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Antinociceptive Effects of Repeated Systemic Injections of Calcitonin in Formalin-Induced Hyperalgesic Rats

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UMENO, H., T. NAGASAWA, N. YAMAZAKI AND Y. KURAISHI. *Antinociceptive effects of repeated systemic injections of calcitonin in formalin-induced hyperalgesic rats.* PHARMACOL BIOCHEM BEHAV **55**(1) 151–156, 1996.—Calcitonin (CT) produces long-lasting analgesia in patients suffering from painful diseases following repeated systemic injections, but there have been only a few contradictory reports on the antinociceptive action of systemic injections of CTs in animal experiments. This study was conducted to elucidate an antinociceptive action of systemic CT in rats. An injection of dilute formalin induced hyperalgesia for about 2 h. Single topical injections of 0.12 and 1.2 U, but not 0.012 U, of [Asu^{1,7}]eel CT (eCT) into the same site of formalin injection inhibited the hyperalgesia. Repeated systemic injections of eCT (4 and 40, but not 0.4, U kg^{-1} day^{-1}) for 7 days inhibited the hyperalgesia, while the single injection was without effects at doses tested. Although the highest dose of eCT (40 U kg^{-1} day^{-1}) inhibited an increase in body weight following repeated injections, lower doses (0.4 and 4 U kg^{-1} day^{-1}) were without effects. The suppression of hyperalgesia following repeated systemic injections of eCT (4 U kg^{-1} day^{-1}) lasted for at least 24 h, and subsided by 3 days following the last eCT injection. These results indicate that the repeated systemic injections of eCT produce a long-lasting inhibition of formalin-induced hyperalgesia in rats. This inhibitory effect is similar to CT analgesia in human subjects in terms of a necessity for repeated administration, effective dose and long-lasting effects.

Formalin-induced hyperalgesia Calcitonin Analgesia Repeated administration Systemic administration Rat

CALCITONIN (CT) is a polypeptide hormone secreted from the parafollicular C cells of the thyroid gland (16,36) and involved in the regulation of calcium homeostasis in blood and its metabolism in the bone (2). Repeated intramuscular or intravenous injections of CTs alleviate pains of osteoporosis (41), advanced cancer (3,5,14), Pagets's disease (40), phantom limb pain (24,25), and migraine (18,48) [for review see (7)]. However, as there have been only a few contradictory reports on the antinociceptive action of systemic injections of CTs in animal experiments, there are few animal models of pain useful for studying the analgesic mechanisms of systemic injection of CT [for review see (7)]. For example, intravenous (11) and intraperitoneal injections (13,27) of CT were shown to suppress writhing responses induced by an intraperitoneal injection of phenylquinone or acetic acid in mice. On the contrary, SC or intraperitoneal injection of CT is without antinociceptive effects in the acetic acid writhing (4) or hot-plate test (27) in mice, respectively. In addition, repeated SC injections of CT were claimed to produce an inhibition (1) or no suppression (39) of nociceptive behaviors of adjuvant arthritic rats.

Recently, it was reported that formalin-induced hyperalgesia of the rat was inhibited by a single injection of CT into the formalin-treated site (21). However, the effects of systemic injection of CT were not examined. Considering the fact that patients are essentially given repeated systemic CT for treatment of pain, we investigated the effects of systemic injection of CT on the formalin-induced hyperalgesia. We report here the long-lasting inhibition of hyperalgesia following repeated systemic injections of CT in rats.

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METHOD

Drugs

[Asu^{1,7}]eel CT (eCT) (31), an eel CT derivative (40 U ml^{-1}, lot. ELC11KK, Asahi Chemical Industry Co., Ltd., Osaka, Japan), was diluted in 0.1 mM sodium acetate buffer containing 0.02% bovine serum albumin (pH 5.5) to get appropriate doses.

Animals

Experiments were carried out using male Sprague–Dawley rats (SLC, Shizuoka, Japan) weighing 90–150 g (4–5 weeks old). They were housed five to seven per cage in a room with controlled temperature (22 ± 1°C), humidity (60%), and artificial light (0700–1900 h) and had free access to food (CE-2; Clea, Tokyo, Japan) and water. The animals were used after several days of acclimation to the housing conditions.

Hyperalgesia and Measurement of Nociceptive Threshold

The guidelines published in a Guest Editorial in *Pain* (51) on ethical standards for investigations of experimental pain in animals were followed. Inflammatory hyperalgesia was induced by subcutaneous (SC) injection of 0.050 ml of 5% formalin (1.85% formaldehyde, Wako, Osaka, Japan) at the dorsal site of the right hind paw of rats (46,50). Nociceptive threshold was measured using an analgesimeter (Ugo Basile, Milan, Italy). The pressure was applied to the same site of the hind paw as formalin injection with a wedge-shaped piston at a loading velocity of 32 g per s and the level of pressure at which rats showed withdrawal response or vocalization was measured as nociceptive threshold. The analgesic test was performed at least three times before the start of experiment to accustom the animals to the testing procedure. The cutoff level of the pressure was 300 g, but all rats tested showed the nociceptive responses under this level.

Experiment 1

Fifty rats were divided into four groups (n = 12–13 per group) at random. Either vehicle or eCT (0.012, 0.12, and 1.2 U) was SC injected into the dorsal site of the right hind paw in a volume of 0.030 ml, and 15 min later formalin was injected into the same site.

Experiment 2

The schedule for the experiment is shown in Fig. 1. Seventy-six rats were divided into seven groups at random and daily given vehicle or eCT in an SC volume of 1.0 ml kg^{-1} at the interscapular level for 7 days. Group 1 was given only vehicle, daily. Groups 2–4 were given vehicle daily for 6 days and then single injection of eCT (0.4, 4, and 40 U kg^{-1}, respectively). Groups 5–7 were given eCT (0.4, 4, and 40 U kg^{-1} day^{-1}, respectively) for 7 days. Dilute formalin was administered 15 min after the last injection of eCT or vehicle.

Experiment 3

The schedule for the experiment is shown in Fig. 1. Twenty-five rats were divided into four groups at random and given vehicle or eCT (4 U kg^{-1} day^{-1}) daily in an SC volume of 1.0 ml kg^{-1} at the interscapular site for 10 days. Group 8 was only given vehicle daily for 10 days. Group 9 was given vehicle for 3 days and then eCT for 7 days. Group 10 was given vehicle for 2 days, eCT for 7 days, and then single shot of vehicle.

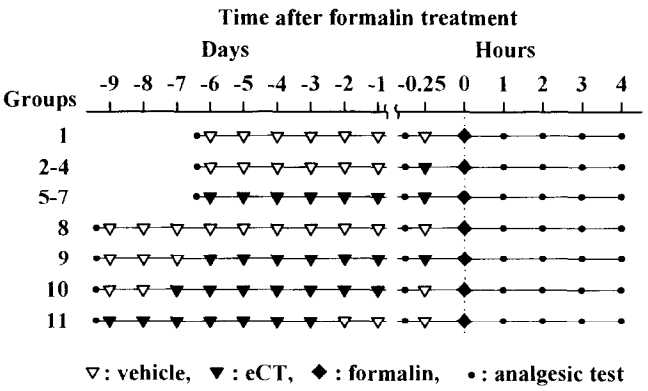

FIG. 1. Schedule of systemic injections of [Asu^{1,7}]eel calcitonin (eCT), formalin treatment, and measurement of nociceptive threshold. The rats of groups 1–7 were subcutaneously given vehicle or eCT (0.4, 4, and 40 U kg^{-1} day^{-1}) into the interscapular region daily for 7 days. The rats of groups 8–11 were subcutaneously given vehicle or eCT (4 U kg^{-1} day^{-1}) daily for 10 days. Dilute formalin was injected into the dorsal side of the hind paw 15 min after the last injection of eCT or vehicle and the nociceptive threshold of formalin-treated site was determined.

Group 11 was given eCT for 7 days and then vehicle for 3 days. Dilute formalin was administered 15 min after the last injection of either eCT or vehicle.

Data Processing

All data are presented as means and SE. Effects of eCT and formalin were analyzed using two-way repeated measures analysis of variance (RM-ANOVA) and one-way RM-ANOVA, respectively. Multiple comparisons against a control were done using post hoc Dunnett's test. The calculation was done using software Sigmastat (Jandel Scientific, San Rafeal, USA) and a value of $p < 0.05$ was considered significant.

RESULTS

Experiment 1: Effects of Local Injection of eCT

We assessed the effects of topical injections of eCT on formalin-induced hyperalgesia. Formalin injection produced a significant, $F(4, 48) = 12.1$, $p < 0.0001$, decrease in the nociceptive threshold of control group from 190.8 ± 7.5 g (before formalin, n = 13) to 75.6 ± 2.7 and 77.8 ± 2.9% of the control level at 1 and 2 h after the injection, respectively (Fig. 2). The threshold almost recovered to the preformalin level after 3 h (Fig. 2). When injected topically into the formalin-treated region 15 min before formalin injection, eCT at doses of 0.12 and 1.2 U, but not 0.012 U, significantly ($p <$ 0.05, Dunnett's test) inhibited formalin-induced hyperalgesia; two-way RM-ANOVA revealed the significant effect of eCT, $F(3, 46) = 10.38$, $p < 0.0001$, and significant interaction between dose and time, $F(12, 184) = 5.08$, $p < 0.0001$. The nociceptive thresholds of the rats given eCT at doses of 0.12 and 1.2 U were significantly, $p < 0.05$, Dunnett's test, higher than those of control rats at 1 and 2 h after formalin injection.

Experiment 2: Effects of Systemic Injections of eCT

First, we examined whether the inhibition of the hyperalgesia would be produced by a single systemic injection of eCT 15 min before formalin treatment (Fig. 3). In control rats

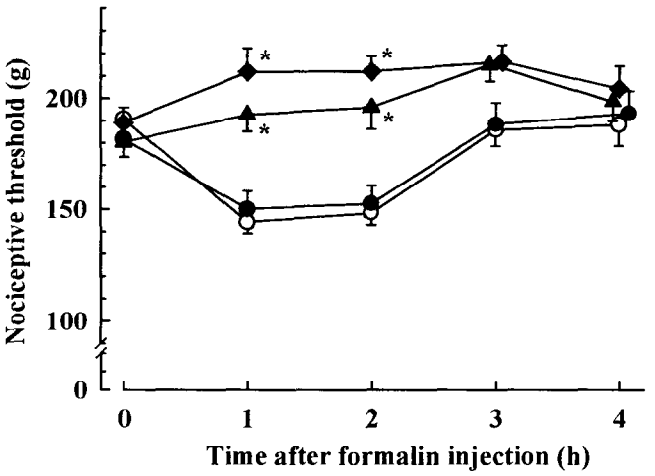

FIG. 2. Effects of single topical injections of [Asu[1,7]]eel calcitonin (eCT) into the hind paw on formalin-induced hyperalgesia. Vehicle or eCT was administered into the dorsal side of the hind paw, and 15 min later dilute formalin was injected into the same region. ○: vehicle (n = 13), ●: eCT (0.012 U, n = 12), ◆: eCT (0.12 U, n = 12), and ▲: eCT (1.2 U, n = 13). Each point represents the means and SE. *$p < 0.05$ when compared with vehicle.

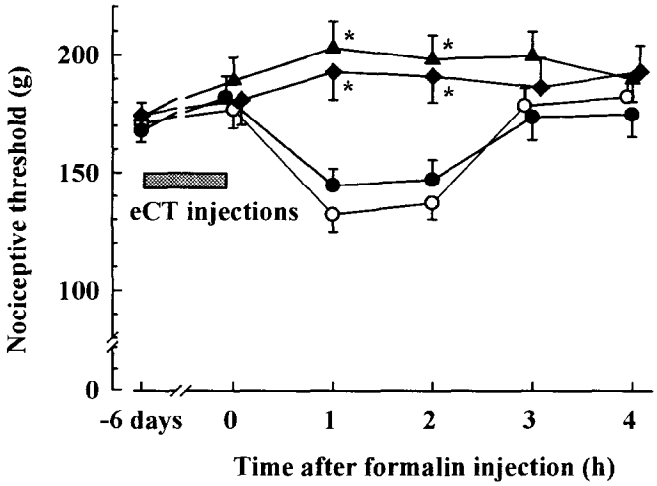

FIG. 4. Effects of repeated systemic injections of [Asu[1,7]]eel calcitonin (eCT) on formalin-induced hyperalgesia. Vehicle or eCT was administered daily for 7 days and dilute formalin was administered 15 min after the last injection of eCT. ○: vehicle (n = 13), ●: eCT (0.4 U kg^{-1} day^{-1}, n = 12), ◆: eCT (4 U kg^{-1} day^{-1}, n = 14), and ▲: eCT (40 U kg^{-1} day^{-1}, n = 12). Each point represents means and SE. *$p < 0.05$ when compared with vehicle.

that were given a 7-day course of vehicle, formalin treatment produced a significant, $F(4, 48) = 13.6$, $p < 0.001$, decrease in the nociceptive threshold, which was 74.6 ± 4.1 and 77.5 ± 4.0% (n = 13) at 1 and 2 h after formalin injection, respectively. Single systemic injection of eCT (0.4, 4, and 40 U kg^{-1}, SC) following the 6-day injections of vehicle produced no significant effects on the formalin-induced hyperalgesia.

Second, we examined the effects of the repeated systemic injection of eCT (Fig. 4). Repeated systemic injections of eCT (4 and 40, but not 0.4, U kg^{-1} day^{-1}) for 7 days produced the significant inhibition of the formalin-induced hyperalgesia; two-way RM-ANOVA revealed the significant effect of eCT, $F(3, 47) = 5.41$, $p = 0.0028$, and significant interaction between dose and time, $F(12, 188) = 4.96$, $p < 0.0001$. A significant suppression was observed 1 and 2 h after formalin injection at eCT doses of 4 and 40 U kg^{-1} day^{-1}. Such repeated injections of eCT did not significantly alter the nociceptive threshold of rats without formalin treatment, $F(3, 47) = 0.166$, $p > 0.05$ for interaction between dose and time.

Table 1 shows changes in body weight of rats that received vehicle or eCT (0.4, 4, and 40 U kg^{-1} day^{-1}) for 7 days. Although repeated administrations of the highest dose of eCT (40 U kg^{-1} day^{-1}) inhibited the increase in the body weight, the lower eCT doses of 0.4 and 4 U kg^{-1} day^{-1} had no significant effects on the growth of body weight for at least 7 days.

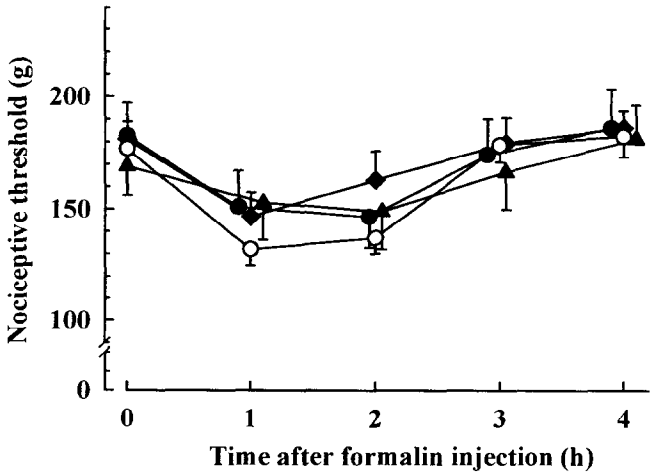

FIG. 3. Effects of single systemic injections of [Asu[1,7]]eel calcitonin (eCT) on formalin-induced hyperalgesia. After a 6-day course of vehicle, rats were given single shot of eCT or vehicle 15 min before formalin treatment. ○: vehicle (n = 13), ●: eCT (0.4 U kg^{-1}, n = 6), ◆: eCT (4 u kg^{-1}, n = 6), and ▲: eCT (40 U kg^{-1}, n = 13). Each point represents the means and SE.

Experiment 3: Duration of Effect of Systemic eCT

In Experiment 2, repeated systemic injections of eCT at a dose of 4 U kg^{-1} day^{-1} suppressed formalin-induced hyperalgesia, in which the last injection of eCT was 15 min before the formalin treatment. In this series of experiments, we examined whether such suppression would be observed 1 and 3 days after discontinuation of a 7-day course of systemic eCT injections (Fig. 5). The significant, $F(3, 21) = 3.88$, $p = 0.0236$, inhibitory effect of eCT (4 U kg^{-1} day^{-1}, SC) on formalin-induced hyperalgesia was revealed by RM-ANOVA. When rats (group 9) were daily given SC injections of eCT for 7 days (after vehicle injections for 3 days) until 15 min before formalin injection, formalin-induced hyperalgesia was significantly ($p < 0.05$, Dunnett's test) inhibited. When rats (group 10) were given vehicle daily for 2 days, eCT for 7 days and then a single injection of vehicle, formalin-induced hyperalgesia was also significantly ($p < 0.05$, Dunnett's test) suppressed. On the other hand, when rats (group 11) were daily administered with eCT for 7 days and then vehicle for 3 days, formalin-induced hyperalgesia was not significantly affected.

TABLE 1

EFFECTS OF DAILY SUBCUTANEOUS INJECTIONS OF [ASU^{1,7}]EEL CALCITONIN (eCT) ON THE BODY WEIGHT OF RATS

Dose of eCT (U kg^{-1} day^{-1})	Body Weight (g) During Repeated Injection of eCT				
	Day 1	Day 3	Day 5	Day 7	*n*
Vehicle	108.5 ± 1.3	122.1 ± 1.4	135.1 ± 2.0	15.27 ± 1.6	13
0.4	107.8 ± 1.6	121.2 ± 1.6	135.3 ± 2.1	151.3 ± 2.2	12
4	110.4 ± 1.2	124.3 ± 1.3	136.7 ± 1.7	152.5 ± 1.7	14
40	106.8 ± 1.5	99.0 ± 1.7*	114.4 ± 1.8*	128.3 ± 1.8*	12

Values are mean ± SE.
*$p < 0.05$ vs. vehicle (Dunnett's test).

DISCUSSION

One important finding in the present experiments is that the repeated systemic injections, but not single injection, of eCT produced a long-lasting inhibition of formalin-induced hyperalgesia. While the highest dose of eCT (40 U kg^{-1} day^{-1}) suppressed an increase in the body weight of rats, which might be due to its inhibitory effect on food and water consumption (38,47), a lower dose of eCT (4 U kg^{-1} day^{-1}) inhibited the hyperalgesia without effects on the increase in body weight. Although, at this stage we cannot rule out the possibility that the suppressive effect of the highest eCT dose (40 U kg^{-1} day^{-1}) was due to its nonspecific and indirect actions, it appears apparent that a lower dose (4 U kg^{-1} day^{-1}) might produce direct inhibition on formalin-induced hyperalgesia. When systemically injected into patients, CTs have been reported to produce analgesia generally at doses of 10 to 300 U day^{-1} (17,40) [for review, see (7)], which are corresponding to 0.2 to 6 U kg^{-1} day^{-1} assuming that body weight is 50 kg. Thus, the effective dose of eCT (4 U kg^{-1} day^{-1}) in rats as observed in the present studies is similar to those in human patients. In the present experiments, eCT was effective for at least 24 h after the last injection. This long-lasting action is also similar to the clinical effects of CTs, which are administered to patients with pains a few times a week (32).

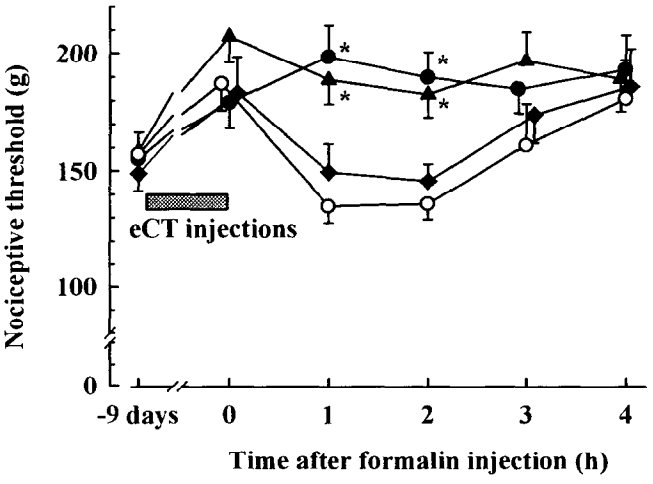

FIG. 5. The duration of suppressive effects of repeated systemic injections of [Asu^{1,7}]eel calcitonin (eCT, 4 U kg^{-1} day^{-1}) on formalin-induced hyperalgesia in rats. ○: vehicle was administered daily for 10 days ($n = 6$). ●: animals were given vehicle for 3 days and then eCT for 7 days ($n = 6$). ▲: animals were given vehicle for 2 days, eCT for 7 days, and then single shot of vehicle ($n = 7$). ◆: animals were given eCT for 7 days and then vehicle for 3 days ($n = 6$). Dilute formalin was administered 15 min after the last injection of vehicle or eCT. Each point represents means and SE. *$p < 0.05$ when compared with vehicle.

A single systemic injection of eCT at doses of 4 and 40 U kg^{-1} did not suppress the formalin-induced hyperalgesia. Thus, it appears that repeated injections of eCT were needed to inhibit this hyperalgesia. In human patients, CT analgesia can be achieved after 1 to 3 weeks of intramuscular injections. Thus, the antinociceptive effects of eCT in rats with formalin-induced hyperalgesia seem to resemble the analgesic effects of CTs in human patients.

The result that the inhibition of hyperalgesia was seen following a 7-day course of eCT suggests that tolerance to this effect was not developed after repeated injections of eCT into rats. This is consistent with the report that 5-day repeated injections of salmon CT did not inhibit the suppressive effects of intracerebroventricular CT on the licking responses of rabbits to electrical stimulation of the tooth pulp (6).

The mechanisms of the inhibitory effect produced by repeated systemic injections of eCT on formalin-induced hyperalgesia are unclear. CT in blood is reduced to half by about 30 min and almost disappears within 4 h after systemic injection (23,33,43,44). Thus, the long-lasting inhibitory effect following repeated administration may possibly be due to long-lasting changes in the neuronal functions.

Regarding the peripheral mechanisms of CT effect, formalin-induced hyperalgesia was inhibited by a topical injection of eCT (0.12 and 1.2 U rat^{-1}) into the same site of formalin injection. These results are similar to the report by another group, where a topical injection of eel CT (200 ng) inhibited formalin-induced hyperalgesia, probably secondary to inflammatory responses (21). It has been reported that CT could reduce the in vitro production of inflammatory mediators, prostaglandins (9). Thus, it appears possible that peripheral actions are at least partly involved in the inhibition of inflammatory pain by CT. However, it has not yet been determined whether repeated systemic injections of CT inhibit the in situ production of inflammatory mediators, including prostaglandins, in the region treated with formalin. Experiments are in progress in our laboratory to elucidate this effect.

There are many reports showing direct central actions of CTs. When administered intracerebroventricularly (8,37,41, 49) or intrathecally (45), CTs induce antinociceptive effects. CTs increase the secretion of β-endorphin and ACTH (26, 42,48) and inhibit that of thyrotropin (30) and prolactin (34). The presence of binding sites for CT in the brain and spinal cord (15,20,22,35) also suggests the ability of this peptide to

act directly on the central nervous system. Several reports have implicated the involvement of serotonergic (10,12,13) and catecholaminergic systems (19) in CT-induced antinociception. In our preliminary experiments, however, reserpine did not affect the suppressive effect of repeated systemic injections of eCT on formalin-induced hyperalgesia (unpublished observation). Although this finding is against the involvement of monoaminergic systems, the present data do not rule out the possibility that the antinociceptive effect of eCT is centrally mediated.

The opioid antagonist naloxone partially reversed the inhibitory effect of intraperitoneal CT (5 and 10 U kg^{-1}) in the acetic acid writhing test (27). Single intraperitoneal injection of CT (2.5 U kg^{-1}) enhances the increasing effect of a κ-receptor agonist on the concentration of plasma corticosterone (29). In addition, CT enhances opioid actions on κ- and δ-receptors in the isolated peripheral tissues (28). These findings suggest the possibility of positive interactions between CT and opioidergic systems. Therefore, it may be possible that opioidergic systems are at least partly involved in the inhibition of hyperalgesia by repeated systemic injections of eCT.

In summary, repeated systemic injections of eCT produce a long-lasting inhibition of formalin-induced hyperalgesia in rats. This inhibitory effect is similar to analgesia of CTs in human patients in terms of a necessity for repeated injections, effective dose, and long-lasting effects. Investigations on the mechanisms of eCT-induced inhibition on formalin-induced hyperalgesia are now in progress in our laboratory.

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