Cocaine- and Amphetamine-Regulated Transcript Peptide Modulation of Voltage-Gated Ca^{2+} Signaling in Hippocampal Neurons

Olena Yermolaieva,[1] Jianguo Chen,[1] Pastor R. Couceyro,[2] and Toshinori Hoshi[1]

[1]Department of Physiology and Biophysics, University of Iowa, Iowa City, Iowa 52242, and [2]Department of Cellular and Molecular Pharmacology, Chicago Medical School, North Chicago, Illinois 60064

Administration of cocaine and amphetamine increases cocaine- and amphetamine-regulated transcript (CART) expression in the rat striatum (Douglass et al., 1995). CART mRNA is highly expressed in different parts of the human and rat brain, including hippocampus (Douglass et al., 1995; Couceyro et al., 1997; Kuhar and Yoho, 1999; Hurd and Fagergren, 2000). The presence of CART peptide 55–102 immunoreactivity in dense core vesicles of axon terminals suggests that the peptide may be released and may act as a neuromodulator (Smith et al., 1997) to induce neurophysiological and behavioral effects. Little is known, however, about CART peptide-responsive cells, receptor(s), or intracellular signaling mechanisms that mediate CART peptide action. Here we show that CART peptide 55–102 inhibits voltage-dependent intracellular Ca^{2+} signaling and attenuates cocaine enhancement of depolarization-induced Ca^{2+} influx in rat hippocampal neurons. The inhibitory effect of CART peptide 55–102 on Ca^{2+} signaling is likely mediated by an inhibition of L-type voltage-gated Ca^{2+} channel activity via a G-protein-dependent pathway. These results indicate that voltage-gated Ca^{2+} channels in hippocampal neurons are targets for CART peptide 55–102 and suggest that CART peptides may be important in physiology and behavior mediated by the hippocampus, such as certain forms of learning and memory.

Key words: hippocampus; neuropeptide; cocaine; CART; calcium; voltage-gated calcium channels

Cocaine- and amphetamine-regulated transcript (CART) peptides are biologically active peptides that mediate feeding and are implicated in psychomotor stimulant drug abuse and stress response (Elias et al., 1998; Couceyro and Lambert, 1999; Kuhar and Dall Vechia, 1999; Kask et al., 2000; Kuhar et al., 2000). CART peptides are processed into shorter biologically active forms from either one or two propeptides in rat (Kristensen et al., 1998; Thim et al., 1998a). CART peptide 55–102 is biologically active and found in the rat brain (Kristensen et al., 1998; Thim et al., 1998a). The human CART cDNA sequence predicts only the shorter propeptide of 116 residues found in the rat (Douglass et al., 1995). (The peptide numbering used here is based on the longer rat propeptide of 102 residues excluding the leader sequence.) The three disulfide bridges (C68–C86, C88–C101, and C74–C94 using rat CART numbering) are present in the physiologically active CART peptide 55–102 (Thim et al., 1998a) and may stabilize its three-dimensional structure, suggesting that disruptions of these disulfide bridges impair biological activity (Kristensen et al., 1998).

CART expression increases in the striatum but particularly in the nucleus accumbens within 1 hr after administration of cocaine or amphetamine (Douglass et al., 1995; Kuhar and Yoho, 1999; Smith et al., 1999; Hurd and Fagergren, 2000). CART mRNA and CART peptides are normally expressed throughout the brain, especially in those areas involved in motivation, reward, and feeding, such as the nucleus accumbens, amygdala, and hypothalamus (Douglass et al., 1995; Couceyro et al., 1997; Koylu et al., 1997, 1998). Consistent with this expression pattern, exogenously applied CART peptides induce many marked behavioral changes, including inhibition of feeding (Lambert et al., 1998a,b; Thim et al., 1998a) and heightened anxiety (Kask et al., 2000).

In addition to the hypothalamus and amygdala, CART peptides are highly expressed in the hippocampus of both rodents and humans (Douglass et al., 1995; Koylu et al., 1998; Hurd and Fagergren, 2000), but their function and the effects of cocaine in the hippocampus are not known. CART mRNA is expressed in granule cells of the dentate gyrus, and CART peptides are found in the dentate gyrus CA3 and CA2 fields (Koylu et al., 1998; Hurd and Fagergren, 2000). The subcellular localization of CART peptides within dense core vesicles of axon terminals (Smith et al., 1997; Couceyro and Lambert, 1999) suggests that the peptides may be released from neurons into the extracellular space. Many neuropeptides, such as neuropeptide Y, which plays a significant role in the regulation of feeding (Flynn et al., 1999), interact with specific receptors on the plasma membrane that are coupled to second messenger systems (Qian et al., 1997). Intracellular Ca^{2+} signaling is intimately involved in synaptic activity and neurophysiological plasticity of hippocampal neurons (Alkon et al., 1998; Magee et al., 1998). Thus, we examined the effects of CART peptide 55–102, a biologically active form of rat CART (Thim et al., 1998a,b), on intracellular Ca^{2+} signals and voltage-gated Ca^{2+} channels in cultured rat hippocampal neurons.

Received May 29, 2001; revised July 18, 2001; accepted July 23, 2001.

This work was supported in part by National Institutes of Health Grants GM57654 (T.H.) and HL14388 (T.H.), the Pharmaceutical Research and Manufacturers Association Foundation (P.R.C.), the Schweppe Foundation (P.R.C.), and the National Alliance for Research on Schizophrenia and Affective Disorders (P.R.C.). We thank Heather Daggett for culturing hippocampal cells.

O.Y. and J.C. contributed equally to this work.

Correspondence should be addressed to Toshinori Hoshi, Department of Physiology and Biophysics, University of Iowa, Bowen Science Building, Iowa City, IA 52242. E-mail: hoshi@hoshi.org.

MATERIALS AND METHODS

Rat hippocampal neurons (embryonic day 18) isolated as described previously (Brewer et al., 1993; Brewer and Price, 1996) were purchased from BrainBits (Springfield, IL). The neurons and undifferentiated PC12 cells were plated at a density of 4×10^5 cells/cm^2 on poly-L-lysine-coated rectangular glass coverslips and used on days 6–12 after plating.

The cells were incubated with 1 μM fura-2 AM (Molecular Probes, Eugene, OR) for 1 hr in the standard recording medium (see below), washed, and mounted in the chamber of the spectrofluorometer (FP-750; Jasco, Tokyo, Japan). The standard recording medium contained (in mM): 140 NaCl, 5 KCl, 1 $MgCl_2$, 1.5 $CaCl_2$, 5 glucose, and 10 HEPES, pH 7.4 (NaOH). High [K^+] medium contained 30 mM KCl in place of NaCl. The chamber was continuously perfused at 7 ml/min. The media were changed, and the drugs were applied through the perfusion system, which took <0.5 sec. Measurements of cytosolic Ca^{2+} concentration were performed as described previously (Grynkiewicz et al., 1985). Fura-2 AM was excited at 340 and 380 nm, and the emission was measured at 510 nm. The background fluorescence was determined after each experiment by quenching fura-2 fluorescence with 10 mM $MnCl_2$ in nominally Ca^{2+}-free medium in the presence of 5 μM ionomycin. The background fluorescence at 340 and 380 nm was subtracted from the respective experimental readings, and the ratio of signals at 340 and 380 was obtained. The data were digitized at 2 sec intervals.

The Ca^{2+} channel openings were recorded in the cell-attached configuration of the patch clamp with an AxoPatch 200A amplifier (Axon Instruments, Union City, CA). The pipette solution contained (in mM): 90 $BaCl_2$, 10 TEA-Cl, and 10 HEPES, pH 7.3 (TEA-OH). The bath solution contained (in mM): 140 potassium gluconate, 10 EGTA, 10 HEPES, 15 NaCl, 3 $MgCl_2$, and 10 glucose, pH 7.4 (KOH). The output of the amplifier was filtered through an eight-pole low-pass filter unit (Frequency Devices, Haverhill, MA) and digitized by an ITC-16 analog-to-digital/digital-to-analog interface (Instrutech, Port Washington, NY) attached to an Apple (Cupertino, Ca) Power Macintosh computer running Pulse (HEKA, Lambrecht, Germany). The data were analyzed using Patch Machine (www.hoshi.org) and IgorPro (WaveMetrics, Lake Oswego, OR).

Two independent sources of CART peptide 55–102 were used in this study: rat CART peptide 55–102 from Peptides International (Louisville, KY; lot 490129) and a recombinant rat CART peptide 55–102 with an N-terminal histidine (HIS) tag (Fritz et al., 2000). After *in vitro* reduction and refolding, the recombinant CART peptide 55–102 HIS tag was biologically active, because it reduced food intake in rats. Inhibition of food intake was not seen if the peptide was not refolded, and an improperly folded inactive peptide was used as a control.

Bay K 8644, staurosporine, genistein, cyclosporine A, and pertussis toxin were obtained from Calbiochem (La Jolla, CA). Other reagents were obtained from Sigma (St. Louis, MO). Experiments were performed at room temperature (20–23°C).

RESULTS

CART peptide 55–102 dose-dependently reduced the amplitude of cytosolic Ca^{2+} signals elicited by K^+ depolarization in fura-2-loaded rat hippocampal cultured neurons (Fig. 1*A,B*). At 1 μM, CART peptide 55–102 decreased the total Ca^{2+} signal during 200 sec depolarization by 48 ± 14% ($n = 4$). The inhibitory effect of CART peptide 55–102 on Ca^{2+} signals elicited by depolarization was long-lasting and continued for at least 30 min after the peptide was washed out. The reduction of Ca^{2+} signals was noticeable at concentrations of CART peptide 55–102 as low as 250 nM and had an EC_{50} of ~600 nM (Fig. 1*B*).

Pretreatment of the neurons with fluorocitrate (0.1 mM for 30 min), a selective glial metabolic poison (Fonnum et al., 1997), altered neither the high K^+-induced Ca^{2+} signals nor the ability of CART peptide 55–102 to inhibit the signals (data not shown; $n = 6$). This suggests that glial cells potentially present in the preparation made an insignificant contribution to the results obtained.

The specificity of the CART peptide 55–102 effects on the Ca^{2+} signal was tested in several ways. We used CART peptide 55–102 from two independent sources synthesized in different ways (see Materials and Methods), and they were equally effective in inhibiting the Ca^{2+} signal. Recombinant rat CART peptide 55–102 purified from bacteria (Fritz et al., 2000) inhibited high-K^+-induced increases in Ca^{2+} signals in hippocampal cultures with a potency similar to that of CART peptide 55–102 made by solid-phase chemical synthesis. The biologically active CART peptide requires appropriate formation of three disulfide bonds (Thim et al., 1998a,b; Kuhar and Dall Vechia, 1999). Inappropriate formation of these bonds eliminates the ability of CART peptide to inhibit food intake (Fritz et al., 2000). Reduction of the disulfide links destabilizes the structure, and the peptide loses its ability to inhibit feeding. We reduced active CART peptide 55–102 with 5 mM dithiothreitol (DTT) for 1 hr at room temperature immediately before use. The DTT-treated CART peptide

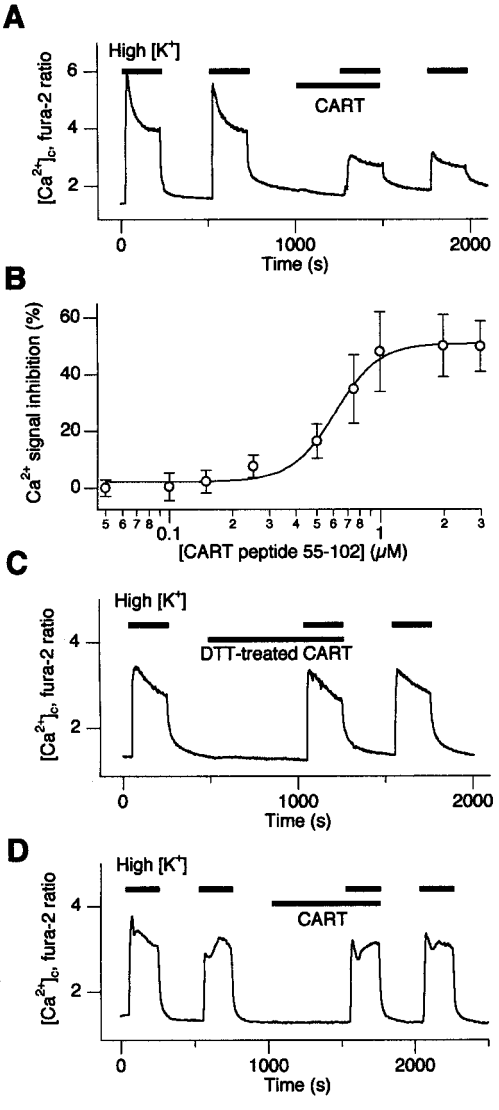

Figure 1. CART peptide 55–102 reduces the depolarization-induced Ca^{2+} signal. *A*, Cytosolic Ca^{2+} signal recorded from fura-2-loaded neurons before, during, and after bath application of CART peptide 55–102 (1 μM). High K^+ (35 mM) medium application is indicated by the *short bars,* and CART peptide 55–102 application is indicated by the *long bar*. *B*, Dose–response relationship between CART peptide 55–102 concentration and its inhibitory effect on Ca^{2+} signal amplitude ($n = 4$). *C*, The effect of CART peptide 55–102 was abolished by previous incubation with DTT. *D*, Ca^{2+} signals in undifferentiated PC12 cells before, during, and after CART peptide 55–102 application.

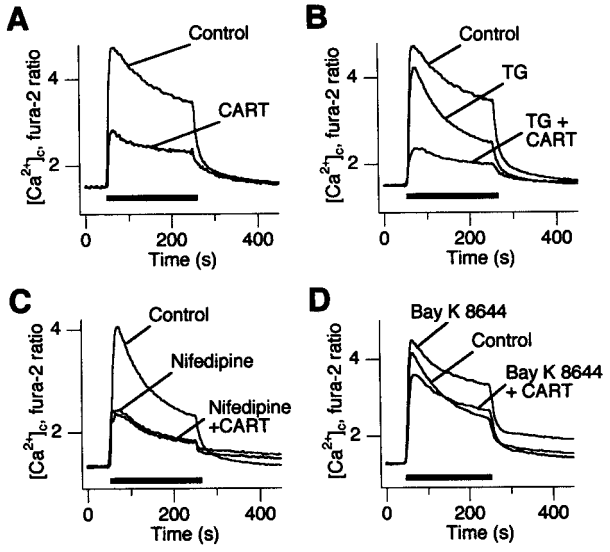

Figure 2. CART peptide 55–102 modifies Ca^{2+} influx in hippocampal neurons. *A*, Depolarization-induced Ca^{2+} signals in hippocampal neurons in the absence and presence of 1 μM CART peptide 55–102 are shown superimposed. *B*, Effects of CART peptide 55–102 (1 μM) on depolarization-induced Ca^{2+} signals in the presence of 1 μM TG. *C*, Effects of CART peptide 55–102 on depolarization-induced Ca^{2+} signals in the presence of 50 μM nifedipine. Nifedipine markedly reduced the Ca^{2+} signal, and the subsequent application of CART peptide 55–102 did not further inhibit the Ca^{2+} signals. The cells were also pretreated with TG. *D*, Depolarization-induced Ca^{2+} signals in the presence of 5 μM Bay K 8644 and CART peptide 55–102 in TG-pretreated cells.

55–102 had no effect on Ca^{2+} signals in hippocampal cultures (Fig. 1*C*). The corresponding control solution with 5 mM DTT left at room temperature for 1 hr without CART peptide failed to affect K^+-induced Ca^{2+} signals (data not shown; $n = 4$). Recombinant CART peptide 55–102 that did not undergo the refolding step (see Materials and Methods) and did not inhibit feeding (Fritz et al., 2000) was also much less effective in reducing the Ca^{2+} signal (15 ± 4%; $n = 4$) than the wild-type CART peptide. These results suggest that the observed inhibition of high-K^+-induced Ca^{2+} signal requires a properly folded peptide. Furthermore, we found that the CART action was tissue-specific. CART peptide 55–102 (up to 1 μM) failed to alter the Ca^{2+} signals in undifferentiated rat pheochromocytoma (PC12) neurosecretory cells (Fig. 1*D*), illustrating its cell-specific action.

The total cytoplasmic Ca^{2+} transient elicited by depolarization results from a number of Ca^{2+} transport mechanisms that balance Ca^{2+} influx and extrusion. CART peptide 55–102 did not significantly alter basal Ca^{2+} levels in the absence of depolarization (Fig. 1*A*). The rate of recovery of intracellular Ca^{2+} concentration after the depolarization-induced influx was also unaffected (Figs. 1*A*, 2*A*). These observations suggest that the peptide may modulate depolarization-activated Ca^{2+} influx rather than affecting a resting Ca^{2+} leak into the cytoplasm or Ca^{2+} extrusion mechanisms.

To assess the contribution of Ca^{2+}-induced Ca^{2+} release from endoplasmic reticulum (ER) Ca^{2+} stores, cells were treated with 5 μM thapsigargin (TG), which depleted intracellular Ca^{2+} stores by inhibiting ER ATPase (Thastrup et al., 1990), in nominally Ca^{2+}-free medium for 15 min. The pretreatment with TG did not markedly alleviate the inhibitory effects of CART peptide 55–102 on depolarization-induced Ca^{2+} signals (Fig. 2, compare *A, B*; $n = 8$). This observation argues against modulation of Ca^{2+}-induced Ca^{2+} release by CART peptide 55–102. In TG-treated cells, the kinetics of Ca^{2+} influx inactivation and the recovery of resting Ca^{2+} levels after depolarization in the presence of CART peptide 55–102 were unchanged (Fig. 2*B*). Thus, the peptide has a negligible effect on plasma membrane Ca^{2+} extrusion mechanisms.

CART peptide 55–102 was unable to inhibit Ca^{2+} signals in hippocampal neurons in the presence of nifedipine, which reduces the opening frequency and duration of voltage-gated L-type Ca^{2+} channels (Hess et al., 1984; Miller, 1987). Application of nifedipine reduced the amplitude of the high-K^+-induced Ca^{2+} signal by ~55% (Fig. 2*C*), indicating that L-type voltage-gated Ca^{2+} channels represent a major contributor to the Ca^{2+} signal measured. In the presence of nifedipine, the Ca^{2+} signals recorded before and after CART peptide 55–102 application were essentially indistinguishable (Fig. 2*C*; $n = 4$). The ineffectiveness of CART peptide 55–102 in the presence of nifedipine suggests that the peptide may inhibit L-type Ca^{2+} channels, although the possibility that the channels are not modulated in the presence of the antagonist cannot be totally ruled out (Dolphin, 1999). In contrast, CART peptide 55–102 was still effective in the presence of Bay K 8644, an L-type Ca^{2+} channel agonist (Fig. 2*D*, compare *Bay K 8644 trace, Bay K 8644 + CART trace*; $n = 5$). These results obtained with nifedipine and Bay K 8644 suggest that CART peptide 55–102 preferentially inhibits L-type Ca^{2+} channels.

We directly examined the action of CART peptide 55–102 on voltage-gated Ca^{2+} channels using the patch-clamp method. Voltage-gated Ca^{2+} channel openings were recorded in the cell-attached configuration using Ba^{2+} as the charge carrier, and CART peptide 55–102 was applied to the bath solution outside the recording pipette. Hippocampal neurons in culture (>6 d) express a high density of L-type Ca^{2+} channels (Porter et al., 1997). We recorded openings with a unitary current amplitude of ~2 pA at −5 mV (Fig. 3*A*, *Control*), which we identified as L-type Ca^{2+} channel openings on the basis of their sensitivity to nifedipine and Bay K 8644, their voltage dependence of activation, and their inactivation kinetics (data not shown). Application of CART peptide 55–102 to the bath markedly reduced the number of openings elicited by depolarization (Fig. 3*A*, *CART*). The unitary current amplitudes were unchanged before and after application of CART peptide 55–102, and the ensemble averages showed that CART peptide 55–102 (1 μM) decreased the mean current amplitude by 80–90% (Fig. 3*A*, *bottom traces*) without noticeably altering the channel kinetics. The inhibitory effect of CART peptide 55–102 was typically observed within 1 min of application (Fig. 3*B*). Similar results were observed in all seven patches examined (Fig. 3*D*). Bay K 8644, which promotes L-type Ca^{2+} channel openings, increased the frequency and the mean open duration of channel openings in the cell-attached configuration (Fig. 3*C*, *Control+Bay K 8644*), confirming that the openings indeed represented L-type Ca^{2+} channel openings. CART peptide 55–102 applied to the bath produced a dramatic reduction in the number of channel openings recorded in the presence of Bay K 8644 (Fig. 3*C*, *CART+Bay K 8644*). These effects were observed in all five patches examined (Fig. 3*E*). Thus, CART peptide modulates the same channels that are upregulated by Bay K 8644, consistent with the idea that CART peptide 55–102 inhibits L-type Ca^{2+} channels. Because in the patch clamp experiments the bath solution contained the Ca^{2+} chelator EGTA, and the recording solution contained Ba^{2+}, it is unlikely that the CART peptide caused noticeable Ca^{2+} influx across the entire

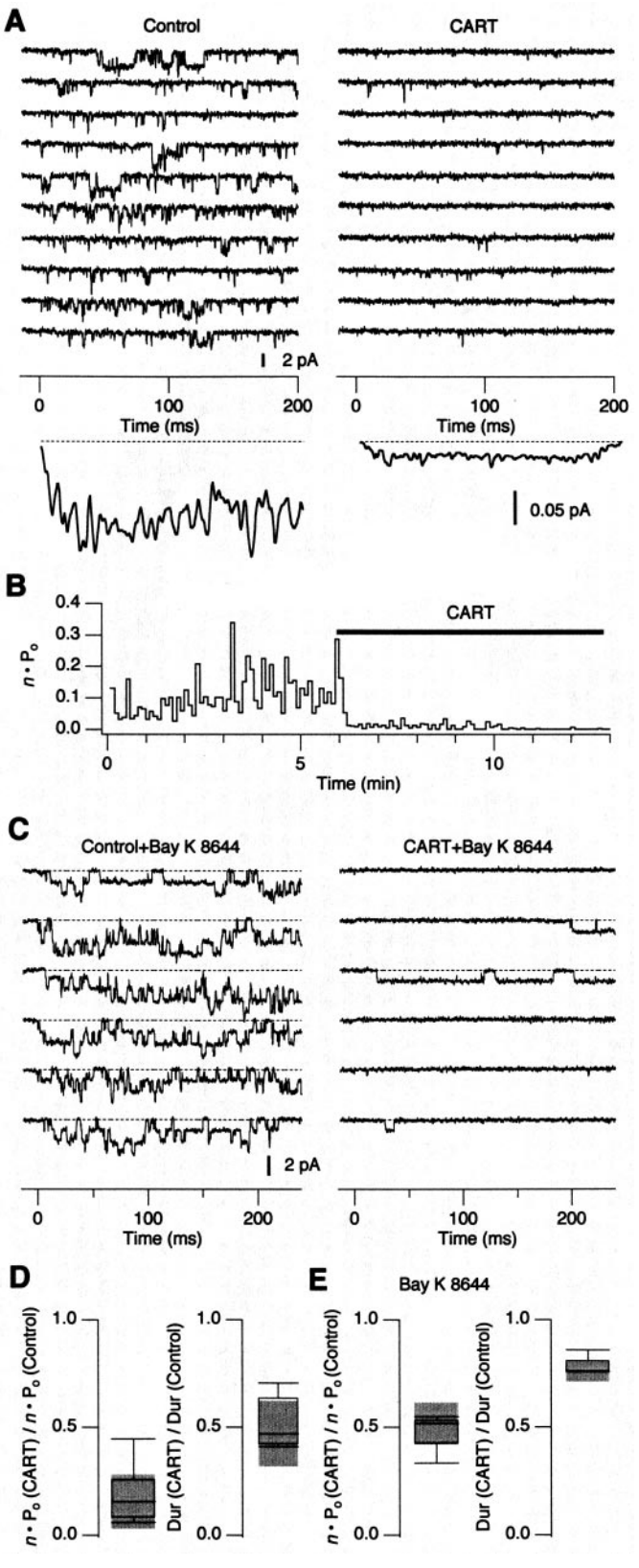

Figure 3. CART peptide 55–102 reduces L-type channel activity. *A*, Representative openings elicited by pulses from −50 to −5 mV in the control condition (*left*) and in the presence of the CART peptide (1 μM; *right*). The corresponding ensemble averages are shown at the *bottom*. *B*, Relative open probability ($n \cdot P_o$) during a representative experiment. The openings were elicited by pulses to −5 mV every 6 sec. The $n \cdot P_o$ value was calculated for each depolarization epoch, and the values are plotted as a function of the epoch number. The CART peptide application period is indicated by the *gray horizontal bar*. *C*, Representative openings elicited by pulses from −50 to −5 mV in the presence of Bay K 8644 (2 μM; *left*) as control and after application of CART peptide 55–102 (1 μM; *right*). *D*, Ratios of the relative open probability ($n \cdot P_o$) after application of the CART peptide (1 μM) to the respective control relative open probability are displayed using a box plot (*left*). The mean and median values in the control condition were 0.14 and 0.11, respectively. The *shaded area* represents the 95% confidence interval of the median. The ratios of the mean open duration (*Dur*) before and after the CART peptide application are also shown using a box plot (*right*). The mean and median values in the control condition were 0.72 and 0.72 msec, respectively. *E*, In the presence of Bay K 8644 (2 μM), the ratios of the open probability ($n \cdot P_o$) before and after the CART peptide application are displayed using a box plot (*left*). The mean and median values in the presence of Bay K 8644 before CART application were 0.44 and 0.54, respectively. *Right*, Ratios of the mean open duration before and after the CART peptide application. The mean and median values in the presence of Bay K 8644 before CART application were 6.8 and 7.7 msec, respectively.

cell membrane, which in turn inhibited the Ca^{2+} channel activity via a Ca^{2+}-dependent inactivation mechanism. The finding that CART peptide 55–102 added to the bath outside the recording pipette reduced the channel activity suggests that the action of CART peptide 55–102 is not mediated by the direct blocking or membrane-delimited effect on the channels but is likely to involve diffusible second messengers.

The above data suggest that the CART peptide receptors may be present in rat hippocampal neurons and that diffusible second messengers are likely to mediate signaling of CART peptides. L-type Ca^{2+} channel activity is subject to modulation by G-proteins, cAMP-dependent protein phosphorylation mechanisms, or both (Dolphin, 1998, 1999; Jones, 1998). We pharmacologically examined which putative second messengers might mediate the action of CART peptide 55–102 on voltage-gated Ca^{2+} channels to reduce the Ca^{2+} signals elicited by depolarization. CART peptide 55–102 inhibited the Ca^{2+} signal in the presence of 100 nM staurosporine, a nonselective serine-threonine protein kinase inhibitor that blocks Ca^{2+}-calmodulin kinase, PKA, PKC, and PKG (Ruegg and Burgess, 1989; Fig. 4*A*; $n = 4$). CART peptide 55–102 also reduced the Ca^{2+} signal in the presence of 5 μM genistein (Akiyama et al., 1987), an inhibitor of protein-tyrosine kinases (Fig. 4*B*; $n = 4$). The inhibitory effect of CART peptide 55–102 was also unaltered by the presence of 5 μM cyclosporine A, which inhibits protein phosphatase 2B, or calcineurin (Fig. 4*C*; $n = 4$). In contrast, incubation of the neurons for 18 hr with 50 ng/ml pertussis toxin, an inhibitor of G_i, G_o, and G_t regulatory proteins (Ui and Katada, 1990; Dolphin, 1998), completely abolished the inhibitory effect of CART peptide 55–102 (Fig. 4*D*; $n = 4$). This sensitivity to pertussis toxin suggests that inhibition of the Ca^{2+} signal by CART peptide 55–102 may be exerted via G_i and G_o proteins, which are known to inhibit of L-type Ca^{2+} channels in some cells (Degtiar et al., 1997; Farrugia, 1997; Zeng et al., 1999).

The ability of cocaine and amphetamine to selectively upregulate CART mRNA in the rat brain (Douglass et al., 1995) suggested that CART peptides may modulate some of the physiological and behavioral actions of stimulant drugs of abuse (Cooper and van der Hoek, 1993; Berke and Hyman, 2000). Cocaine has been shown to increase neuronal excitability and to enhance Na^+ channel activity (Zhai et al., 1997) and cytosolic Ca^{2+} levels (Onaivi et al., 1996) in hippocampus neurons. The drug also potentiates L-type Ca^{2+} channel activity in cardiac myocytes

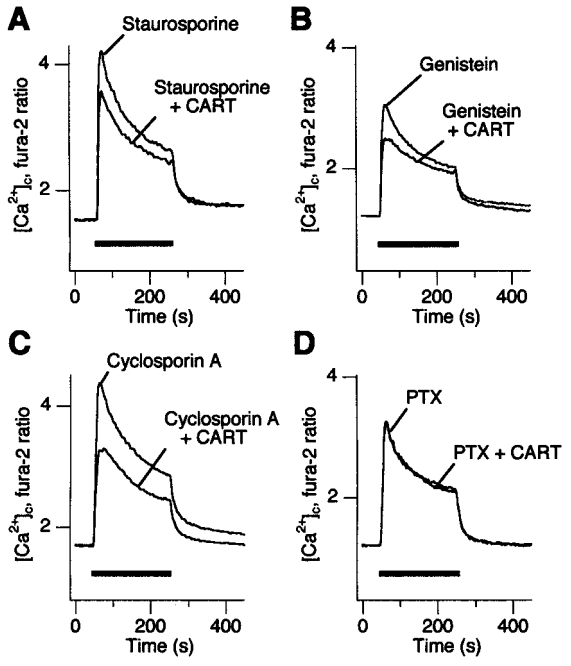

Figure 4. Signal transduction pathways mediating CART peptide 55–102 action. *A*, Ca^{2+} signals in response to high-K^+-induced depolarization were measured using fura-2 fluorescence in TG-pretreated rat hippocampal cells. Ca^{2+} signals are shown in the presence of 100 nM staurosporine, after a 5 min pretreatment, and then after addition of 1 μM CART peptide. *B*, Ca^{2+} transients in neurons pretreated with 5 μM genistein with and without CART peptide 55–102. *C*, Ca^{2+} signals after a 5 min pretreatment and in the continuous presence of 5 μM cyclosporine A and after addition of CART peptide. *D*, Ca^{2+} transients in hippocampal neurons pretreated with pertussis toxin (*PTX*; 50 ng/ml, 18 hr) and in the presence of CART peptide.

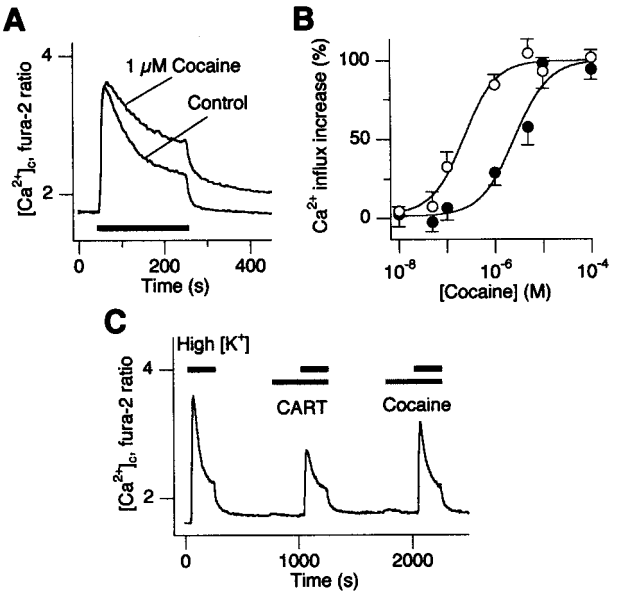

Figure 5. CART peptide decreases sensitivity of hippocampal neurons to cocaine. *A*, K^+ depolarization-induced Ca^{2+} transients in the presence of 0 and 1 μM cocaine (TG-pretreated cells). *B*, Increase in total Ca^{2+} influx in response to K^+ depolarization in the presence of increasing cocaine concentrations in control conditions (*open symbols*) and after a single 5 min application of 1 μM CART peptide (*filled symbols*) in TG-pretreated hippocampal cells. *C*, The depolarization-induced Ca^{2+} transient is reduced in the presence of 1 μM CART peptide 55–102 and partially restored by 5 μM cocaine.

(Premkumar, 1999). We found that cocaine dose-dependently potentiated intracellular Ca^{2+} signals produced by K^+ depolarization in cultured hippocampal neurons (Fig. 5*A*,*B*). CART peptide 55–102 (1 μM) attenuated the enhancement of the Ca^{2+} signal caused by cocaine and shifted the dose–response curve to the right, increasing the EC_{50} for cocaine from 0.23 to 2.6 μM (Fig. 5*B*). Moreover, application of 5 μM cocaine partially reversed inhibition of Ca^{2+} transients observed in the presence of CART peptide 55–102 (Fig. 5*C*; $n = 5$).

DISCUSSION

Previous studies have elucidated a role for CART peptides in a variety of physiological effects, including inhibition of feeding and increasing anxiety. The cellular mechanism of the CART action was, however, for the most part unknown. We show here that CART peptide 55–102 reduces the depolarization-induced intracellular Ca^{2+} signal by inhibiting voltage-gated Ca^{2+} channels in cultured rat hippocampal neurons. The results presented here indicate that CART peptide 55–102 is a neuropeptide modulator of voltage-gated Ca^{2+} channels in hippocampal neurons. Several lines of evidence indicate that this observation is of biological relevance. First, the observed inhibition of the Ca^{2+} signal requires a biologically active form of CART. Rat CART peptide 55–102 contains three disulfide links to stabilize the biologically active structure (Thim et al., 1998a; Kuhar and Dall Vechia, 1999). Disruption of these disulfide links by reducing agents, such as DTT, is known to destabilize the structure and to render the peptide ineffective in inhibiting feeding (Fritz et al., 2000). Our results show that application of the reducing agent DTT to solid-phase synthesized CART peptide 55–102 or omitting the refolding step in the synthesis of recombinant CART virtually eliminates the ability of CART peptide 55–102 to reduce the depolarization-induced Ca^{2+} signal in hippocampal neurons. Second, the effect of CART peptide 55–102 is dose-dependent (Fig. 1*B*), and the concentration of CART 55–102 required to inhibit the Ca^{2+} signal is within a range expected for a peptide neuromodulator. We estimated that the EC_{50} value of the CART action was ~600 nM. Other neuromodulator peptides have similar effective concentration ranges. For example, the inhibition of presynaptic Ca^{2+} entry in rat hippocampus by neuropeptide Y has an EC_{50} value of ~1 μM (Qian et al., 1997). Opioid peptides, such as enkephalin, inhibit voltage-gated Ca^{2+} channels effectively within a concentration of range of 1–10 μM (Hernandez-Guijo et al., 1999). Third, the action of CART peptide 55–102 is tissue-specific, consistent with the idea that some cells contain specific receptors designed for CART peptides. We observed robust inhibition of depolarization-activated Ca^{2+} signals in cultured rat hippocampal neurons, even in the presence of a glial-specific poison. In contrast, CART peptide 55–102 did not alter the Ca^{2+} signal in PC12 cells derived from adrenal chromaffin cells, even though they do contain a variety of voltage-gated Ca^{2+} channels, including L-type Ca^{2+} channels (McCullough et al., 1998). These Ca^{2+} measurements, therefore, suggest that the rat hippocampal neurons may contain specific CART peptide receptors that mediate the inhibitory action of the peptide on Ca^{2+} signaling. The rat hippocampus may represent a good source of CART peptide receptors for biochemical and molecular studies.

Both cytosolic Ca^{2+} measurements and electrophysiological results indicate that CART peptide 55–102 inhibits voltage-gated Ca^{2+} channel openings. Although CART peptide 55–102 inhibits

the depolarization-induced Ca^{2+} signal, it alters neither the basal Ca^{2+} level nor the recovery time course after depolarization. These observations show that Ca^{2+} influx through voltage-gated Ca^{2+} channels is inhibited by CART peptide 55–102. The single-channel data corroborate this idea; CART peptide 55–102 applied to the bath decreases the frequency and the mean open duration of the Ca^{2+} channel openings. We suggest that CART peptide 55–102 preferentially inhibits L-type Ca^{2+} channels for the following reasons. First, the CART-induced inhibition of the Ca^{2+} signal is abolished by nifedipine, an L-type Ca^{2+} channel inhibitor. If L-type channels are already inhibited by nifedipine, CART peptide 55–102 should not have any effect. However, this observation alone does not definitively argue that CART inhibits L-type Ca^{2+} channels, because some modulatory pathways of voltage-gated Ca^{2+} channels may not operate normally in the presence of dihydropyridines (Dolphin, 1999). Second, our single-channel recordings show that the channel openings were prolonged by Bay K 8644, and those long openings were in turn inhibited by CART peptide 55–102. These results strongly argue that CART peptide 55–102 inhibits voltage-gated L-type Ca^{2+} channels, and their inhibition contributes to the reduced Ca^{2+} signal observed with CART peptide 55–102. It is possible that other Ca^{2+} channel types are also modulated by CART peptide 55–102; however, our experiment did not address this possibility.

The inhibitory modulation of L-type Ca^{2+} channels by CART peptide 55–102 is likely to involve a diffusible intracellular second messenger and not likely to be solely mediated by a membrane-delimited system. Application of the peptide to the bath inhibited the channel activity recorded within the patch-clamp pipette (Fig. 3*A*). The results obtained using pharmacological inhibitors of various intracellular signaling cascades implicate a pertussis toxin-dependent G-protein system in the CART action. Staurosporine, genistein, and cyclosporine A, which have been shown to alter serine-threonine kinase-, tyrosine kinase-, and phosphatase 2B-dependent signaling pathways, did not alter the inhibitory action of CART peptide 55–102 on the Ca^{2+} signal (Fig. 4*A–C*). L-type Ca^{2+} channels are often upregulated by peptide neuromodulators via G-protein-dependent pathways (Dolphin, 1999). Our results indicate, however, that CART peptide 55–102 inhibits L-type Ca^{2+} channels in a pertussis toxin-sensitive manner (Fig. 4*D*). This sensitivity to pertussis toxin suggests that inhibition of the Ca^{2+} signal by CART peptide 55–102 may be exerted via G_i and G_o proteins (Ui and Katada, 1990; Dolphin, 1998). Although less common than upregulation, G-protein-dependent inhibition of L-type Ca^{2+} channels is not totally unprecedented. A similar mechanism of L-type Ca^{2+} channel inhibition has been demonstrated for the action of neuropeptide Y, somatostatin, galanin (Degtiar et al., 1997; Zeng et al., 1999), prostaglandins (Yamamoto et al., 1999), opioids, and catecholamines (Hernandez-Guijo et al., 1999). The long-lasting nature of the CART action may be surprising, considering the putative G-protein-dependent mechanism. One possible explanation is that CART peptide 55–102 may be difficult to wash out and that the observed long-lasting effect may not reflect the molecular nature of the underlying intracellular signaling cascade.

The exact physiological processes and behaviors modulated by CART peptide 55–102 effects on Ca^{2+} homeostasis in the hippocampal neurons remain to be investigated. Our studies with cocaine do offer some clues. Cocaine increases the total amount of depolarization-induced Ca^{2+} influx into the cultured hippocampal neurons, and this effect is antagonized by application of CART peptide 55–102 (Fig. 5*A*,*B*). The hippocampus has been implicated in the association of environmental cues and the affective states produced by drugs (White, 1996; Vorel et al., 2001). Electrical stimulation of the hippocampus elicits cocaine seeking in rats after extinction of cocaine self-administration behavior (Vorel et al., 2001), suggesting that hippocampal neurons are deeply involved in the learning and memory mechanisms associated with cocaine and possibly CART peptide action. Because CART peptides are rapidly produced in response to cocaine application at least in some cells (Douglass et al., 1995; Hurd and Fagergren, 2000), it is possible that the CART-mediated inhibition of depolarization-induced Ca^{2+} flux represents a homeostatic feedback response to counteract the enhanced Ca^{2+} entry induced by cocaine. The CART-mediated inhibition of voltage-gated Ca^{2+} channels described here may contribute to the drug tolerance observed with repeated use and also to the withdrawal symptoms and conditioned responses acquired during drug use (Gawin, 1991; Hyman, 1996; Breiter et al., 1997). Although cocaine does upregulate L-type Ca^{2+} channels in cardiac myocytes (Premkumar, 1999), it should be noted that our results do not directly address whether cocaine increases the total influx of Ca^{2+} in hippocampal neurons by modulating the hippocampal L-type Ca^{2+} channels.

The cytosolic Ca^{2+} measurements and single-channel measurements suggest that hippocampal neurons may possess membrane receptors for CART and that application of CART peptide 55–102 inhibits voltage-gated Ca^{2+} channels in a G-protein-dependent manner. CART peptides inhibit food intake and may mediate the central action of leptin (Elias et al., 1998; Thim et al., 1998a). Our observations further expand the role of CART peptides in the brain, but the exact behaviors affected remain unknown. It is quite possible that CART peptides help shape the neuronal plasticity associated with learning and memory mediated by the hippocampus.

REFERENCES

Akiyama T, Ishida J, Nakagawa S, Ogawara H, Watanabe S, Itoh N, Shibuya M, Fukami Y (1987) Genistein, a specific inhibitor of tyrosine-specific protein kinases. J Biol Chem 262:5592–5595.

Alkon DL, Nelson TJ, Zhao W, Cavallaro S (1998) Time domains of neuronal Ca^{2+} signaling and associative memory: steps through a calexcitin, ryanodine receptor, K^+ channel cascade. Trends Neurosci 21:529–537.

Berke JD, Hyman SE (2000) Addiction, dopamine, and the molecular mechanisms of memory. Neuron 25:515–532.

Breiter HC, Gollub RL, Weisskoff RM, Kennedy DN, Makris N, Berke JD, Goodman JM, Kantor HL, Gastfriend DR, Riorden JP, Mathew RT, Rosen BR, Hyman SE (1997) Acute effects of cocaine on human brain activity and emotion. Neuron 19:591–611.

Brewer GJ, Price PJ (1996) Viable cultured neurons in ambient carbon dioxide and hibernation storage for a month. NeuroReport 7:1509–1512.

Brewer GJ, Torricelli JR, Evege EK, Price PJ (1993) Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free medium combination. J Neurosci Res 35:567–576.

Cooper SJ, van der Hoek GA (1993) Cocaine: a microstructural analysis of it effects on feeding and associated behavior in the rat. Brain Res 608:45–51.

Couceyro PR, Lambert PD (1999) CART peptides: therapeutic potential in obesity and feeding disorders. Drug News Perspect 12:133–136.

Couceyro PR, Koylu EO, Kuhar MJ (1997) Further studies on the anatomical distribution of CART by in situ hybridization. J Chem Neuroanat 12:229–241.

Degtiar VE, Harhammer R, Nurnberg B (1997) Receptors couple to L-type calcium channels via distinct G_o proteins in rat neuroendocrine cell lines. J Physiol (Lond) 502:321–333.

Dolphin AC (1998) Mechanisms of modulation of voltage-dependent calcium channels by G proteins. J Physiol (Lond) 506:3–11.

Dolphin AC (1999) L-type calcium channel modulation. Adv Second Messenger Phosphoprotein Res 33:153–177.

Douglass J, McKinzie AA, Couceyro P (1995) PCR differential display identifies a rat brain mRNA that is transcriptionally regulated by cocaine and amphetamine. J Neurosci 15:2471–2481.

Elias CF, Lee C, Kelly J, Aschkenasi C, Ahima RS, Couceyro PR, Kuhar MJ, Saper CB, Elmquist JK (1998) Leptin activates hypothalamic CART neurons projecting to the spinal cord. Neuron 21:1375–1385.

Farrugia G (1997) G-protein regulation of an L-type calcium channel current in canine jejunal circular smooth muscle. J Membr Biol 160:39–46.

Flynn MC, Plata-Salaman CR, French-Mullen JM (1999) Neuropeptide Y-related compounds and feeding. Physiol Behav 65:901–905.

Fonnum F, Johnsen A, Hassel B (1997) Use of fluorocitrate and fluoroacetate in the study of brain metabolism. Glia 21:106–113.

Fritz T, Franklin M, Couceyro PR (2000) Generation of CART peptides with in vivo biological activity. Soc Neurosci Abstr 26:1151.

Gawin FH (1991) Cocaine addiction: psychology and neurophysiology. Science 251:1580–1586.

Grynkiewicz G, Poenie M, Tsien RY (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem 260:3440–3450.

Hernandez-Guijo JM, Carabelli V, Gandia L, Garcia AG, Carbone E (1999) Voltage-independent autocrine modulation of L-type channels mediated by ATP, opioids and catecholamines in rat chromaffin cells. Eur J Neurosci 11:3574–3584.

Hess P, Lansman JB, Tsien RW (1984) Different modes of Ca channel gating behaviour favoured by dihyropyridine Ca agonists and antagonists. Nature 311:538–544.

Hurd YL, Fagergren P (2000) Human cocaine- and amphetamine-regulated transcript (CART) mRNA is highly expressed in limbic- and sensory-related brain regions. J Comp Neurol 425:583–598.

Hyman SE (1996) Addiction to cocaine and amphetamine. Neuron 16:901–904.

Jones SW (1998) Overview of voltage-dependent calcium channels. J Bioenerg Biomembr 30:299–312.

Kask A, Schioth HB, Mutulis F, Wikberg JE, Rago L (2000) Anorexigenic cocaine- and amphetamine-regulated transcript peptide intensifies fear reactions in rats. Brain Res 857:283–285.

Koylu EO, Couceyro PR, Lambert PD, Ling NC, DeSouza EB, Kuhar MJ (1997) Immunohistochemical localization of novel CART peptides in rat hypothalamus, pituitary and adrenal gland. J Neuroendocrinol 9:823–833.

Koylu EO, Couceyro PR, Lambert PD, Kuhar MJ (1998) Cocaine- and amphetamine-regulated transcript peptide immunohistochemical localization in the rat brain. J Comp Neurol 391:115–132.

Kristensen P, Judge ME, Thim L, Ribel U, Christjansen KN, Wulff BS, Clausen JT, Jensen PB, Madsen OD, Vrang N, Larsen PJ, Hastrup S (1998) Hypothalamic CART is a new anorectic peptide regulated by leptin. Nature 393:72–76.

Kuhar MJ, Dall Vechia SE (1999) CART peptides: novel addiction- and feeding-related neuropeptides. Trends Neurosci 22:316–320.

Kuhar MJ, Yoho LL (1999) CART peptide analysis by Western blotting. Synapse 33:163–171.

Kuhar MJ, Adams LD, Hunter RG, Dall Vechia S, Smith Y (2000) CART peptides. Regul Pept 89:1–6.

Lambert PD, Couceyro P, Koylu EO, Ling NC, DeSouza EB, Kuhar MJ (1998a) A role for novel CART peptide fragments in the central control of food intake. Neuropeptides 31:620–621.

Lambert PD, Couceyro PR, McGirr KM, Dall Vechia SE, Smith Y, Kuhar MJ (1998b) CART peptides in the central control of feeding and interactions with neuropeptide Y. Synapse 29:293–298.

Magee J, Hoffman D, Colbert C, Johnston D (1998) Electrical and calcium signaling in dendrites of hippocampal pyramidal neurons. Annu Rev Physiol 60:327–346.

McCullough LA, Egan TM, Westfall TC (1998) Neuropeptide Y inhibition of calcium channels in PC12 pheochromocytoma cells. Am J Physiol 274:C1290–C1297.

Miller RJ (1987) Multiple calcium channels and neuronal function. Science 235:46–52.

Onaivi ES, Bishop-Robinson C, Motley ED, Chakrabarti A, Chirwa SS (1996) Neurobiological actions of cocaine in the hippocampus. Ann NY Acad Sci 801:76–94.

Porter NM, Thibault O, Thibault V, Chen KC, Landfield PW (1997) Calcium channel density and hippocampal cell death with age in long-term culture. J Neurosci 17:5629–5639.

Premkumar LS (1999) Selective potentiation of L-type calcium channel currents by cocaine in cardiac myocytes. Mol Pharmacol 56:1138–1142.

Qian J, Colmers WF, Saggau P (1997) Inhibition of synaptic transmission by neuropeptide Y in rat hippocampal area CA1: modulation of presynaptic Ca^{2+} entry. J Neurosci 17:8169–8177.

Ruegg UT, Burgess GM (1989) Staurosporine, K-252 and UCN-01—potent but nonspecific inhibitors of protein-kinases. Trends Pharmacol Sci 10:218–220.

Smith Y, Koylu EO, Couceyro P, Kuhar MJ (1997) Ultrastructural localization of CART (cocaine- and amphetamine-regulated transcript) peptides in the nucleus accumbens of monkeys. Synapse 27:90–94.

Smith Y, Kieval J, Couceyro PR, Kuhar MJ (1999) CART peptide-immunoreactive neurons in the nucleus accumbens in monkeys: ultrastructural analysis, colocalization studies, and synaptic interactions with dopaminergic afferents. J Comp Neurol 407:491–511.

Thastrup O, Cullen PJ, Drobak BK, Hanley MR, Dawson AP (1990) Thapsigargin, a tumor promoter, discharges intracellular Ca^{2+} stores by specific inhibition of the endoplasmic reticulum Ca^{2+}-ATPase. Proc Natl Acad Sci USA 87:2466–2470.

Thim L, Kristensen P, Larsen PJ, Wulff BS (1998a) CART, a new anorectic peptide. Int J Biochem Cell Biol 30:1281–1284.

Thim L, Nielsen PF, Judge ME, Andersen AS, Diers I, Egel-Mitani M, Hastrup S (1998b) Purification and characterisation of a new hypothalamic satiety peptide, cocaine and amphetamine regulated transcript (CART), produced in yeast. FEBS Lett 428:263–268.

Ui M, Katada T (1990) Bacterial toxins as probe for receptor-G_i coupling. Adv Second Messenger Phosphoprotein Res 24:63–69.

Vorel SR, Liu X, Hayes RJ, Spector JA, Gardner EL (2001) Relapse to cocaine-seeking after hippocampal theta burst stimulation. Science 292:1175–1177.

White NM (1996) Addictive drugs as reinforcers: multiple partial actions on memory systems. Addiction 91:921–949.

Yamamoto T, Habuchi Y, Tanaka H, Suto F, Morikawa J, Kashima K, Yoshimura M (1999) EP receptor-mediated inhibition by prostaglandin E_1 of cardiac L-type Ca^{2+} current of rabbits. Am J Physiol 277:H1369–H1374.

Zeng N, Athmann C, Kang T, Walsh JH, Sachs G (1999) Role of neuropeptide-sensitive L-type Ca^{2+} channels in histamine release in gastric enterochromaffin-like cells. Am J Physiol 277:G1268–G1280.

Zhai J, Wieland SJ, Sessler FM (1997) Chronic cocaine intoxication alters hippocampal sodium channel function. Neurosci Lett 229:121–124.