer extension assay [9], is a consensus site for the neuron specific silencer SNOG [85]. The SNOG element is present in a number of neuronally expressed genes downstream of the TATA box. As cortistatin is not expressed in peripheral tissues a repressor such as SNOG may account for this inhibition. In contrast, a SNOG element is not present in sequence within 1600 bp of the somatostatin transcription start site [25], which may be explain the expression of somatostatin in peripheral tissues and not cortistatin. Features in common for both cortistatin and somatostatin promoters include a CREB like element in similar positions and a GATA/homeo/homeo arrangement, which may be responsible for the co-expression of these genes in certain cortical interneurons.

3. Regulation of cortistatin mRNA expression

The pattern of accumulation of cortistatin mRNA during development was examined using Northern blot and in situ hybridization. The 600 nucleotide band corresponding to preprocortistatin was observed in rats initially between P5 and P10, achieving maximal levels by P15, and subsequently reducing slightly in intensity into adulthood [20]. By in situ hybridization preprocortistatin mRNA was detected in the cortical plate, subiculum and stratum oriens of the hippocampus starting at P0. By P5 the hippocampal and cortical preprocortistatin expression had increased with transcript observed in both the stratum oriens and the pyramidal layer. The distribution of cortistatin mRNA remains essentially the same up to adulthood with peak expression recorded at P16 and slightly declining thereafter. A population of preprocortistatin expressing cells was observed in the dentate hilar region with maximal expression at P10–12 and reduction to barely detectable levels in the adult. The role of this temporally induced preprocortistatin was examined by application of rCST14 in this region and measurement of the excitability of dentate granule cells and efficacy of local inhibitory circuits in the dentate gyrus. Application of rCST14 did not alter either of these parameters in adult rats but suppressed PS amplitudes by at least 40% in P15 animals [20].

Preprocortistatin mRNA expression is regulated in response to the circadian cycle and sleep perturbations. In rats experiencing a normal day/night cycle, preprocortistatin mRNA levels showed a circadian fluctuation with highest levels recorded at circadian time 21 (3 a.m.), which is late in the rat active time period. Preprocortistatin levels were increased 4-fold in rats which were deprived of sleep for 24 h by gentle handling, with recovery to normal levels after 8 h recovery. REM sleep deprivation by placing a rat on a small platform above water did not increase preprocortistatin mRNA levels compared to control rats under the same conditions except on a larger platform [18]. These data, in conjunction with the sleep modulatory effects of cortistatin (see below), reliably classify cortistatin as an endogenous regulator of sleep whose concentration is increased as the pressure for sleep increases. Thus cortistatin fulfills most of the requirements of a sleep factor: it is endogenously expressed, synthesized by cortical GABAergic interneurons, increases during sleep deprivation, selectively enhances slow wave sleep, and depresses neuronal activity.

4. Localization of cortistatin

Using the technique of in situ hybridization, cortistatin mRNA has been shown to be essentially restricted in its expression to the cortex and hippocampus (Fig. 4A). This is in contrast to the expression of its relative somatostatin which is widely expressed in central and peripheral tissues [59,71]. The brain specific nature of cortistatin expression was originally indicated by Northern blot: cortistatin mRNA was found in rat brain but not in adrenal gland, liver, spleen, thymus, ovary, testes or anterior pituitary tissues [19]. More recently, Fukusumi et al. [26] failed to detect cortistatin transcripts in a wide range of human peripheral tissues by Northern blot but observed cortistatin expression in the central nervous system (CNS). However, cortistatin cDNA sequences have been cloned from human peripheral tissues including fetal heart, fetal lung, prostate,

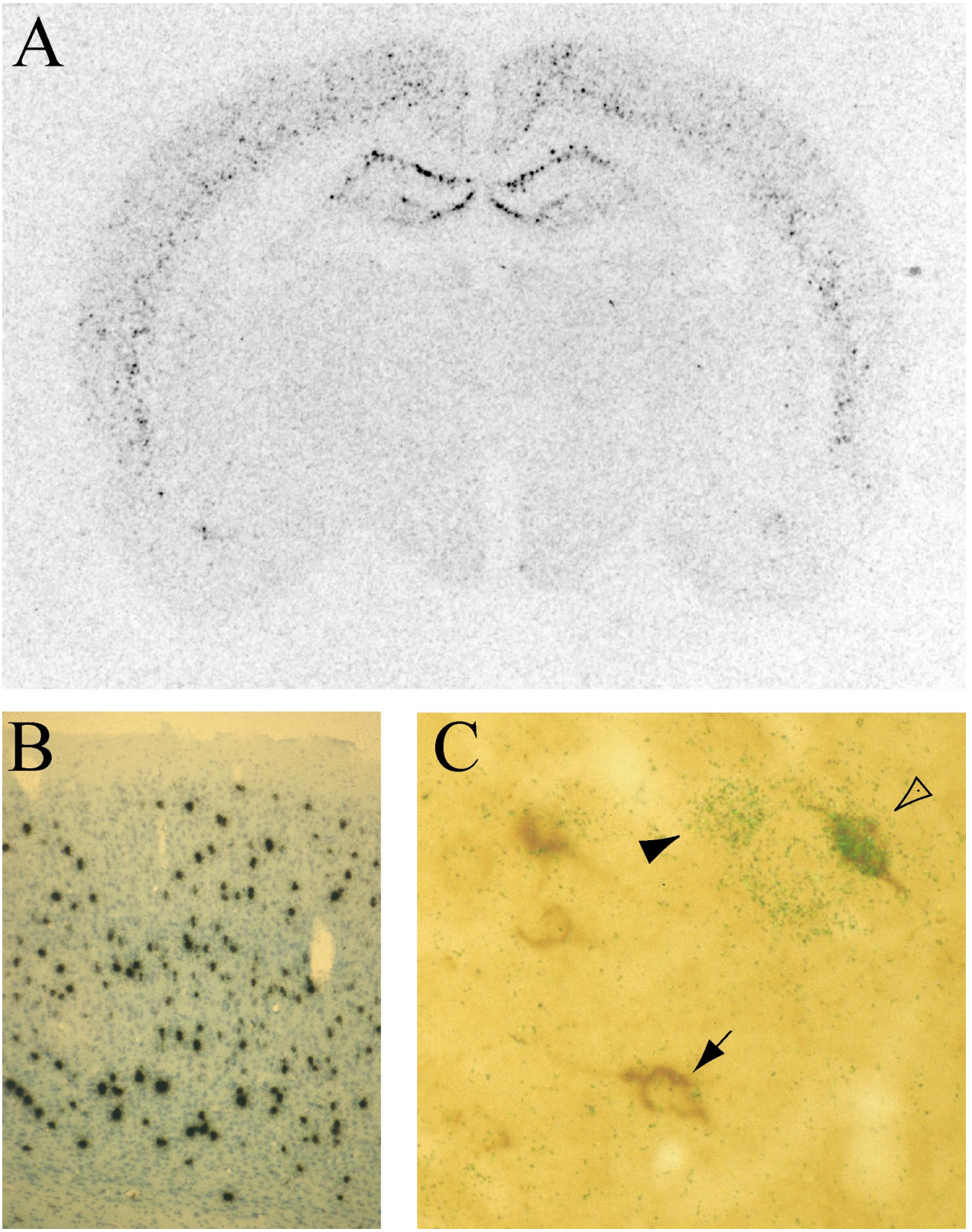

Fig. 4. (A) In situ hybridization detection of cortistatin mRNA in sparse GABAergic interneurons of the cerebral cortex and hippocampus from a coronal section of a rat brain. (B) Enlarged view of cortistatin mRNA positive cells in the rat visual cortex (clusters of black silver grains), and (C) a double labeled cortical rat brain section showing cortistatin in situ hybridization and somatostatin immunohistochemistry. Cells expressing cortistatin mRNA (filled arrowhead), somatostatin immunoreactivity (arrow) and signal from both peptides (empty arrowhead) are indicated.

colon and many tumors, as revealed by their presence in the expressed sequence tag database (Genbank).

In situ hybridization experiments have been used to determine the cellular expression of cortistatin transcripts in the rat and mouse [19–21]. In rat, cortistatin was found to be primarily expressed in scattered neurons of the cerebral cortex and hippocampus (Fig. 4B) with weak signals observed in the striatum and olfactory bulb but not in the thalamus, midbrain, cerebellum, spinal chord or hypothalamus (an important site of somatostatin expression). In the brain the highest number of cortistatin containing cells was observed in the cerebral cortex, in particular layers II–III and VI. Within the cortex the number of cortistatin expressing cells varies, with highest levels found in the visual/temporal cortex and lowest levels in the upper layers such as the somatosensory cortex. Cortistatin containing neurons were also detected in the piriform cortex and entorhinal area. In the hippocampus cortistatin is found in the subiculum, and stratum oriens and pyramidal layer of the CA1 region, with low levels also observed in the CA3 region and granule cell layer. The distribution of cortistatin in the mouse brain is similar to the rat: mouse cortistatin is not found in a range of peripheral tissues and is most abundant in the cerebral cortex and hippocampus. Minor differences include the observation of cortistatin transcripts in the amygdala and periventricular hypothalamic area of the mouse brain.

Colocalization studies indicate that cortistatin is only expressed in GABAergic interneurons, as all cortistatin labeled cells are also labeled with probes against glutamic acid decarboxylase (GAD) mRNAs [20]. This demonstrates the GABAergic nature of cortistatin expressing cells. Somatostatin is also expressed in GABAergic interneurons and displays a partially overlapping expression pattern with cortistatin (Fig. 4C). However, less than half of the cortistatin expressing neurons also contain the somatostatin peptide, and approximately a quarter of somatostatin containing cells express cortistatin mRNA. Thus populations of neurons exist in the brain, which contain one or both types of statin [20]. Cortistatin also colocalizes with the calcium binding proteins parvalbumin, especially in layers II and III of the visual and somatosensory cortices, and calbindin in the deep layers of the cortex. These data support the possibility that a distinct subset of cortical neurons may be defined by expression of preprocortistatin.

5. Structure of the cortistatin peptide

The putative 112 amino acid protein encoded by the preprocortistatin gene contains multiple sites at which endoproteolysis may occur. Recently Puebla et al. [65] have shown that preprocortistatin is indeed cleaved at the two C-terminal dibasic cleavage sites, KK and KR, to produce rCST14 and rCST29 analogously to SST14 and SST28. However, evidence of cleavage at both C-terminal dibasic sites to produce rCST14 and rCST13 was not found. The ratio of rCST14:rCST29:preprocortistatin in tissue culture cells was found to be 41:55:4.5 and the ratio of secreted peptides rCST14:rCST29 was shown to be approximately 2:1. Thus, although the two peptides are produced approximately equally the rCST14 form is preferentially released compared to rCST29. The enzymes that process cortistatin are not known, but the cells used in the above mentioned studies (AtT20) contain the convertases PC1 and PC2 [27] which are also highly expressed in the cerebral cortex [70], a major site of cortistatin expression. These enzymes have also been implicated in preprosomatostatin processing [27,60] and hence are suitable candidate enzymes for in vivo processing of preprocortistatin. rCST14 shares 11 of its 14 residues with SST14, including the two cysteine residues that render the peptide cyclic. As for somatostatin, cyclic rCST14 does not posses a particular conformation in solution, as determined by circular dichroism and nuclear magnetic resonance spectroscopy [15]. The 29/28mer and 14mer processing products are the physiologically relevant peptides from the preprocortistatin and preprosomatostatin genes. Structurally, within the N-terminal 15/14 residues of the 29/28mer statin peptides there is little similarity between cortistatin and somatostatin. Only one residue is conserved between these peptides in this region in rat and human peptides (two are conserved in the mouse), and there are no identifiable regions of structurally or functionally homologous amino acids. Conversely, the amino acid sequence in this region is highly conserved between somatostatins cloned from different species with, for example, one amino acid difference between human and chicken, and two differences between human and frog clones [13,80]. The role, if any, that this region plays in cortistatin signaling in vivo remains to be determined, although the significant structural differences may mediate physiologically relevant differences in CST29 and SST28 function.

The 14mer cortistatin and somatostatin peptides are remarkably similar in structure with rodent peptides sharing 11 of their 14 residues. The major structural differences lie outside of the cyclic portion of the peptide. The N-terminal, extra-cyclic residues in CST14 are a Pro (rat and mouse) or Asp-Arg-Met-Pro (human), whereas in SST14 there is an Ala-Gly pair in this position. The C-terminal portion of SST14 ends in the Cys that forms part of the Cys–Cys loop, whereas a Lys residue is predicted to be C-terminal of this Cys for all of the known CST14 peptides. It is not clear if this extracyclic C-terminal CST14 Lys residue is present in the mature peptide as it represents an attractive site for proteolysis by carboxypeptidase enzymes, although radio-immunoassay of HPLC fractions of brain extracts identify a peptide with an identical elution profile to CST14 [65]. Inside the Cys–Cys loop rat and mouse CST14 peptides are identical in sequence with the human form containing one differ-

ence: an Arg follows the N-terminal Cys residue, which is a Lys in the other CST14 peptides. The intracyclic loop of CST14 peptides differs from that of SST14 with a Ser residue present two positions N-terminal of the C-terminal Cys, which is a Thr residue in SST14. Some of these structural differences have been shown to have pharmacological and physiological significance for cortistatin (see below).

6. Cortistatin pharmacology

Given the apparent structural homologies between cortistatin and somatostatin the first target receptors tested for CST14 binding were the somatostatin receptors. Somatostatin binds to five different known receptors, named SSTR1-5, which are members of the 7-transmembrane G-protein coupled receptor superfamily. The receptors are widely distributed in the brain, and periphery, with distinct patterns of expression [38,59,71]. SSTRs transduce ligand-binding into cellular effects via G protein linked modulation of many secondary-messenger systems including adenylyl cyclase, Ca^{2+} and K^{+} ion channels, Na^{+}/H^{+} antiporter, guanylate cyclase, phospholipase C, Phospholipase A2, MAP kinase, and serine, threonine and phosphotyrosyl protein phosphatase [59]. Initially, synthetic CST14 was shown to displace ^{125}I-SST14 binding to GH4 pituitary cells, a model cell system to study SSTR binding, with a similar K_d to SST14 itself [19]. Subsequently rCST14 and hCST17 have been shown to displace ^{125}I-SST14 binding to each of the five cloned SSTRs expressed in transfected cell lines, with affinities in the low nanomolar range similar to those of SST14 [15,26,81] (Table 1). ^{125}I-CST14 was shown to bind to all five cloned human SSTRs expressed in transfected cell lines with similar affinities to ^{125}I-SST, and none of the receptors preferentially bound one of the statins over the other [75]. CST14 has also been shown to effectively agonize SSTR(s) expressed by GH4 cells, as measured by inhibition of vasoactive intestinal peptide (VIP) or thyroid-releasing hormone (TRH) induced cAMP accumulation, with indistinguishable efficacy from SST14 [19]. GH4 cells are thought to primarily express SSTR1, and cortistatin has further been shown to agonize SSTRs2-5 in transfected CHO cells [26].

Several studies have examined the peptide structures necessary for cortistatin binding to SSTRs. Fukusumi et al. [26] showed that hCST17, hCST15 and hCST13 all displaced ^{125}I-SST14 binding to SSTRs1-5 with similar efficacy. Surprisingly this indicates that the four N-terminal extracyclic residues of hCST17 are not important for mediating a detectable pharmacological difference between hCST17 and SST14. Using the rationale that the residues contained within the Cys–Cys loop of cortistatin and somatostatin only differ by one amino acid whereas the extracyclic residues are distinct, Criado et al. [15] examined the role of the extracyclic residues in cortistatin pharmacology. With the cyclic moiety of the peptide utilizing a sequence obtained from an octreotide-like analogue of SSTRs, sandostatin, the extracyclic residues were systematically modified. It was shown that both the N-terminal Pro and C-terminal Lys are necessary to elicit cortistatin's unique physiological effects on sleep and locomotor activity. With only the N-terminal Pro present a cortistatin-like effect on locomotor activity but not on sleep was observed and if only the C-terminal Lys is present the compound behaves like somatostatin. Analog compounds with the N-terminal proline bound with nanomolar affinities to SSTR3 and 5, but not to SSTRs 1, 2 and 4. Interestingly, SSTR5 mRNA is expressed only at low levels in the brain, while SSTR3 mRNA is expressed abundantly in the cortex and hippocampus. This leads to the possibility that cortistatin-like effects in vivo may be mediated through SSTR3.

7. Cortistatin electrophysiology

The first observation of the electrophysiological effects of cortistatin was superfusion of rCST14 on the hippocampal slice preparation [19]. The rCST14 hyperpolarized current- and voltage-clamped hippocampal neurons as evinced by inhibition of action potential firing, in a similar manner to SST14. The effect of rCST29 on the same preparation was indistinguishable from that of rCST14 (de Lecea, unpublished data). As a means to explain the mechanism of cortistatin-induced inhibition, the effect of

Table 1
Binding selectivities of cortistatin, somatostatin and a cortistatin analog to the five cloned somatostatin receptors

	IC_{50} (nM)					Reference
	SSTR1	SSTR2	SSTR3	SSTR4	SSTR5	
rCST14	1.7–5.0	0.09–1.8	0.3–3.8	0.2–18.2	0.3–1.9	[14,25,58]
rCST29	2.8	7.1	0.2	3.0	13.7	[59]
hCST17	0.25–7.0	0.6–0.9	0.4–0.6	0.5–0.6	0.3–0.4	[25,58]
Compound III[a]	>1000	>1000	105	>1000	60.4	[14]
SST14	0.1–2.26	0.2–1.3	0.3–1.6	0.3–1.8	0.2–0.9	[73]
SST28	0.1–2.2	0.2–4.1	0.3–6.1	0.3–7.9	0.05–0.4	[73]

[a] Compound III=PCYWKVCK (N-terminal Pro-, C-terminal Lys CST analog, see text).

rCST14 on the M-current (I_m), a non-inactivating voltage dependent K^+ current observed in hippocampal neurons [32], was examined. rCST14, like SST [56,73], increased the amplitude of the I_m relaxation concomitantly with an outward steady-state current. These observations indicate that cortistatin may act on hippocampal neurons through similar mechanisms to somatostatin. More recent characterization of the influence of rCST14 and SST on I_m and other currents in CA1 pyramidal neurons from the rat showed that rCST14 and somatostatin both augment similar inhibitory K^+ currents: I_m and leak currents in the hippocampal slice preparation. The rCST14 effect was slow in onset and could be augmented by SST suggesting that the two statins act at the same receptors. However, rCST14 was able to rapidly activate the hyperpolarization-activated cationic *h*-current in these cells whereas SST was unable to activate this current (P. Schweitzer, Pers. Com.). These observations suggest that cortistatin acts at both SSTRs and as yet unidentified cortistatin specific receptor(s).

The inhibitory effects of cortistatin have been observed in vivo. Field potentials, elicited in the rat hippocampal CA1 region by commissural pathway stimulation, resulted in a characteristic population spike (PS), which could be significantly decreased by microiontophoretic application of rCST14 and SST14. In contrast, application of rCST14 to the adult rat dentate gyrus had no effect on PS amplitudes measured in response to stimulation of the angular bundle of the perforant path [20], presumably due to a lack of cortistatin receptors. Interestingly, cysteamine induced release of somatostatin potentiates the PS response of dentate granule cells to perforant path stimulation [79].

One of the earliest behavioral effects of cortistatin application was a modification of sleep patterns. As acetylcholine (ACh) is known to be an important neurotransmitter involved in regulation of sleep, with low cortical ACh levels associated with slow-wave sleep and high concentrations associated with wakefulness [74], modulation of cholinergic activity by cortistatin was examined by de Lecea et al. [19]. The interaction of rCST14 and ACh on rat hippocampal CA1 neurons was examined using an evoked paired-pulse (PP) stimulation paradigm mediated in part by hippocampal interneurons. ACh application was shown to antagonize the inhibitory phase of PP responses whereas application of rCST14 alone had no effect on the PP responses. However, coapplication of rCST14 and ACh abolished the ACh effect, indicating that cortistatin acts as an antagonist of the ACh induced reduction of the PP response inhibitory phase. The effects of somatostatin on this paradigm were the same as ACh indicating that cortistatin and somatostatin may have different functions in the brain. As a follow up experiment, the investigators examined the effect of rCST14 on ACh-induced desynchronization of electroencephalogram (EEG) in the cerebral cortex, in order to determine any interaction between cortistatin and the cholinergic systems that regulate cortical function. They showed the EEG baseline of the rats in the study was predominantly slow waves (0.5–4 Hz) and that iontophoretic application of ACh desynchonized this EEG, an effect that could be prevented by co-application of rCST14. The N-terminal Pro sandostatin based analogs also prevented the ACh desynchronization of cortical EEG [15]. Given the established relationship between cholinergic input to the cortex and learning and memory [34,87] it is a possibility that cortistatin's modulation of cholinergic activity in the cortex may have profound effects on these higher cognitive functions. Indeed the effect of cortistatin on memory has been examined using a footshock-avoidance training paradigm on mice [24]. ICV injection of rCST14 relatively soon after a training period was shown to reduce memory retention of the learned activity The authors propose this effect to be mediated by rCST14 modulation of post-learning memory processing.

The effect of cortistatin on cells in the locus coeruleus (LC), a group of noradrenergic neurons in the brainstem with a role in modulation of REM sleep, was examined by Connor et al. [14]. Application of rCST14 to rat LC neurons in the brain slice preparation elicited an inwardly rectifying K^+ current indistinguishable from that elicited by somatostatin. This current was shown to be mediated through the same mechanism for cortistatin and somatostatin as high concentrations of either statin cross-desensitized the other. The SSTR subtype which is proposed to mediate inhibition of LC neurons was pharmacologically characterized as the SSTR2 subtype [12]. Thus it is possible that cortistatin exerts an in vivo effect via SSTR2. More recent data supports the involvement of SSTR2 as a mediator of cortistatin responses. SST14 has been demonstrated to enhance glutamate sensitivity of hypothalamic neurons in culture via SSTR1 or decrease the glutamate sensitivity via SSTR2 [28,41]. In the same assay system rCST14 was shown to decrease the glutamate sensitivity similarly to SST14, but CST14 did not enhance the glutamate sensitivity of these cells [81]. These data, along with the observation that SST14 could not potentiate the glutamate sensitivity after rCST14 had been applied to the cells, suggest that CST14 acts as an agonist at SSTR2 and is a possible antagonist/inverse agonist at SSTR1. Thus in addition to its inhibitory effects observed in the cortex, hippocampus and LC, cortistatin is also inhibitory in the hypothalamus. However, the physiological significance of these findings is yet to be demonstrated as cortistatin mRNA expression is undetectable in the LC and relatively low in the hypothalamus.

8. Behavioral and neurobiological effects of cortistatin

Cortistatin was originally shown to have sleep promoting properties and effects on animal locomotor behavior.

Infusion of up to 6 nmol rCST14 into rat brain ventricles induced hypoactivity in the treated rat population. The EEG for rCST14 treated animals showed a significant increase in cortical slow waves (1–4 Hz), and polygraphic recording indicated that the rCST14 injected animals spent significantly more time in slow wave sleep and less time in paradoxical (REM) sleep than control injected animals [19] (Fig. 5). These results have since been replicated

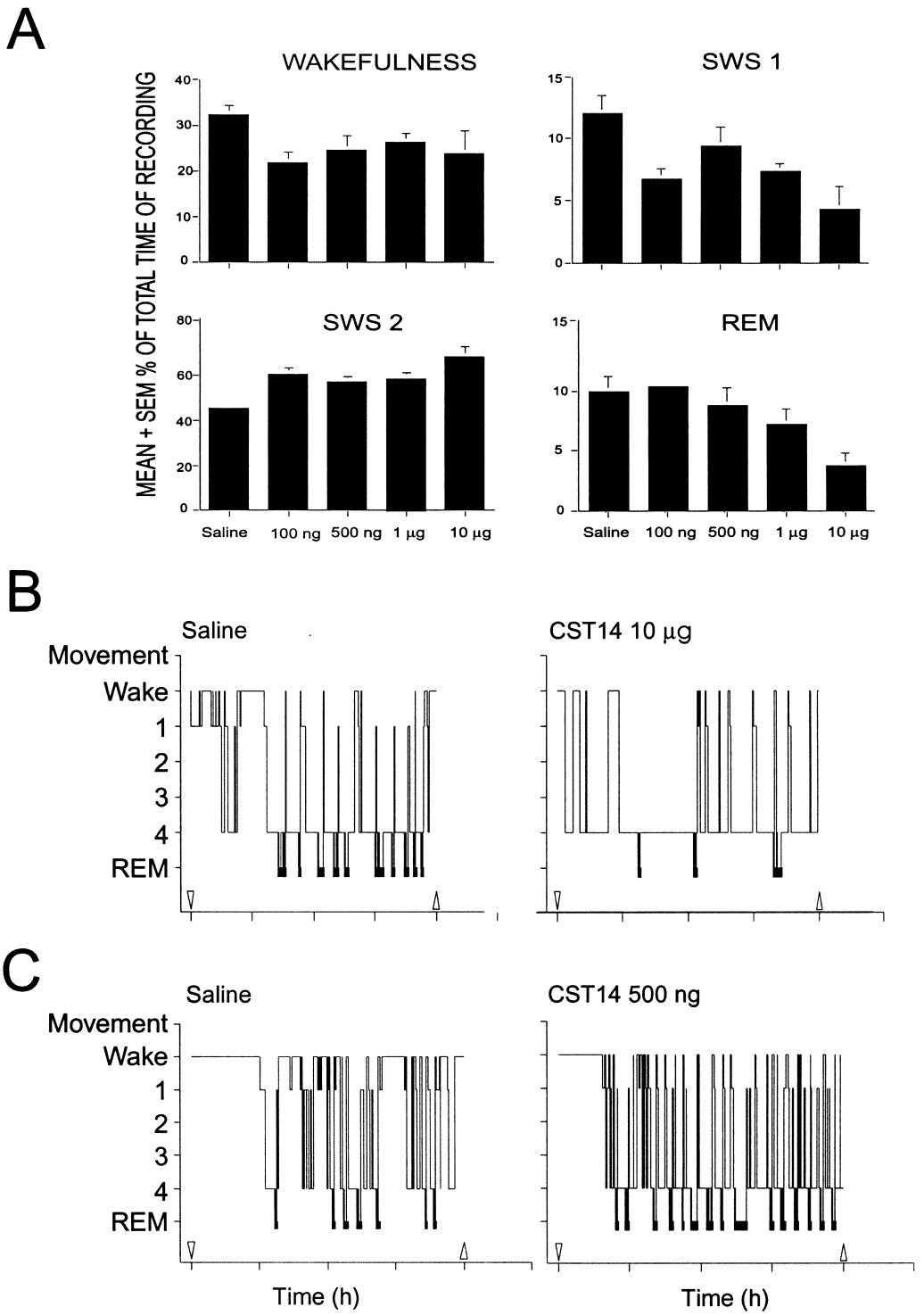

Fig. 5. The results of intracerebroventricular injection of CST14 on the sleep/wake patterns of the rat. (A) CST14 increases the time spent in slow-wave sleep 2 (SWS). (B) Traces show representative hypnographic recordings from a rat treated with saline and 10 µg CST14. This dose of peptide enhanced slow-wave sleep and decreased REM (paradoxical) sleep. (C) Hypnographs from EEG recordings of a rat under reverse light/dark cycle injected with either saline or 500 ng CST14. The peptide increased both slow-wave sleep and REM sleep in rats that had already experienced their normal physiological amount of sleep.

using hCST17 [26]. The cortistatin-induced changes to sleep patterns are in contrast to those reported for somatostatin. Somatostatin injection into rat brain ventricles induced hypermotility [4,83] and EEG analysis of somatostatin-injected rats indicated that these rats spend increased time under REM sleep compared to control rats [5,6,16]. High doses of injected rCST14 induced barrel rotation behavior and seizures in rats, which are characteristic of somatostatin injection ([67], de Lecea et al., unpublished observations). This suggests that under saturating conditions cortistatin may bind to SSTRs and induce somatostatin-like behaviors, which mask the cortistatin-specific induced behaviors.

The behavioral effects of cortistatin on sleep are closely mimicked by the sandostatin based cortistatin analog containing both N-terminal Pro and C-terminal Lys residues (mentioned above), but not by any of the other cortistatin analogs tested to date [15]. Interestingly, although the cortistatin analog with a C-terminal Lys but without the N-terminal Pro did not have an effect on sleep patterns, it was able to replicate the cortistatin effect of inducing hypoactivity in injected rats. This effect was also reproduced by injection of the N-terminal Pro and C-terminal Lys containing analog. As the cortistatin mimicking analogs are proposed to bind to SSTR3 in vivo, this receptor would be an interesting candidate as a mediator of cortistatin-specific effects which could be simply tested with recent progress in the development of SSTR knock out mouse lines.

9. Sleep promoting properties

The observation that cortistatin is mainly expressed in the cortex, and in particular, in local circuit inhibitory neurons, led to the hypothesis that the endogenous peptide may have a role in modulating cortical activity. Indeed, ICV injection of nanomolar amounts of CST14 significantly increased the number and duration of slow-wave sleep episodes both in normal and reversed light/dark cycles.

How does cortistatin promote sleep? Initial experiments have shown that application of CST14 antagonizes the effects of ACh on cortical and hippocampal excitability. Cholinergic cells from the basal forebrain are known to be critical for the maintenance of fast activity of the cortical EEG. Maximal ACh release is associated with the natural cortical activation of active wake and REM sleep states [10,35,47]. Blockade of cholinergic receptors results in diminished cortical activation [43] and lesions of the basal forebrain produce decreased cortical activation in parallel with a decrease in acetylcholine release [42,78]. Furthermore, the activating influence of ACh in the cerebral cortex has been shown to involve the depolarization and excitation of cortical neurons [50], resulting in a shift of cortical activity from very slow to fast activity [53]. In order to induce sleep, an endogenous substance would have to overcome or antagonize the effects of ACh release in the cortex. As noted above, CST has been shown to antagonize the effects of acetylcholine on cortical activity, an activity that may be necessary for cortical synchronization.

In vivo and in vitro experiments in anesthetized rats have demonstrated that CST has a direct inhibitory effect on pyramidal neurons. CST causes hyperpolarization of hippocampal neurons, and blocks the increase in firing rate induced by NMDA in the neocortex (J. Criado, unpublished results). Release of CST from interneurons would inhibit spontaneous activity of pyramidal cells that is also associated with slow-wave sleep.

Cortistatin may also enhance slow-wave sleep by influencing the activity of neuronal networks. Spindle waves are sleep-related rhythmic oscillations that occur as spontaneous 7–14 Hz activity that last 1–3 s and recur approximately once every 5–20 s (reviewed in [49]). The generation of these oscillations resides in a reciprocal interaction between GABAergic neurons of the thalamic reticular nuclei and thalamocortical cells [77]. The 5–20 s refractory period in between spindle waves is associated with a small after-depolarization in thalamocortical cells that is caused by activation of the hyperpolarization-activated cation current I_h during the oscillatory activity [44]. Activation and subsequent deactivation of I_h in thalamocortical cells thus appears to be a key mechanism through which synchronized oscillations are terminated and prevented within defined intervals. The *h*-current has also been shown to be important for the establishment of rhythmicity in other neuronal systems [45]. Computational and experimental data have suggested that intracortical mechanisms may be responsible for synchronizing oscillations over cortical distances of several millimeters through cortex–thalamus–cortex loops, thus providing a possible cellular mechanism to explain the genesis of large-scale coherent oscillations in the thalamocortical system [22]. As described above, cortistatin, but not somatostatin, enhances the amplitude of I_h on hippocampal slices. Activation of I_h by cortistatin in cortical neurons may thus have a prominent role in regulating the synchronous activity of networks of cortical cells that accompany slow-wave sleep.

10. Cortistatin and neuroprotection

Cortistatin has also been demonstrated to be neuroprotective against kainate-induced neurotoxicity in rat brains. Injection of the excitotoxic seizure inducing compound kainic acid (KA) caused seizure behavior in injected rats, which could be significantly reduced by prior ICV injection of rCST14. KA also caused significant neuronal death in the CA1 and CA3 regions of the hippocampus, an effect that could be blocked by application of rCST14 [7]. The neuroprotective properties of cortistatin are in line with expectations from previous studies using somatostatin in KA treated rats. The somatostatin agonist, octreotide,

reduced seizure activity after KA injection [63,84], and induction of somatostatin mRNA and release has been observed following KA injection in rats [33,76]. However, unlike somatostatin, expression of cortistatin mRNA is not upregulated in response to KA induced seizure paradigms in vivo [9]. These results suggest that cortistatin is not used as a native neuroprotective agent but infusion of high concentrations of cortistatin produces neuroprotective effects through activation of the somatostatin signaling system. This is another example of the difficulties involved in assessing the natural neurobiological roles of cortistatin. Effects distinct or opposite compared to somatostatin may be assumed to be cortistatin specific, but effects which are in accordance with those found for somatostatin may be due to saturation of SSTRs, an event that may not occur in vivo.

11. Cortistatin in the somatostatin-like Cys–Cys loop family of peptides

Cortistatin shares many structural and functional properties with somatostatin. The amino acid sequences of the bioactive peptides, gene structures, partial coexpression, activation of common receptors and signaling pathways, and inhibition of neurons via activation of the *M*-current all indicate a duplication of function between these two peptides. Indeed the lack of a significant phenotype in mice lacking the gene for somatostatin [36] suggests that cortistatin at least partly duplicates somatostatin function and is capable of assuming somatostatin's neurobiological role. However, recently it has been shown that cortistatin mRNA expression is not increased in somatostatin deficient mice [66]. As somatostatin makes up an estimated 97% of brain cortistatin/somatostatin immunoreactivity (as measured using cortistatin/somatostatin cross reacting antibodies) if cortistatin compensates for somatostatin loss its expression would be expected to increase significantly. The lack of cortistatin expression upregulation in the somatostatin knock out mouse suggests that the role of cortistatin is perhaps not as a somatostatin back-up. This hypothesis is supported with the observation that kainic acid injection induces increased expression of somatostatin but not of cortistatin [9], and the many distinct functional activities of cortistatin described above.

Cortistatin and somatostatin are members of a wider group of approximately 20 families of neuropeptides with varying degrees of sequence, structural and evolutionary relatedness including opioid, vassopressin, oxytocin, corticotropin-releasing factor (CRF), urotensin-II, melanin concentrating hormone (MCH) and related peptides [13,17]. The receptors for these neuropeptides are also structurally and functionally related [17,46] with some degree of cross talk between neuropeptides and receptors for other neuropeptide families. For instance, the recently identified G-protein-coupled receptor for urotensin II, formerly known as GPR14, also binds and is activated by somatostatin and somatostatin analogs albeit at lower affinities than urotensin II itself [57]. Somatostatin has also been shown to have low affinities for opioid receptors and somatostatin analogues bind opioid receptors in the nanomolar range [48,61]. However, it has been shown that cortistatin does not bind to either the GPR14/urotensin II receptor [57] or μ-, κ-, and δ-opioid receptors [14]. Whether cortistatin binds to other known neuropeptide receptors in addition to those for somatostatin remains to be determined.

12. Does cortistatin have a specific receptor?

It is clear that cortistatin is capable of activating all known somatostatin receptors. However, it has not yet been shown that the observed in vitro or artificially induced activation of somatostatin receptors reflects in vivo events. Additionally, a large body of experimental evidence, discussed above, actually points towards the in vivo effects of cortistatin being mediated by receptors which are distinct from the classically defined somatostatin receptors. In summary this evidence includes: ICV injection of cortistatin causes hypomotility in rats whereas injection of somatostatin causes hypermotility; somatostatin application to the hippocampus and cortex increases cortical excitability whereas this is depressed by cortistatin application; ICV injection of cortistatin increases slow wave sleep without affecting REM sleep whereas somatostatin injection increases REM sleep; and cortistatin is able to activate a cation selective *h*-current in hippocampal neurons which is not activated or antagonized by somatostatin. These differences in somatostatin and cortistatin physiology do not provide conclusive evidence for a cortistatin specific receptor as they may be realized based on cortistatin's affinity for somatostatin receptors which could be modified by a number of recently described mechanisms. Receptor-activity-modifying proteins (RAMPs) could interact with somatostatin receptors to affect their cortistatin/somatostatin selectivity or the result of ligand-binding. Differential RAMP expression has been shown to be capable of modifying the calcitonin-receptor-like receptor (CRLR) from a calcitonin-gene-related peptide receptor to an adrenomedullin receptor [52]. G-protein coupled receptor dimerization has been shown to affect signal transduction for the gamma-aminobutyric acid GABA(B) receptor system: heterologous expression of GABA(B)R2 alone mediated inhibition of adenylyl cyclase but inwardly rectifying K^+ channels were only activated when GABA(B)R1 and GABA(B)R2 were coexpressed [40]. These two G-protein coupled receptors were previously shown to form dimers [86]. Additionally, the G-protein coupled β2-adrenergic receptor has recently been shown to also interact with the Na^+/H^+-exchanger regulatory factor (NHERF), a regulatory protein for the Na^+/H^+

exchanger type 3 (NHE3), to control Na^+/H^+ exchange [31].

Recently, it has been shown that differential biological actions of peptides may depend, in part, on their abilities to induce endocytosis. SST14 causes internalization of SSTRs 1, 2 and 3, and does not cause internalization of SSTR4 [39,68,72]. It is possible that application of CST14 elicits internalization of SSTR4 and does not affect trafficking of the other SSTRs. Analogously to morphine and methadone on μ receptors [69], differences in receptor internalization may account for the observed differences in the physiological effects of CST14 and SST14.

If cortistatin's effects are mediated via a specific receptor, rather than post-translational modification of SSTRs, it is of interest to consider the somatostatin receptor-like orphan G-protein coupled receptors which have been so far been identified. Four orphan receptors show significant homology to the SSTRs: GPR7 (40% to SSTR5), GPR8 (45% to SSTR5), GPR14 (28% to SSTR4) and GPR24/SLC-1 (32% to SSTR5) [46]. GPR14 has recently been shown to be the receptor for urotensin II and to not bind cortistatin [57], and GPR24/SLC-1 has been shown to be the receptor for melanin-concentrating hormone [11]. Thus GPR7 and GPR8 [58] are potentially of interest as cortistatin specific receptors. The imminent completion of sequencing the human genome will considerably facilitate the identification of potential cortistatin receptor targets.

Whether cortistatin effects are mediated via SSTRs or cortistatin-specific receptors the neurobiological effects of cortistatin are of considerable importance. Cortistatin does not appear to be an alternative somatostatin. Cortistatin regulates the onset of sleep by inhibition of the cortical desynchronizing properties of released ACh and accumulates as the need for sleep increases. The inhibition of cholinergic input to the cortex by cortistatin may also play a role in processes such as learning and memory that require ACh input in cortical areas. The development of cortistatin knock out mice will likely provide further insights into the biology of this neuropeptide.

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