Intracerebroventricular Injection of $^{125}$I-Salmon Calcitonin in Rats: Fate, Anorexia and Hypocalcemia*

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Abstract—Intracerebroventricular (i.c.v.) injection of 19 pmol/rat or more of salmon calcitonin (sCT) or iodinated sCT suppressed spontaneous intake of food and water in a dose-dependent manner. Tail-whipping was a peculiar behavior which concomitantly developed, but no analgesia ensued from the doses tested (up to 62 pmol/rat). It was examined how the rise and fall pattern of these behavioral effects would correlate with the dispositional pattern of $^{125}$I-sCT. When the radioactive peptide was injected in anorectic doses via the i.c.v. route, the radioactivity was found to distribute throughout the brain, but not uniformly. In rats which showed a marked anorexia and tail-whipping behavior, distribution occurred in such a manner that it could be interpreted to reflect the regional and subcellular distribution pattern of sCT-specific binding sites. Even 3 hr after injection, the hypothalamus, the smallest region, retained the highest radioactivity corresponding to about 1% of the dose and at least one half of which was identified as the intact iodo-sCT. To be noted is the finding that sCT injected centrally will quickly enter the systemic circulation and peripherally induced long-lasting hypocalcemia, since the anorectic dose of sCT is considerably higher than the dose needed for the peripheral effect. It is concluded that most of the sCT after i.c.v. injection leaks into the systemic circulation, but the rest is retained rather selectively around the receptor in hypothalamic nuclei for a long time, leading to day-long suppression of feeding and drinking behavior.

Salmon calcitonin (sCT) when administered by intracerebroventricular (i.c.v.) injection in rats inhibits feeding and drinking (1-4). The duration of the effects appears to be dose-related, and after high doses, the absence of feeding and drinking may persist longer than 24 hr. Since these activities are not shared with the 1 to 10 or 11 to 32 synthetic fragment of sCT, it is highly possible that the intact fish hormone can survive in brain tissue for at least as long as the effects last.

In order to test such a possibility, some anorectic doses of $^{125}$I-labeled sCT were injected into the lateral ventricle of rats. Hours later, animals were divided into two groups depending on the degree of their behavioral responses (tail-whipping and
anorexia); the major groups responded well and the minor one was poor in responding. First, the distribution pattern of the radioactivity among the subcellular fractions of some brain regions was compared between the two groups. Secondly, the survival rate of the peptide in the hypothalamus was estimated by high performance liquid chromatography, since the hypothalamus has been suggested to contain a receptor linked to sCT's anorectic effect (5-11) and in the major group, the highest radioactivity was retained by this region. Thirdly, the rate with which the main portion of the peptide entered the systemic circulation was paralleled by the amount of labeled iodine found in the serum and deduced from the temporal pattern of hypocalcemia.

Materials and Methods

Peptides: sCT (4500 M.R.C. U/mg), porcine calcitonin (pCT, 170 M.R.C. U/mg), and sCT-(11-32) were the natural or synthetic products from Armour Pharmaceutical Co. (Kankakee, IL, U.S.A.).

Animals: Male Wistar rats weighing 250-350 g were used. Food and water were provided ad libitum.

Preparation and purification of iodinated sCT: Salmon calcitonin was iodinated at 25°C for 10 min with a mixture of 125I and 127I in 20 mM potassium phosphate buffer (pH 6.0) by the lactoperoxidase method of Marchalonis (12). The molar ratio of 125I to 127I was varied so that the resulting specific activity (Ci/mmol) would be 20, 50 or 100. The ratio of hydrogen peroxide/the total of iodide/sCT was 1.1/1.1/1.0. The reaction was stopped by adding acetonitrile and trifluoroacetic acid to make a solution that is 28.6% and 0.1%, respectively, in each. The peptide was adsorbed by Unisil Q C18 (30 mg for up to 15 nmole of sCT) upon incubation at 25°C for 5 min. Repeated washing of the silica powder with an aqueous 28.6% acetonitrile solution containing 0.1% trifluoroacetic acid removed unreacted iodide. Labeled peptide was then eluted with aqueous 40% acetonitrile containing 0.1% trifluoroacetic acid. Removal of acetonitrile by evaporation was followed by dilution with an appropriate buffer or, for i.c.v. injection, by lyophilization and then dissolution in saline. In some experiments, the labeled peptide in the eluate was further purified by high performance liquid chromatography on a Unisil Q C18 column (4.6×250 mm), for which the mobile phase was aqueous 38% acetonitrile containing 0.1% trifluoroacetic acid.

I.c.v. injection of iodinated sCT and its localization in brain regions and in subcellular fractions: Under pentobarbital anesthesia, rats were cannulated as described previously (4). The peptide was dissolved in saline to give a concentration of 19 or 62 pmol/10 μl, and 10 μl per rat of the solution was injected at a rate of 4 μl/min usually between 17:00 and 18:00 before spontaneous feeding began. Three hr after injection, unless otherwise specified, the animal was killed by cervical dislocation and four brain regions (cerebral cortex, cerebellum, pons plus medulla oblongata, and hypothalamus) were dissected out according to the procedure of Glowinski and Iversen (13). Each region was homogenized with 0.32 M sucrose containing 10 μM of sCT-(11-32) for further fractionation. The subcellular fractionation was carried out as described by Jones and Matus (14). The P1 and the P2 fractions corresponded to their nuclear and mitochondrial pellets, respectively. The P3 fraction was prepared by further centrifugation of the final supernatant at 1.0 × 105 × g for 60 min. The distribution pattern of acetylcholinesterase activity among these fractions generally paralleled that described by them.

Recovery and identification of iodinated sCT from the hypothalamus: Three hr after i.c.v. injection of iodinated sCT (62 pmol/rat, 1.4 × 106 cpm), rats which clearly showed both “tail whipping” behavior* and no intake of food and water were selected. Six hypothalami were collected and processed simultaneously to obtain an acetone powder. The powder was extracted at 4°C for 30 min by mixing with 20 ml of 1 N acetic acid containing 1 mg of sCT. The extract was centrifuged at 15,000 × g at 4°C for 10 min, and the supernatant was centrifuged again

* See Results section below.
at 1.0×10^5×g at 4°C for 120 min. The lyophilizate of the final supernatant was dissolved in 3 ml of 10 mM ammonium acetate buffer (pH 4.5), and the solution was then applied onto a CM-Sephadex C-25 column (5 ml in volume) equilibrated with the same buffer. The column was eluted with a gradient consisting of 25 ml each of the above buffer and 1 M ammonium acetate buffer (pH 5.9). The radioactive peaks were pooled and lyophilized. The residue was dissolved in aqueous 38% acetonitrile in 0.1% trifluoroacetic acid and applied onto a Unisil Q C18 column (4.6×250 mm). The absorbancy at 280 nm and the radioactivity of the eluent were monitored.

Measurements: Analgesia was evaluated by the yeast-paw test as described by Randall and Selitto (15). The protein was quantified by the procedure of Lowry et al. (16) using bovine serum albumin as the standard. The assay method of acetylcholinesterase activity was that of Ellman et al. (17). When blood samples were taken, rats were bled through the orbital sinus under ether anesthesia, and the calcium concentration in serum was estimated by the method of Gitelman (18).

Results

Behavioral effects of sCT and iodinated sCT: Following i.c.v. injection of sCT in a dose of 19 pmole or more, both food and water intakes were depressed in a dose-dependent manner (Fig. 1), resulting in a sharp drop of body weight as measured 24 hr later. Another noteworthy effect was the induction of a behavior, for which "tail-whipping" would be the best description. The animal flips its tail widely, strongly hitting the cage which makes a crashing sound. The behavior developed soon after injection, and after the highest dose (62 pmol/rat), it usually lasted longer than 24 hr as the anorexia did. The appearance of the behavior can be correlated with that of anorexia, as compared in Table 1. In contrast, no detectable analgesia developed after sCT at doses up to 62 pmol/rat.

Table 2 shows effects of 62 pmol/rat of sCT and I-sCT on feeding and drinking. Tail-whipping also developed in both groups. Iodinated sCT (62 pmol/rat) was almost equipotent with the same dose of sCT in producing these responses.

Regional and subcellular distribution of i.c.v. 125I-l-sCT: A low dose of iodinated sCT (19 pmol/rat) was also able to induce the tail-whipping behavior as well as anorexia in most of the rats. Three hr after injection, the distribution pattern of the radioactivity in the brain was compared between the behav-

Table 1. Correlation between anorectic effect and tail-whipping response induced by i.c.v. injection of sCT (62 pmol/rat)

|                   | Anorectic effect* | Total |
|-------------------|-------------------|-------|
|                   | +                 | -     |       |
| Tail-whipping response** | 54               | 0     | 54    |
|                   | 1                 | 5     | 6     |
| Total             | 55                | 5     | 60    |

*: perfect anorexia for 6 hr after injection. **: presentation at 6 hr after injection. The correlation was significant by the χ²-test (P<0.01).
positive and negative groups. In the positive group, the radioactivity per mg tissue protein was the highest in the hypothalamus and the lowest in the cerebral cortex (Table 3). The pattern seems to closely reflect the distribution of the sCT-specific binding site estimated in vitro (Fig. 2). In the negative group, distribution to the cerebral cortex was higher than those to the other regions (Table 3).

### Table 2. Effects of sCT and I-sCT on food and water intake in rats

| Peptide | i.c.v. dose (pmol/rat) | n | Food intake (g/6 hr) | Food intake (g/24 hr) | Water intake (g/24 hr) |
|---------|------------------------|---|---------------------|----------------------|----------------------|
| Control |                        | 6 | 7.2±1.5             | 20.1±2.1             | 31.3±3.3             |
| sCT     |                        | 62| 0                   | 0.8±0.5              | 2.9±1.0              |
| l-sCT   |                        | 12| 0.8±0.1             | 5.1±0.9              |

### Table 3. Regional and subcellular distribution of the radioactivity in the brain of the rat after i.c.v. injection of 125I-sCT (19 pmol/rat, 4.0 х 10⁵ cpm)

| Fraction          | Hypothalamus | Cerebellum | Medulla oblongata plus pons | Cerebral cortex |
|-------------------|--------------|------------|-----------------------------|----------------|
|                   | cpm/mg protein |           |                             |                |
| Tail-whipping group* |              |           |                             |                |
| Homogenate        | 636±81       | 154±9     | 189±15                      | 87±18          |
| P₁                | 264±45       | 60±6      | 58±7                        | 44±7           |
| P₂                | 682±97       | 177±7     | 229±27                      | 73±17          |
| P₃                | 2408±372     | 161±7     | 715±105                     | 153±20         |
| No tail-whipping group** |        |           |                             |                |
| Homogenate        | 106±20       | 29±11     | 31±7                        | 379±56         |
| P₁                | 44±8         | 11±5      | 8±2                         | 158±30         |
| P₂                | 114±19       | 33±13     | 36±7                        | 273±45         |
| P₃                | 402±72       | 28±4      | 113±22                      | 381±71         |

*: Each value is the mean±S.E. of 5 rats. **: Each value is the mean±S.E. of 6 rats.

Fig. 2. Comparison of regional distribution of 125I-sCT binding sites estimated in vitro and in vivo. The in vitro binding (□) was estimated as described before (7) using the membrane fraction of each brain region. The data for the in vivo distribution were taken from Table 3 for the rats showing tail-whipping (■) and for the negative group (□).
In vivo uptake of the radioactivity by the subcellular fractions occurred in the same order for both groups, P3 > P2 > P1, with the only exception being that of the cerebellum (Table 3), where P2 > P3. Within the subfractions of P2, the label was found to be most concentrated in the synaptosomal fraction (Table 4). For comparison, the subcellular distribution pattern of sCT-specific binding sites estimated in vitro is given in Table 5.

The dose was increased to 62 pmol/rat and distribution measured in the positive group at varying times. The results are summarized in Table 6. In these brain region's, except for the cerebellum, the logarithm of the radioactivity in the homogenate when plotted against after injection was found to be linear. Table 7 compares the radioactivity found in four different tissues. When plotted similarly, the decay data for the hypothalamus again fell on a straight line, while those for the kidney and the serum seem to give concave curves. As expected, 125I was gradually accumulated by the thyroid in a time-dependent manner.

In two groups of rats showing tail-whipping, the survival of 125I-sCT in the hypothalamus was estimated 3 hr after injection. Of the radioactivity injected, 0.86 and 1.1% were recovered per region, of which 73 and 45%, respectively, were iodos-C. The survival rate was not measured later. However, if it is assumed that the ratio of 125I-sCT/total 125I in the hypothalamus would not change during this experiment, the half life of 125I-sCT in this region is calculated to be about 12 hr from the data in Table 6.

Entering of i.c.v. sCT into the systemic circulation and resulting hypocalcemia:





Table 4. Distribution of the radioactivity in the P2 subfractions from the brain of the rat showing tail-whipping 3 hr after i.c.v. injection of 125I-sCT (19 pmol/rat, 4.0×10⁵ cpm)

| Subfraction | Hypothalamus (cpm/mg protein) | Midbrain (cpm/mg protein) | Medulla oblongata plus pons (cpm/mg protein) |
|-------------|-------------------------------|---------------------------|------------------------------------------------|
| Myelin      | 1463±287                      | 325±6                     | 551±87                                           |
| Synaptosome | 4410±336                      | 1094±104                  | 1984±226                                         |
| Mitochondria| 570±65                        | 256±12                    | 276±12                                           |

Each value is the mean±S.E. of three groups, each consisting of 3 rats.

Table 5. Subcellular distribution of 125I-sCT binding sites in some regions of rat brain*

| Fraction                  | Specific binding** (cpm/mg protein) | ACh esterase** (μmol/min/mg) |
|---------------------------|-------------------------------------|------------------------------|
| Prepared in 0.32 M sucrose|                                     |                              |
| 9×10³×g, 20 min. pellet   | 1601±202                            | 37.4±9.4                     |
| 1×10⁵×g, 60 min pellet    | 2546±403                            | 68.7±4.8                     |
| P1                       | 1217±408                            | 36.8±3.3                     |
| P2                       | 2276±368                            | 50.2±2.7                     |
| P2 subfractions          |                                     |                              |
| myelin                   | 661±243                             | 23.5±4.1                     |
| synaptosome               | 7184±1479                           | 139.2±29                     |
| mitochondria             | 1219±233                            | 17.2±3.6                     |
| Prepared in 50 mM Tris-HCl|                                     |                              |
| 1.1×10⁴×g, 10 min. pellet| 2690±355                            | 66.8±10.7                    |

*: Four brain regions (hypothalamus, midbrain, pons and medulla oblongata) were combined for processing. **: The mean±S.E. of 3 groups, each consisting of 5 rats.
start, the radioactivity appeared in serum, and thereafter, it kept gradually increasing at least up to 3 hr (Fig. 3 and Table 7).

Parallel with the appearance of the radioactivity in serum, hypocalcemia developed possibly due to the systemic hormone action of iodo-sCT. As pictured in Fig. 4, the serum Ca²⁺ level first decreased progressively with time up to 3 hr, and then it gradually returned to the normal value. The duration of this effect was dose-dependent and apparently correlates with that of anorexia. However, the relationship between hypocalcemia and anorexia is not simply the cause and effect, because hypocalcemia induced by subcutaneous injection of the same dose of iodo-sCT was not accompanied with anorexia.

| Table 6. Regional and subcellular distribution of the radioactivity in the brain of the rat showing tail-whipping at varying times after i.c.v. injection of ¹²⁵I-sCT (62 pmol/rat, 1.4x10⁶ cpm) |
|---------------------------------|------------------|------------------|------------------|------------------|
| Fraction | Hypothalamus | Cerebellum | Medulla oblongata plus pons | Cerebral cortex |
|----------|--------------|------------|------------------|------------------|
| a) 3 hr after injection* | 894±44 | 249±37 | 322±27 | 140±14 |
| Homogenate | P₁ | 522±33 | 178±23 | 155±26 | 97±6 |
| P₂ | 1172±53 | 278±65 | 524±47 | 92±12 |
| P₃ | 3222±216 | 235±40 | 944±80 | 176±19 |
| b) 15 hr after injection** | 520±100 | 18±1 | 150±29 | 53±15 |
| Homogenate | P₁ | 344±98 | 27±5 | 100±12 | 53±20 |
| P₂ | 780±144 | 33±6 | 367±56 | 44±13 |
| P₃ | 1958±367 | 41±11 | 612±136 | 113±25 |
| c) 30 hr after injection* | 244±46 | 7±0.7 | 52±13 | 21±6 |
| Homogenate | P₁ | 172±36 | 10±2 | 45±10 | 22±4 |
| P₂ | 376±62 | 21±10 | 137±36 | 23±3 |
| P₃ | 928±152 | 21±2 | 319±71 | 57±4 |

*: Each value is the mean±S.E. of 5 rats. **: Each value is the mean±S.E. of 4 rats.

| Table 7. Distribution of the radioactivity in some tissues at varying times after i.c.v. injection of ¹²⁵I-sCT (62 pmol/rat, 1.4x10⁶ cpm) |
|---------------------------------|------------------|------------------|------------------|
| Tissue | Radioactivity 3 | 15 | 30 hr after injection |
|------------------|------------------|------------------|------------------|
| Hypothalamus | 7463±416* | 4332±429** | 1583±194* |
| Kidney | 29530±906 | 4019±867 | 1264±175 |
| Thyroid gland | 5045±976 | 24680±1410 | 29683±4614 |
| Serum | 3386±744 | 820±49 | 512±81 |

*: Each value is the mean of S.E. of 5 rats. **: Each value is the mean of S.E. of 4 rats.

Discussion

In 1970, Copp (19) noted that sCT is a biologically stable peptide and in the physiological milieu of mammals, will survive for many hours. When Pecile et al. (20) first reported the central action of sCT, they noticed that sCT-induced antinociception in rabbits would last longer than 2 hr. Later, Fraioli et al. (21) who injected sCT into the lumbar subarachnoid space of terminal cancer patients observed that pain amelioration persisted as long as 2–5 days. These observations imply that in the central nervous system too, the peptide can survive for a long time.

When ¹²⁵I-sCT was injected in anorectic
doses via the i.c.v. route, the radioactivity was found to distribute throughout the brain, but not uniformly. In rats which developed tail-whipping and complete anorexia, the radioactivity was highest in the hypothalamus and lowest in the cerebellum. Even 3 hr after injection, the hypothalamus, the smallest region, retained the highest radioactivity corresponding to about 1% of of the dose, and at least one half of this was identified as the intact iodo-sCT. These results demonstrate an interesting long half-life for sCT in the hypothalamus, where the receptor which mediates the anorectic effect of sCT has been suggested to reside (2, 6–8, 10, 11). In contrast, the group which showed a poor response characteristically had a low level of radioactivity, possibly due to inadequate injection.

Tail-whipping is a novel behavior possibly described herein for the first time. Though it is unknown how sCT causes the behavior, the behavior seems to correlate well with anorexia in its appearance and also in its duration. Thus, we employed the behavior as the basis on which to judge adequacy of injection. Recently, it has been reported that systemically administered calcitonins in man may induce nausea and vomiting as common side effects (22, 23). Though tested in a wide range of doses (0.06–3.1 nmol/kg), sCT administered subcutaneously has so far failed to induce tail-whipping in rats. Furthermore, the behavior has never been produced by pCT or hCT, even when injected centrally. Thus, it is not likely that the behavior results from nausea.

It should be noted that sCT injected centrally will quickly enter the systemic circulation. Though the identity of the radioactive substance in the blood was not checked, by assuming the survival rate of iodo-sCT as 50%, the peptide concentrations (nM) at 1, 2, 3, 15 and 30 hr are calculated to be 0.035, 0.047, 0.075, 0.018 and 0.011, respectively. This temporal pattern is considered to be reflected in that of hypocalcemia, but not precisely; for example, the extent of hypocalcemia measured at 1 and 15 hr after sCT injection is in the same range, while the circulating level of iodo-sCT is supposed to be lower in 15 hr. The cause of this discrepancy is unknown.

It has been reported that the Ca-uptake by hypothalamic slices in vitro is inhibited by sCT (2, 24), but not by pCT, and that this effect is specific to this brain region (24). According to in vitro autoradiography which allowed more detailed mapping of the sCT-binding site (10, 11), some nuclei in the
anterior and intermediate hypothalamus were heavily labeled, suggesting their possible involvement in sCT-induced anorexia. The present results clearly show that among the brain regions tested, the hypothalamus has the highest ability to retain iodo-sCT, not only in vitro but also in vivo. The peptide was also found to be retained by the medulla oblongata plus pons to a high extent, for which the relation with the analgesic effect of sCT has been suggested, though no antinociception developed with the doses employed in this experiment. Thus, it may be concluded that sCT given centrally is selectively retained around the receptor in those hypothalamic nuclei, possibly affecting the Ca²⁺-flux, which then leads to inhibition of feeding and drinking behavior.

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