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European Journal of Pharmacology 399 (2000) 165–169

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Decrease of hindpaw withdrawal latency by cocaine- and amphetamine-regulated transcript peptide to the mouse spinal cord

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Received 18 May 2000; accepted 19 May 2000

Abstract

Immunohistochemical studies with the use of an antiserum against the cocaine- and amphetamine-regulated transcript (CART) peptide-(55–102) showed an abundance of CART-immunoreactive fibers in the mouse dorsal horn laminae I and II. A few CART-positive somata were scattered in the dorsal horn and around the central canal. Intrathecal injection of the CART peptide-(55–102) at doses 3, 10 and 100 ng caused a dose-dependent and significant decrease of paw withdrawal latency; whereas, saline injection was without significant effect. Our results provide the first evidence that CART-immunoreactive fibers are present in the dorsal horn and that the peptide administered intrathecally produces hyperalgesia, as assessed by paw withdrawal latency in mice.

Keywords: Cocaine; Amphetamine; Dorsal horn; Hyperalgesia

1. Introduction

Cocaine- and amphetamine-regulated transcript (CART) was identified by polymerase chain reaction differential display and found to be up-regulated in the rat striatium following acute administration of cocaine and amphetamine (Douglass et al., 1995). Immunohistochemical and in situ hybridization studies showed that CART-immunoreactivity or mRNA is widely distributed in the rat brain, spinal cord and adrenal medulla (Douglass et al., 1995; Couceyro et al., 1997; Koylu et al., 1997, 1998). Western Blot analysis of several rat brain regions and longitudinal intestinal muscle strips revealed at least six different CART peptides with molecular weight ranging from 4 to 14 kDa (Kuhar and Yoho, 1999). The two lower molecular weight peptides CART-(55–102) and CART-(62–102) are thought to be the endogenous, physiologically active fragments (Kuhar and Dall Vechia, 1999; Kuhar and Yoho, 1999).

A dense network of CART-immunoreactive fibers has been detected in superficial layers of the rat dorsal horn (Koylu et al., 1998). The biological role of CART peptides with respect to dorsal horn neurons and their behavioral correlate are not known. Here, we report the presence of CART-immunoreactive fibers in superficial layers of the dorsal horn and hyperalgesic behaviors upon intrathecal injection of the CART peptide-(55–102) to the mouse spinal cord.

2. Materials and methods

2.1. Animals

Male ICR mice weighing 25–30 g (Sasco, Omaha, NE) were used. Animals were housed five per cage in a room maintained at 22 ± 0.5°C with an alternating 12-h light–dark cycle. Food and water were available ad libitum. Animals were used only once. This study was conducted in

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0014-2999/00/$ - see front matter
PII: S0014-2999(00)00374-5

accordance of the guidelines of the Animal Care and Use Committee of respective institutions.

2.2. Intrathecal administration

Intrathecal injection was performed according to the method described by Hylden and Wilcox (1980) using a 25-μl Hamilton syringe with a 30-gauge needle. The injection volume was 5 μl.

2.3. Hindpaw withdrawal test

The hindpaw withdrawal latency was determined by a thermal stimulus apparatus (Model 336; IITC, Woodland Hills, CA), as described in detail by Hargreaves et al. (1988). Mice were placed individually in a set of 8 clear plastic cages ($10.5 \times 6.5 \times 10.0$ cm^3 each) on an elevated glass surface and allowed to acclimatize for 60 min. A radiant heat stimulus was applied from underneath the glass floor with a high-intensity projector lamp bulb. The heat stimulus was focused on the plantar surface of each hindpaw. The intensity of the heat stimulus was adjusted to derive an average baseline latency of paw withdrawal of approximately 9 s in naive animals. A 20-s cut-off was used to prevent tissue damage. Paw withdrawal latency was determined as the average of two measurements per paw. Left and right hindpaws were tested alternately in no less than 2 min.

2.4. Immunohistochemistry

Immunohistochemical procedures used in this study were similar to those described earlier (Dun et al., 1993; Narita et al., 1996). Mice anesthetized with a combination of ketamine (70 mg/kg, i.p.) and xylazine (15 mg/kg, i.p.) were perfused intracardially with 0.1 M phosphate-buffered saline (PBS) followed by freshly prepared 4% paraformaldehyde in PBS. Spinal cords were removed, postfixed in the same fixative for 2 h, and cryoprotected in 30% sucrose–PBS overnight. Transverse 40 μm spinal sections prepared by a Vibratome were processed for CART-immunoreactivity using the avidin–biotin complex method, as described previously (Dun et al., 1993; Narita et al., 1996). The CART-antiserum, a rabbit polyclonal directed against the rat CART peptide-(55–102), was the same used in our recent study (Dun et al., 2000). The specificity of CART-antisera was characterized by radioimmunoassay and Western blot (see product information, Phoenix Pharmaceuticals, Mountain View, CA). Sections were first treated with 3% H_2O_2 to quench endogenous peroxidase, washed and blocked with 10% normal goat sera, and incubated in CART-antisera (1:10,000 dilution with 0.1% Triton X-100 and 1% bovine serum albumin in PBS) for 48 h at 4°C with gentle agitation. After thorough rinsing, sections were incubated with biotinylated anti-rabbit immunoglobulin G (1:150, Vector Laboratories) for 2 h. Sections were rinsed with PBS and incubated in avidin–peroxidase complex for 1 h (1:100, Vector Laboratories). After several rinses in Tris-buffered saline, sections were developed in diaminobenzidine–H_2O_2 solution and washed for 2 h with Tris-buffered saline. Sections were mounted on slides with 0.25% gel alcohol, air-dried, dehydrated with absolute alcohol followed by xylene and cover-slipped with Permount.

In control experiments, spinal sections were processed with CART-antisera pre-absorbed with the peptide (10 μg/ml) overnight.

2.5. Peptide and statistical data analyses

CART peptide-(55–102) was from Phoenix Pharmaceuticals. The behavioral data are presented as mean ± S.E.M. at different time points after the injection. Statistical analysis of differences between groups was assessed with analysis of variance (ANOVA) followed by Newman–Keuls test; $P < 0.05$ was considered significant.

3. Results

3.1. Distribution of CART-immunoreactivity in mouse spinal cord

Distribution of CART-immunoreactivity was examined in three spinal cords. Similar to the rat spinal cord (Koylu et al., 1998), dense networks of CART-immunoreactive fibers were observed in the superficial layers of the dorsal horn. For example, an abundance of CART-fibers was present in laminae I and II of the lumbar section and much fewer CART-positive fibers were detected in the deeper laminae (Fig. 1). In addition to fibers, several CART-positive cell bodies, generally of small diameter, were seen in lamina X (Fig. 1B) and superficial layers of the dorsal horn (Fig. 1C). This pattern of distribution of CART-immunoreactivity was observed throughout the cervical, thoracic, lumbar and sacral segments. In addition to CART-fibers, CART-positive somata were observed in the intermediolateral cell column of mouse thoracolumbar segments (not shown). These were similar to that described for the rats (Koylu et al., 1998; Dun et al., 2000).

In control experiments, CART-immunoreactivity was not detected in any of the spinal sections processed with CART-antisera pre-absorbed with the peptide overnight (Fig. 1D).

3.2. Effects of intrathecal administration of CART peptide-(55–102) on hindpaw withdrawal response

Intrathecal injection of CART peptide-(55–102) at 3, 10 and 100 ng produced a dose-dependent reduction of the latency of thermal paw withdrawal responses (Fig. 2). At the dose of 1 ng, the peptide did not cause a significant

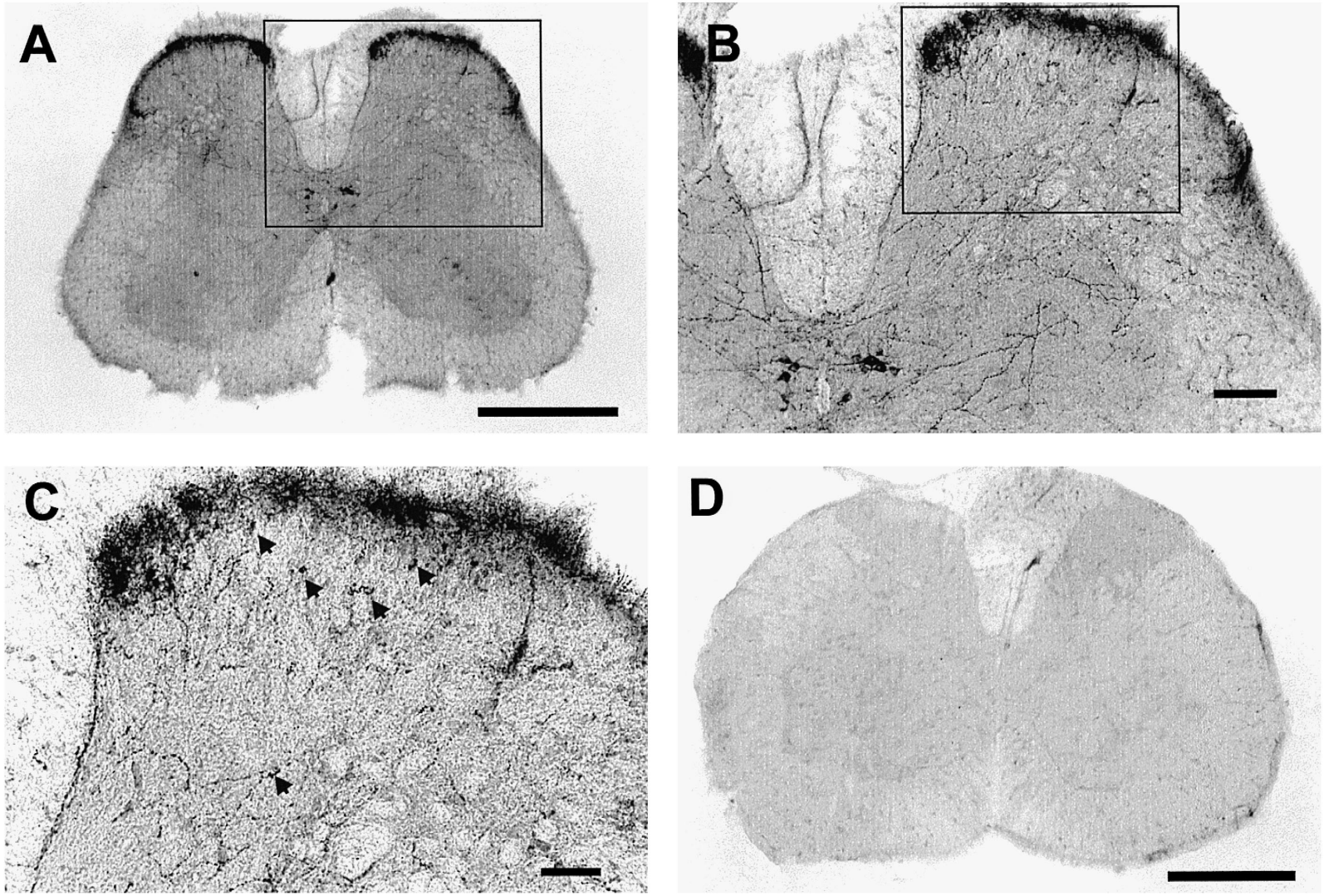

Fig. 1. Photomicrographs of mouse lumbar spinal sections labeled with CART-antisera or CART-antisera pre-absorbed with the peptide overnight. (A) Low magnification showing CART-immunoreactive fibers are concentrated in laminae I and II of the dorsal horn; deeper laminae receive much fewer CART-fibers. (B) Higher magnification of the area outlined in A where varicose CART-immunoreactive fibers are concentrated in the superficial layers and fewer in deeper laminae; several CART-positive somata are seen around the central canal (cc). (C) Higher magnification of the area outlined in B where several small diameter CART-positive somata, as indicated by arrows, are seen in the dorsal horn. (D) A section of lumbar spinal cord labeled with CART-antisera pre-absorbed with the peptide (10 μg/ml) overnight; immunoreactivity is not seen in this section. Scale bar: 500 μm for (A) and (D); 100 μm and 50 μm for B and C, respectively.

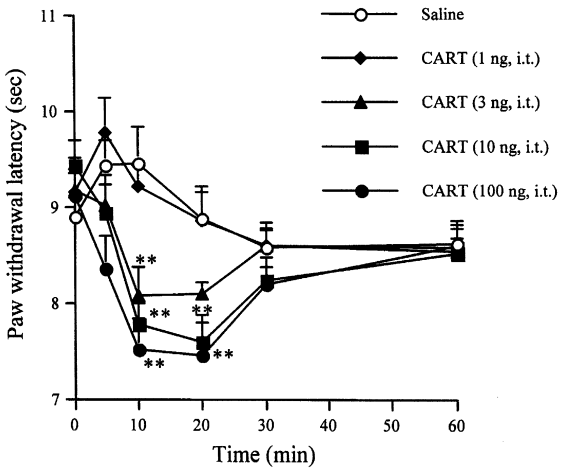

Fig. 2. Time- and dose-dependent decrease of hindpaw withdrawal latency by intrathecal injection of CART peptide-(55–102) in mice. Ordinate is paw withdrawal latency in seconds and abscissa is time in minutes after intrathecal administration of CART. At the doses of 3, 10 and 100 ng, CART caused a significant decrease of paw withdrawal latency as compared to saline injected animals; 1 ng CART produced no significant change of paw withdrawal latency. Each point represents the averaged responses of seven or eight animals; * * denotes statistical significance, $P < 0.05$.

change of paw withdrawal latency as compared to that of saline injected animals (Fig. 2). The withdrawal latency decreased in 5 min, reached a maximal in 10 to 20 min, and returned to baseline level in about 30 min. Injection of CART at doses higher than 100 ng did not cause any further reduction of the withdrawal latency (data not shown). Intrathecal injection of saline caused a slight, but not significant, increase of the withdrawal latency 5 and 10 min post-injection (Fig. 2).

4. Discussion

CART is a family of novel peptides (Kuhar and Dall Vechia, 1999). Immunohistochemical and in situ hybridization studies reveal an extensive distribution of CART in various regions of the rat brain, implying that the peptide may have a multiple function at different levels of the neuraxis (Koylu et al., 1998). Currently the only reported biological action of CART relates to a reduction of food intake upon intracerebroventricular injection in rats (Lambert et al., 1998). With respect to the rat spinal cord, an abundance of CART-immunoreactive fibers is detected in laminae I and II, with much fewer fibers in the deeper laminae (Koylu et al., 1998). A similar pattern of distribution of CART-immunoreactivity is noted in the mouse spinal cord. The origin of CART-fibers observed in the dorsal horn has not been studied. They may arise from dorsal root ganglion cells and/or from neurons in the supraspinal structure. Another question that needs to be addressed in a future study is whether or not CART peptide is released from fibers in the dorsal horn.

Concentration of CART-fibers in the superficial layers of the dorsal horn raises the possibility that the peptide(s) may be involved in the processing of sensory information. In our behavioral study, the CART peptide-(55–102) upon intrathecal injection produced a significant and consistent decrease of paw withdrawal latency, which is indicative of a state of hyperalgesia. Viewed in this context, the action of CART differs from putative hyperalgesic peptides substance P and pituitary adenylate cyclase-activating polypeptide in that CART did not show any algesic action, such as licking, biting and scratching of injection site (Hylden and Wilcox, 1981; Dirig and Yaksh, 1996; Narita et al., 1996). Little is known of the site and mechanism of the hyperalgesic action of CART. As the peptide was administered intrathecally, dorsal horn neurons are likely to be the primary target upon which CART produces its hyperalgesic behaviors. It remains to be determined whether CART produces hyperalgesia by interacting with receptors or binding sites located on dorsal horn neurons or by releasing other biologically active substances within the dorsal horn or both.

Acknowledgements

This study was supported by NIH Grants NIDA 03811(LFT) and NS18710 and NS39646 (NJD) from the Department of Health and Human Services.

References

Couceyro, P.R., Koylu, E.O., Kuhar, M.J., 1997. Further studies on the anatomical distribution of CART by in situ hybridization. J. Chem. Neuroanat. 12, 229–241.

Dirig, D.M., Yaksh, T.L., 1996. Thermal hyperalgesia in rats evoked by intrathecal substance P at multiple stimulus intensities reflects an increase in the gain of nociceptive processing. Neurosci. Lett. 220, 93–96.

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

Dun, N.J., Dun, S.L., Wu, S.Y., Forstermann, U., Schmidt, H.H.H.W., Tseng, L.F., 1993. Nitric oxide synthase immunoreactivity in the rat, mouse, cat and squirrel monkey spinal cord. Neuroscience 54, 843–857.

Dun, N.J., Dun, S.L., Kwok, E.H., Yang, J., Chang, J.-K., 2000. Cocaine- and amphetamine-regulated transcript-immunoreactivity in the rat sympatho-adrenal axis. Neurosci. Lett. 283, 97–100.

Hargreaves, K., Dubner, R., Brown, F., Flores, C., Joris, J., 1988. A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. Pain 32, 77–88.

Hylden, J.L., Wilcox, G.L., 1980. Intrathecal morphine in mice: a new technique. Eur. J. Pharmacol. 67, 313–316.

Hylden, J.L., Wilcox, G.L., 1981. Intrathecal substance P elicits a caudally-directed biting and scratching behavior in mice. Brain Res. 217, 212–215.

Koylu, E.O., Couceyro, P.R., Lambert, P.D., Ling, N.C., DeSouza, E.B., Kuhar, M.J., 1997. Immunohistochemical localization of novel CART peptides in rat hypothalamus, pituitary and adrenal gland. J. Neuroendocrinol 9, 823–833.

Koylu, E.O., Couceyro, P.R., Lambert, P.D., Kuhar, M.J., 1998. Cocaine- and amphetamine-regulated transcript peptide immunohistochemical localization in the rat brain. J. Comp. Neurol. 391, 115–132.

Kuhar, M.J., Dall Vechia, S.E., 1999. CART peptides: novel addiction- and feeding-related neuropeptides. Trends Neurosci. 22, 316–320.

Kuhar, M.J., Yoho, L.L., 1999. CART peptide analysis by western blotting. Synapse 33, 163–171.

Lambert, P.D., Couceyro, P.R., McGirr, K.M., Dall Vechia, S.E., Smith, Y., Kuhar, M.J., 1998. CART peptides in the central control of feeding and interactions with neuropeptide Y. Synapse 29, 293–298.

Narita, M., Dun, S.L., Dun, N.J., Tseng, L.F., 1996. Hyperalgesia induced by pituitary adenylate cyclase-activating polypeptide in the mouse spinal cord. Eur. J. Pharmacol. 311, 121–126.