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Distinct Neurochemical Populations in the Rat Central Nucleus of the Amygdala and Bed Nucleus of the Stria Terminalis: Evidence for Their Selective Activation by Interleukin-1β

HEIDI E.W. DAY,[1]* EILEEN J. CURRAN,[2] STANLEY J. WATSON, JR.,[1] AND HUDA AKIL[1]

[1]Mental Health Research Institute, University of Michigan, Ann Arbor, Michigan 48109
[2]Amgen, Inc., Thousand Oaks, California 91320

ABSTRACT

The lateral division of the central nucleus of the amygdala (CEAl) and the oval nucleus of the bed nucleus of the stria terminalis (BSTov) have been linked closely anatomically and functionally. To determine whether these regions may be subdivided further on a neurochemical basis, dual in situ hybridization was used to determine the colocalization of corticotropin-releasing hormone (CRH), enkephalin (ENK), or neurotensin (NT) with glutamic acid decarboxylase isoforms 65 and 67 [used concurrently as a marker for γ-aminobutyric acid GABA] in these nuclei. It was found that, for both regions, each peptide invariably was localized in a GABAergic cell. Although there was a similar overlap in the distribution of NT with ENK in the BSTov and CEAl, it was observed that CRH and ENK rarely were colocalized in either nucleus. To determine whether these distinct neuronal populations could be activated differentially, male rats were given a systemic injection of interleukin-1β (IL-1β; 5 μg/kg, i.p.), a stimulus that results in a robust increase in c-fos mRNA expression in the BSTov and CEAl. The neurochemical identity of these activated neurons showed striking similarities between the BSTov and the CEAl; All IL-1β-responsive cells were GABAergic, the majority of c-fos- positive cells expressed ENK mRNA (BSTov, 81%; CEAl, 94%), and some expressed NT mRNA (BSTov, 23%; CEAl, 22%), whereas very few expressed CRH mRNA (BSTov, 4%; CEAl, 1%). These data provide evidence for the existence of discrete neural circuits within the BSTov and CEAl, and the similarities in the patterns of neurochemical colocalization in these nuclei are consistent with the concept of an extended amygdala. Furthermore, these data indicate that intraperitoneal IL-1β recruits neurochemically distinct pathways within the BSTov and CEAl, and it is suggested that this differential activation may mediate specific aspects of immune, limbic, and/or autonomic processes. J. Comp. Neurol. 413:113–128, 1999.

Indexing terms: corticotropin-releasing hormone; enkephalin; neurotensin; γ-aminobutyric acid; c-fos

A significant body of work has demonstrated both anatomical and functional similarities between the lateral division of the central nucleus of the amygdala (CEAl) and the oval nucleus of the bed nucleus of the stria terminalis (BSTov: Ju and Swanson, 1989; Ju et al., 1989; Swanson 1992; equivalent to the dorsal lateral subnucleus of Moga et al., 1989). Indeed, together with the substantia inominata, it has been proposed that these structures form the central extended amygdala (for review, Alheid et al., 1995). Although it is not the purpose of this article to review all of the anatomical connections of these nuclei, it is worth noting the strong topographic projections from the CEA to the BST (Sun et al., 1991). Furthermore, both structures are connected reciprocally with the parabrachial nucleus

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*Correspondence to: Dr. H.E.W. Day, Mental Health Research Institute, 205 Zina Pitcher Place, Ann Arbor, MI 48109-0720.
E-mail: heididay@umich.edu

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(Moga and Gray, 1985; Moga et al., 1989; Bernard et al., 1993; Krukoff et al., 1993; Alden et al., 1994) and the nucleus tractus solitarius (NTS; Ricardo and Koh, 1978; Gray and Magnuson, 1987), and they share a number of other common efferent and afferent inputs, as outlined in more detail below. Although the functional significance of activation of these regions is not clear, together, they are thought to be involved in the coordination of central autonomic function (Saper, 1995). Furthermore, the BST and CEA have been associated with the elicitation and integration of behavioral and possibly emotional responses to anxiety, fear, and stress (Davis, 1992; Gallagher and Chiba, 1996; Maren and Fanselow, 1996).

With such a diverse array of complex functions ascribed to the BST and CEA, it is not surprising that these nuclei receive inputs from a wide variety of sources. For example, cortical areas (Hurley et al., 1991; Sun et al., 1994; McDonald and Mascagni, 1997), thalamic areas (Moga et al., 1995), hypothalamic areas (Cullinan and Zaborsky, 1991; Canteras et al., 1994; Vertes et al., 1995), and brainstem areas (Ricardo and Koh, 1978; Bernard et al., 1993; Krukoff et al., 1993; Alden et al., 1994) as well as other amygdaloid nuclei (Weller and Smith, 1982; Canteras et al., 1995; Savander et al., 1995, 1996) have been shown to project to the BST and the CEA. To achieve functional differentiation, there is a considerable degree of anatomical specificity within the BST and CEA in the targets of these different inputs. For example, a subset of cortical areas (Sun et al., 1994; McDonald and Mascagni, 1997), thalamic areas (Moga et al., 1995), and brainstem areas (Ricardo and Koh, 1978; Krukoff et al., 1993) project to the CEAl. Within the CEAl, although it appears that inputs from these different origins overlap broadly, one way in which further functional separation may be gained is by targeting distinct neurochemical subpopulations within the nucleus. Although these neurons may be different functionally, because they are in close proximity to one another, their roles may become integrated and coordinated. In addition, if these patterns of segregation appear consistently across other components of the central extended amygdala, then this would lend support to the proposed coherent nature and functional relevance of these areas.

Several studies have been published describing the distribution of various peptides in the BST and the CEA (Wray and Hoffman, 1983; Cassell et al., 1986; Ju et al., 1989; Moga et al., 1989; Shimada et al., 1989). Although these studies have demonstrated similarities in the expression of a number of peptides between the BSTov and the CEAl, they also have indicated that, within these subnuclei, there is differential peptide expression. The few colocalization studies that have been published indicate that there may be neurochemically distinct neuronal populations within the BSTov and the CEAl (Oertel et al., 1983; Shimada et al., 1989; Veinante et al., 1997). Furthermore, these two nuclei are known to be highly γ-aminobutyric acidergic (GABAergic; Sun and Cassell, 1993), and there is some evidence from immunocytochemical studies on adjacent sections to suggest that GABAergic neurons of the BSTov and the CEAl synthesize either corticotropin-releasing hormone (CRH) or enkephalin (ENK; Veinante et al., 1997).

In this study, a double in situ hybridization (ISH) technique was used to extend these findings in experimentally naïve adult male rats. The degree of colocalization of ENK, neurotensin (NT), or CRH mRNA in GABAergic neurons of the BSTov and the CEAl was determined initially. Furthermore, we determined whether or not CRH or NT mRNA was expressed in enkephalinergic cells. These studies revealed a highly discrete pattern of peptide colocalization, and we went on to ask whether these neurochemically distinct neuronal populations of the BSTov and CEAl could be activated selectively by an intraperitoneal injection of interleukin-1β (IL-1β). This stimulus is known to elicit c-fos mRNA expression in the BSTov and CEAl (Brady et al., 1994; Day and Akil, 1996), which is thought to reflect synaptic or transcriptional activation. The extent of colocalization of IL-1β-induced c-fos mRNA in GABAergic neurons and in cells that express ENK, NT, or CRH mRNA in the BSTov or CEAl was established. Preliminary data from this work were presented previously in abstract form (Day et al., 1996).

MATERIALS AND METHODS

Animals

All animal procedures were approved by University of Michigan Committee on Use and Care of Animals. Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA), weighing 250–300 g were used. Rats were housed five or six per cage under conditions of constant temperature and humidity on a 12-hour light/dark cycle (lights on at 0600) with access to food and water ad libitum. Six rats that were naïve to handling or other treatment regimens were used for neurochemical colocalization studies. Animals that received saline (n = 5) or IL-1β injections (n = 6) were handled daily for 1 week prior to the experiment.

Administration of IL-1β

IL-1β (human, recombinant) was obtained from Bachem (Torrance, CA). The manufacturer's specifications stated that endotoxin levels were < 0.1 ng per μg of IL-1β. The specific activity was approximately 5×10^7 U/mg protein. Animals were given an intraperitoneal (i.p.) injection of IL-1β, (5 μg/ml/kg; n = 6) or an equivalent volume of 0.9% sterile, pyrogen-free saline (n = 5) and were returned to their home cage. Injections were given 2–3 hours after the onset of the light period.

Tissue preparation

Animals were rapidly decapitated directly after being removed from the home cage for naïve rats or 30 minutes after injection of IL-1β or saline. Brains were removed and frozen in isopentane cooled to between −40°C and −50°C and were stored at −80°C. Sections (10 μm thick) were cut on a cryostat (Bright, Huntingdon, United Kingdom) through the BST (approximately 0.20 to −0.80 mm from Bregma) and CEA (approximately −1.80 to −3.60 mm from Bregma) and thaw mounted onto polylysine-coated slides. Tissue was air dried and stored at −80°C until processing for ISH.

Double ISH histochemistry

The method used for double ISH was adapted from that described by Curran and Watson (1995). The specificity of the probes was confirmed in control experiments by using sense probes or tissue that had been pretreated with RNase A (200 μg/ml) for 1 hour at 37°C prior to hybridiza-

tion with antisense probes. No specific hybridization was observed for any of the controls.

Probes

cRNA probes complementary to c-fos (680 mer; courtesy of Dr. T. Curran, St. Jude Children's Research Hospital, Memphis, TN), glutamic acid decarboxylase 65 (GAD65; 230 mer; courtesy of Dr. N.J.K. Tillakaratne, University of California), GAD67 (210 mer; courtesy of Dr. A. Tobin, University of California-Los Angeles), ENK (693 mer; courtesy of Dr. J. Douglass, Amgen, Inc., Thousand Oaks, CA), NT (587 mer; courtesy of Dr. P. Dobner, University of Massachusetts), and CRH (770 mer; courtesy of Dr. R. Thompson, University of Michigan) were used.

To generate ^{35}S-labeled cRNA probes, linearized plasmid (1 μg) was incubated at 37°C for 2 hours in 1 × transcription buffer (Gibco BRL, Grand Island, NY), 75 μCi α-[^{35}S]-UTP (≥ 1,000 Ci/mmol; 20 mCi/ml; Amersham, Arlington Heights, IL), 100 μCi α-[^{35}S]-CTP (800 Ci/mmol; 40 mCi/ml; Amersham), 400 μM ATP, 400 μM GTP, 10 mM dithiothreitol (DTT), 20 U RNase inhibitor, and 6 U appropriate RNA polymerase. To generate a nonradioactive labeled probe, digoxigenin-UTP (Dig-UTP; Boehringer Mannheim, Indianapolis, IN) was used. Linearized plasmid (1 μg) was incubated at 37°C for 2 hours in 1 × transcription buffer, 400 μM ATP, 400 μM CTP, 400 μM GTP, 80–260 μM UTP, 140–320 μM Dig-UTP (total UTP + Dig-UTP = 400 μM), 10 mM DTT, 20 U RNase inhibitor, and 6 U appropriate RNA polymerase. The resulting probes were separated from free nucleotides on a Sephadex G50–50 column. The fractions containing Dig-UTP-labeled probe were determined by performing a small-scale color reaction (see below) on 1 μl each fraction blotted onto Nytran membrane.

Before hybridization, brain sections were placed in 4% phosphate-buffered paraformaldehyde and fixed for 1 hour at room temperature. Sections were rinsed three times in 2 × standard saline citrate (SSC; 1 × SSC = 150 mM sodium chloride and 15 mM sodium citrate). Slides were then placed in a solution of 0.1 M triethanolamine with 0.25% acetic acid for 10 minutes. The tissue was rinsed in water and dehydrated through a series of alcohols.

Dig-UTP- and [^{35}S]-labeled probes were diluted together in hybridization buffer (50% formamide; 10% dextran sulfate; 3 × SSC; 50 mM sodium phosphate buffer, pH 7.4; 1 × Denhardt's solution; 0.1 mg/ml yeast tRNA; and 10 mM DTT) to yield an approximate concentration of 2–2.5 × 10^6 c.p.m./65 μl. Appropriate dilutions for nonradioactive probes were estimated from pilot experiments. The following probe combinations were run for the BSTov and the CEAl: Dig-GAD 65/67 (used concomitantly as a marker for GABAergic neurons) with [^{35}S]-ENK, [^{35}S]-NT, or [^{35}S]-CRH in naïve animals; Dig-ENK, Dig-NT, or Dig-CRH with [^{35}S]-GAD 65/67 in naive animals; Dig-ENK with [^{35}S]-NT or [^{35}S]-CRH in naive animals; Dig-c-fos with [^{35}S]-GAD 65/67, [^{35}S]-ENK, [^{35}S]-NT, or [^{35}S]-CRH in animals treated with IL-1β, 5 μg/kg i.p. Diluted probe (65 μl) was placed on each slide, and the sections coverslipped. Slides were placed in plastic trays lined with filter paper dampened with 50% formamide/50% water. Trays were sealed and incubated at 55°C for 16 hours. Coverslips were floated off in 2 × SSC, and slides were rinsed an additional three times in 2 × SSC. Sections were incubated in RNase A (200μg/ml) at 37°C for 60 minutes and rinsed in 2 × SSC, 1 × SSC, 0.5 × SSC, and 0.1 × SSC. Tissue was washed to a final stringency of 0.1 × SSC at 65°C for 60 minutes Slides were then cooled to room temperature in 0.1× SSC.

After this posthybridization treatment, sections were processed for detection of the nonradioactive probe. Sections were rinsed in 0.1 M phosphate buffer, pH 7.4, and incubated for 2–4 hours at room temperature in a blocking solution (0.25% carrageenan, 0.5% Triton-X 100, 0.1 M phosphate buffer, pH 7.4). Sections were then incubated at room temperature overnight with an antibody against Dig conjugated to alkaline phosphatase (sheep anti-Dig-AP, Fab fragments; Boehringer Mannheim) diluted 1:20,000 in blocking solution. Sections were then washed three times in 0.1 M phosphate buffer, twice in Tris-buffered saline, and once in alkaline substrate buffer (ASB; 100 mM Tris base, 50 mM NaCl, 50 mM $MgCl_2$, pH 9.5). The color reaction was carried out in the dark at room temperature in ASB containing 5% polyvinyl alcohol (Sigma, St. Louis, MO), 0.025% levamisole (Sigma), 0.45% 4-nitro blue tetrazolium chloride (NBT; Boehringer Mannheim) and 0.35% 5-bromo-4-chloro-3-indoyl-phosphate, 4-toluidine salt (BCIP; Boehringer Mannheim). The color reaction took 6–24 hours, depending on the probe, and sections were examined under a microscope to determine reaction completion. Slides were then washed extensively in water. Sections were incubated in 0.1 M glycine and 0.5% Triton-X 100, pH 2.2, for 10 minutes at room temperature to remove the antibody (Hauptmann and Gerster, 1994) and then washed in water. Finally, sections were fixed in 2.5% glutaraldehyde for 1 hour, washed in water, and air dried. The glycine and glutaraldehyde steps were found to help prevent the increase in color background observed after the development of emulsion dipped slides (see below). Sections initially were exposed to x-ray film (Biomax-MR; Eastman-Kodak, Rochester, NY) for 1–3 days, then dipped in emulsion (Ilford KD-5; Polysciences, Warrington, PA), and stored in light-tight boxes at 4°C for 2–14 days. After development (Kodak D-19) of dipped slides, sections were either coverslipped with an aqueous mounting medium (Polysciences), which enabled the visualization of white matter tracts that were used as anatomical landmarks, or dehydrated in alcohols and then mounted in a xylene-based mounting medium (Permount) for photomicrographs.

Data analysis

The cellular distribution of different mRNA combinations from dual ISH experiments was determined by using a Leica microscope (Leitz DMR; Wetzlar, Germany). Nonradioactive riboprobes were visualized under brightfield as a blue-purple precipitate, whereas radioactive probes were visualized under darkfield by using silver grain distribution. Sections at 60–160 μm intervals from three to six animals per group were analyzed throughout the rostral-caudal extent of the BSTov and the CEAl. Cell profile counts were determined at ×40 magnification with the aid of an eye-piece grid (500 μm × 500 μm divided into 100 squares). No attempt was made to establish absolute numbers of cells within these structures. Rather, the numbers of cell profiles counted for each animal were used to calculate the relative percent colocalization of mRNA for that animal. The mean ± S.E.M. percent colocalization for each probe combination with respect to the nonradioactive probe (see Technical considerations, below) is reported in

TABLE 1. Coexpression of Enkephalin, Neurotensin, Corticotropin-Releasing Hormone, and Glutamic Acid Decarboxylase 65/67 mRNAs in the Oval Nucleus of the Bed Nucleus of the Stria Terminalis and the Lateral Division of the Central Nucleus of the Amygdala[1]

Dig-marker	[35S]marker	Dig only (no. cells)	[35S] only (no. cells)	Doubles (no. cells)	Double w.r.t. Dig (%)	S.E.M.	No.
BSTov							
Expression of GAD65/67 mRNA in cells containing ENK, NT, or CRH mRNA							
ENK	GADs	0	nc	781	100	0	3
NT	GADs	0	nc	151	100	0	3
CRH	GADs	0	nc	427	100	0	4
Expression of ENK, NT, or CRH mRNA in cells expressing GAD65/67 mRNA							
GADs	ENK	632	nc	432	41	3.1	3
GADs	NT	505	98	360	43	4.1	3
GADs	CRH	1,913	142	587	24	3.2	3
Expression of NT or CRH mRNA in cells expressing ENK mRNA[2]							
ENK	NT	900	321	103	10	0.9	3
ENK	CRH	947	382	10	1	0.4	3
CEAl[3]							
Expression of GAD65/67 mRNA in cells containing ENK, NT, or CRH mRNA							
ENK	GADs	0	2,045	4,507	100	0	4
NT	GADs	0	nc	309	100	0	4
CRH	GADs	0	nc	464	100	0	4
Expression of ENK, NT, or CRH mRNA in cells expressing GAD65/67 mRNA							
GADs	ENK	1,639	nc	5,328	77	4.1	4
GADs	NT	2,450	215	1,116	32	0.9	3
GADs	CRH	2,241	161	698	24	1.5	3
Expression of NT or CRH mRNA in cells expressing ENK mRNA[2]							
ENK	NT	6,777	1,425	629	9	1.0	5
ENK	CRH	2,581	729	13	0.5	0.1	4

[1]Total cell counts for double in situ hybridization experiments in the oval nucleus of the bed nucleus of the stria terminalis (BSTov) and the lateral division of the central nucleus of the amygdala (CEAl) for the expression of mRNA encoding glutamic acid decarboxylase isoforms 65 and 67 (GADs) in cells expressing enkephalin (ENK), neurotensin (NT), or corticotropin-releasing hormone (CRH) mRNA; and the expression of mRNAs encoding the peptides ENK, NT, or CRH in cells expressing GAD65/67 mRNA. Note that "no. cells" represents the *total* number of cells counted for all animals. The percent of colocalization was calculated relative to the digoxigenin (Dig) probe for each animal separately, according to the following formula: no. doubles ÷ (no. doubles + no. Dig-only) × 100. The mean percentage colocalization relative to the Dig-labeled probe was calculated from these values. w.r.t., with respect to; S.E.M., standard error of the mean; nc, not counted.
[2]The expression of mRNAs encoding the peptides NT or CRH in ENK mRNA-containing neurons.
[3]Note that cell counts were taken within the area of peak expression for each peptide mRNA, but there was significant variation in the percentage of colocalization across the region due to the differential mRNA distribution.

TABLE 2. Coexpression of c-fos mRNA With Enkephalin, Neurotensin, Corticotropin-Releasing Hormone, or Glutamic Acid Decarboxylase 65/67 mRNAs in the Oval Nucleus of the Bed Nucleus of the Stria Terminalis and Lateral Division of the Central Nucleus of the Amygdala 30 Minutes after Interleukin-1β (5 μg/kg, i.p.)[1]

Dig-marker	[35S]marker	Dig only (no. cells)	[35S] only (no. cells)	Doubles (no. cells)	Double w.r.t. Dig (%)	S.E.M.	No.
BSTov							
Fos	GADs	3	1,791	769	100	0.1	4
Fos	ENK	110	1,767	473	81	1.4	4
Fos	NT	401	1,043	119	23	0.5	4
Fos	CRH	787	1,316	34	4	1.4	4
CEAL							
Fos	GADs	0	nc	4,168	100	0	6
Fos	ENK	133	nc	2,168	94	1.3	4
Fos	NT	1,814	2,012	468	22	1.8	5
Fos	CRH	1,728	806	17	1	0.5	3

[1]Total cell counts for double in situ hybridization experiments in the oval nucleus of the bed nucleus of the stria terminalis (BSTov) and the lateral division of the central nucleus of the amygdala (CEAl) for the expression of glutamic acid decarboxylase isoforms 65 and 67 (GADs), enkephalin (ENK), neurotensin (NT), or corticotropin-releasing hormone (CRH) mRNA in c-fos mRNA-positive cells 30 minutes after the administration of interleukin-1β (5 μg/kg, i.p.). Note that "no. cells" represents the *total* number of cells counted for all animals. The percent of colocalization was calculated relative to the digoxigenin (Dig) probe for each animal separately according to the following formula: no. doubles ÷ (no. doubles + no. Dig-only) × 100. The mean percentage colocalization relative to the Dig-labeled probe was calculated from these values. w.r.t., with respect to; nc, not counted.

Tables 1 and 2. The cell numbers reported in Tables 1 and 2 reflect the *total* numbers of cell profiles counted for all animals combined. The grid was centered over the area of peak expression of mRNA labeled with the nonradioactive probe within the boundaries of the nucleus. Neuroanatomical boundaries of the BSTov, CEAl, medial CEA (CEAm), and capsular CEA (CEAc) were determined according to the atlas of Swanson (1992) by using white matter tracts as a guide. It should be noted that the type of tissue processing involved with the dual ISH technique precluded further regional compartmentalization within the CEA. Both the left and right BSTov were included in the analysis. For the CEAl, analysis was limited to the right side. Previous quantitative results did not indicate differences in c-fos mRNA expression between the left and right sides (Day and Akil, 1996), and results from the current study did not suggest qualitative differences in the neurochemical identity of activated cells between the two sides.

Photography and image processing

With the exception of Figure 5, photomicrographs were taken by using a Zeiss Axiophot microscope (Thornwood, NY), using Kodak Tmax-100 film (for darkfield images) or Kodak Ektachrome Tungsten film (for brightfield and double-exposure dual ISH photomicrographs). Slides and negatives were converted to digital form by using a Polaroid Sprint Scanner and Adobe Photoshop software (version 4.0; Adobe Systems, Mountain View, CA) on a Macintosh computer (Apple Computers, Cupertino, CA). Images were adjusted by using one "sharpen" filter and increased brightness and contrast to allow photographic-quality prints to be generated. Color images also under-

went a color transformation (image: variations: "more blue") to provide optimal contrast between silver grains, Dig-labeled cells, and background. Each image in a composite set was processed in an identical manner. Figure 5 was generated digitally. Brightfield and darkfield images were captured separately with a Sony CCD video camera (DXC-970MD; Sony Corp., Tokyo, Japan) attached to a Leica (Leitz DMR) microscope. Brightfield and darkfield images were then overlaid to produce a single image by using the MCID image-analysis system (MCID, Ontario, Canada). The composite was formed within Adobe Photoshop. Brightness and contrast were altered to the same degree for each picture within the composite to generate a photographic-quality print. Schematic diagrams were adapted from the atlas of Swanson (1992) by using Adobe Illustrator (version 7.0; Adobe Systems).

RESULTS

Initial studies indicated that the nonradioactive ISH technique was less sensitive than the radioactive ISH technique, in that the numbers of cells observed to be labeled after hybridization with a Dig-labeled probe were less than those observed with a similar [^{35}S]-labeled probe. For example, sections from the striatum of one animal were labeled concurrently with Dig-ENK and [^{35}S]-ENK (Fig. 1A). Every cell that was positive for Dig-ENK also was positive for [^{35}S]-ENK (485 cells counted). In contrast, only 94% of cells that were positive for [^{35}S]-ENK also were positive for Dig-ENK (33 cells were positive for [^{35}S]-ENK alone). Hence, the results of the current study are expressed with respect to the Dig-labeled probe (Tables 1, 2). For probe combinations in which results were required with respect to both probes, two series of sections were analyzed; for example, Dig-ENK with [^{35}S]-GAD65/67 and Dig-GAD65/67 and [^{35}S]-ENK.

Expression of peptide mRNA in GABAergic neurons of the BSTov and CEAl

The general patterns of expression of CRH, ENK, NT, GAD65, and GAD67 mRNAs were consistent with previous reports (Harlan et al., 1987; Alexander et al., 1989; Esclapez et al., 1994; Makino et al., 1994). All of these mRNAs were expressed within the BSTov (Fig. 2) and the

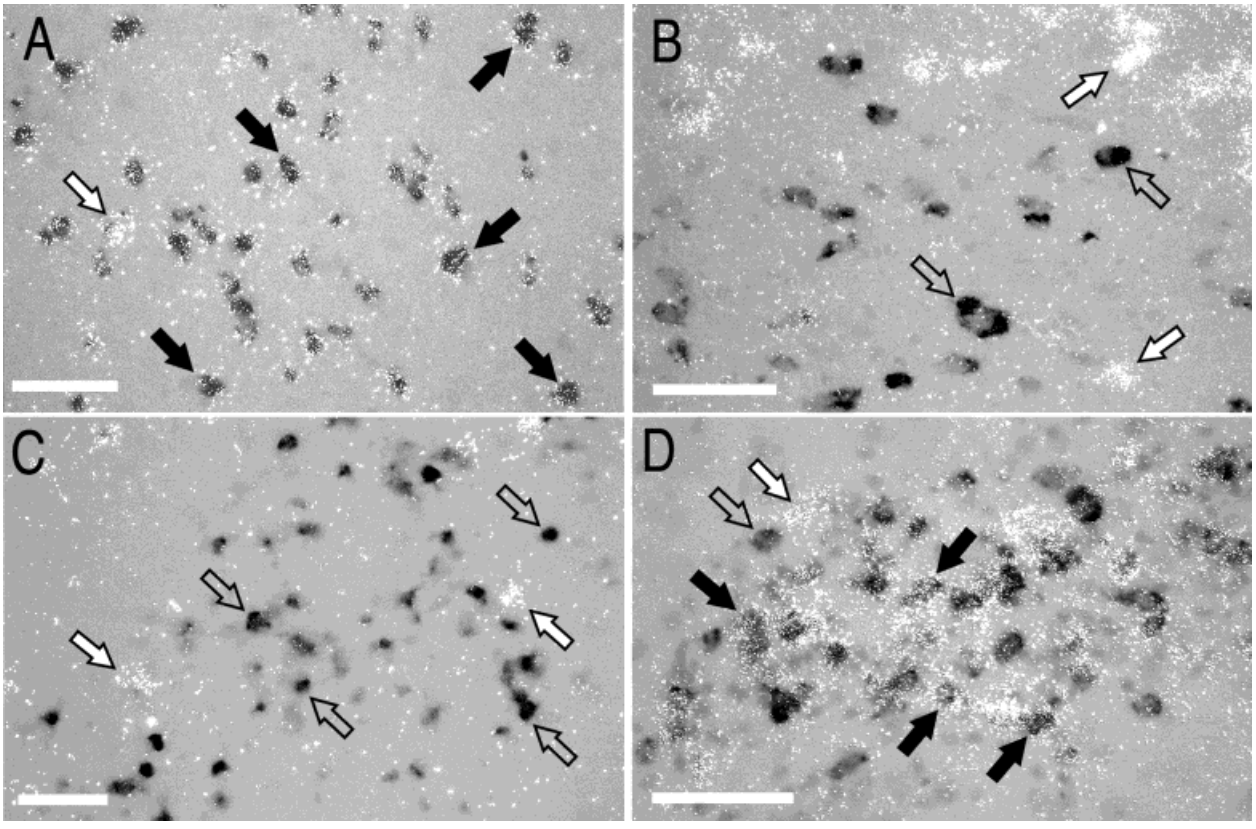

Fig. 1. Photomicrographs (double exposure) of control double in situ hybridization experiments using the following probes: **A:** Digoxigenin-enkephalin (Dig-ENK) + [^{35}S]-ENK in the striatum demonstrating the extent of colocalization of the two labeled probes with one another. **B:** Dig-ENK + [^{35}S]-glutamic acid decarboxylase 65/67 (GAD65/67) in the paraventricular nucleus of the hypothalamus (PVH) demonstrating the lack of colocalization of ENK and γ-aminobutyric acid (GABA) in this nucleus compared with that observed in the oval nucleus of the bed nucleus of the striatum (BSTov) and the lateral division of the central nucleus of the amygdala (CEAl). **C:** Dig-Fos + [^{35}S]-GAD65/67 in the PVH 30 minutes after interleukin-1β (IL-1β; 5 μg/kg, i.p.) demonstrating the lack of colocalization of c-fos mRNA and GABA in this nucleus compared with that observed in the BSTov and CEAl. **D:** Dig-Fos + [^{35}S]-corticotropin-releasing hormone (CRH) in the PVH 30 minutes after IL-1β (5 μg/kg, i.p.) demonstrating the significant colocalization of c-fos and CRH mRNA in this nucleus compared with that observed in the BSTov and CEAl. Open arrows indicate cells hybridized with the Dig-labeled probe only. Solid white arrows indicate cells hybridized with the [^{35}S]-labeled probe only. Solid black arrows indicate double-labeled cells. Scale bars = 50 μm.

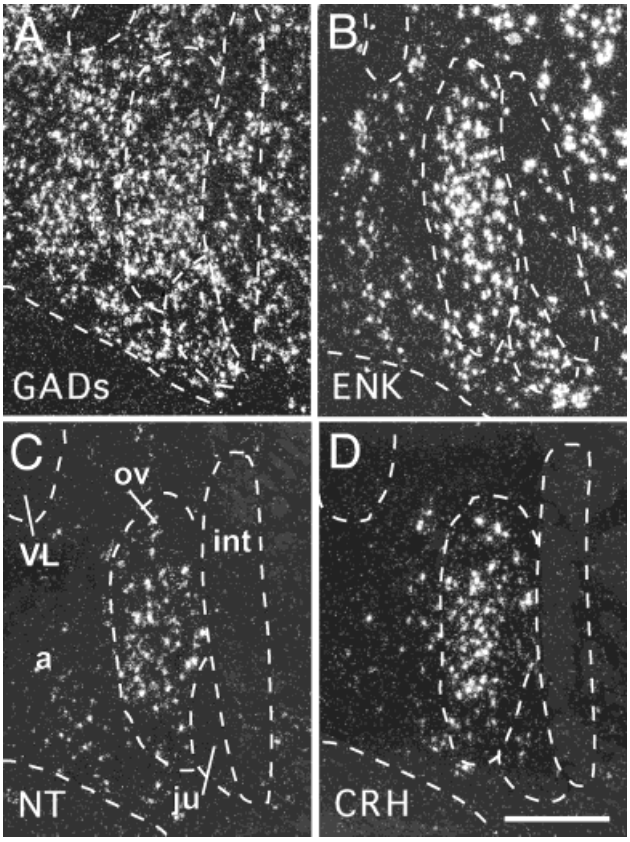

Fig. 2. Distribution of mRNA encoding GAD65/GAD67 (**A**), ENK (**B**), neurotensin (NT; **C**), and CRH (**D**) in the BSTov. Dashed lines delineate anatomical subdivisions as indicated in C. Divisions of the bed nucleus of the stria terminalis (BST): a, anterior; ju, juxtacapsular nucleus; ov, oval nucleus; int, internal capsule; VL, lateral ventricle. Scale bar = 200 μm.

CEAl (Fig. 3), with GAD65/67 and ENK mRNAs more abundant than CRH and NT mRNAs. GABAergic and peptidergic neurons exhibited specific patterns of expression within these nuclei. GAD65 and GAD67 mRNAs were observed throughout the BST and CEA, with high levels in both the lateral and medial divisions (Figs. 2B, 3B). Expression of ENK mRNA was high throughout the BSTov (Fig. 2C) and the CEAl (Fig. 3C), but some labeled cells also were observed in the CEAm. Expression of NT mRNA was moderate in the BSTov and CEAl (Figs. 2D, 3D). Expression of NT mRNA was highest in the dorsal and medial aspects of the CEAl, with some cells also observed in the CEAm (Fig. 3D). For CRH mRNA, expression within the BSTov was greater toward the medial extent of this nucleus (Fig. 2E). Within the CEA, CRH mRNA was localized mainly to the lateral division, with expression greatest within the central region of the CEAl (Fig. 3E). Expression of CRH mRNA was observed throughout the

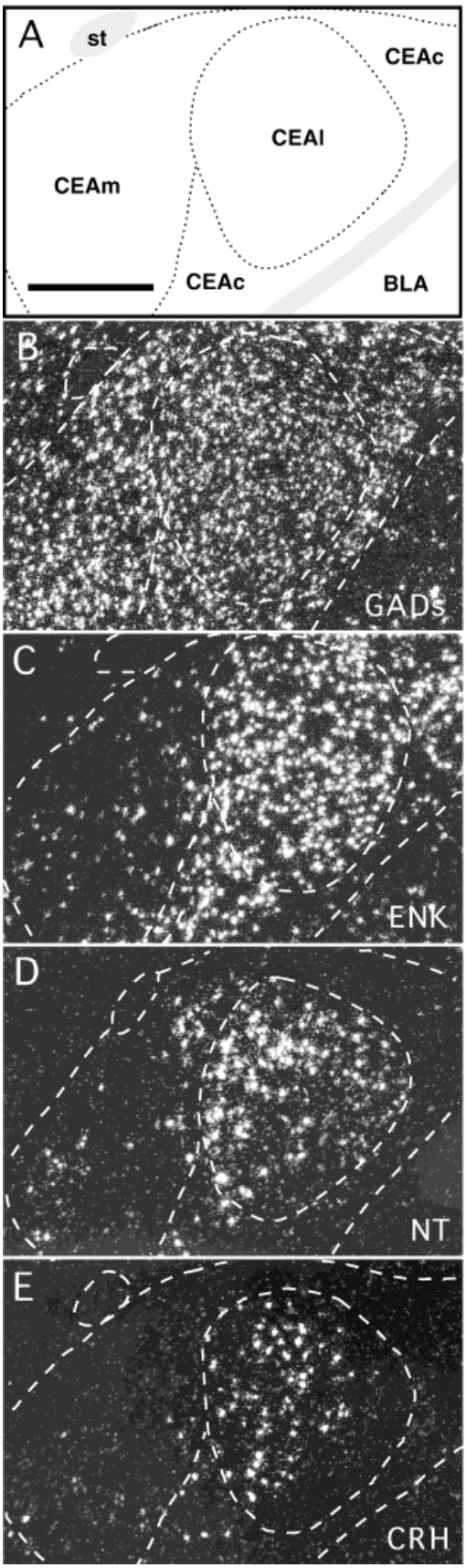

Fig. 3. **A:** Schematic diagram of the CEA. **B–E:** Distribution in the CEA of mRNAs encoding GAD65/GAD67 (B), ENK (C), NT (D), and CRH (E). Dashed lines delineate anatomical subdivisions as indicate in A. CEAl, lateral division of the CEA; CEAm, medial division of the CEA; CEAc, capsular division of the CEA; st, stria terminalis; BLA, basolateral amygdaloid nucleus. Scale bar = 300 μm.

rostral-caudal extent of the CEAl but was highest at midlevels, caudal to the paraventricular nucleus of the hypothalamus (PVH).

Within the CEAl and BSTov, every cell that expressed either CRH, ENK, or NT mRNA (Dig-labeled probe) also expressed GAD65/67 mRNA ([^{35}S]-labeled probe; Fig. 4). The 100% colocalization of the peptide mRNAs with GAD65/67 is unlikely to be due to a nonspecific interaction of the probes. For example, within the reticular nucleus of the thalamus, GAD65/67 mRNA did not colocalize with either CRH, ENK, or NT mRNA (data not shown). Furthermore, within the PVH, neither CRH nor ENK mRNA was coexpressed with GAD65/67 mRNAs (shown for Dig-ENK with [^{35}S]-GAD65/67 in Fig. 1B). However, not all GABAergic cells (Dig-labeled GAD65/67 mRNA) also contained a particular peptide mRNA ([^{35}S]-labeled probe). The percentage of colocalization varied, particularly across the CEA, because of the differential distributions of each of the peptide mRNA species. However, the greatest colocalization for both the BSTov and the CEAl was observed for ENK, with up to 75% of GABAergic neurons containing this peptide (BSTov: 41% ± 3.1%; n = 3; CEAl: 77% ± 2.1%; n = 4). Fewer GABAergic cells expressed NT or CRH. Within the area of peak expression of CRH in both the BSTov and the CEAl, ≈25% of neurons that were positive for GAD65/67 also expressed CRH mRNA (BSTov: 24% ± 3.2%; n = 3; CEAl: 24% ± 1.5%; n = 3). The ratio was slightly higher at the peak of NT expression (BSTov: 43% ± 4.1%; n = 3; CEAl: 32% ± 0.9%; n = 3). The expression of NT or CRH in ENK-containing cells revealed a highly specific pattern of colocalization (Fig. 5). There was some colocalization of NT in ENK mRNA-containing cells (BSTov: 10% ± 0.9%; n = 3; Fig. 5A; CEAl: 9 ± 1.0%; n = 5; Fig. 5B). However, extremely few ENK-positive cells

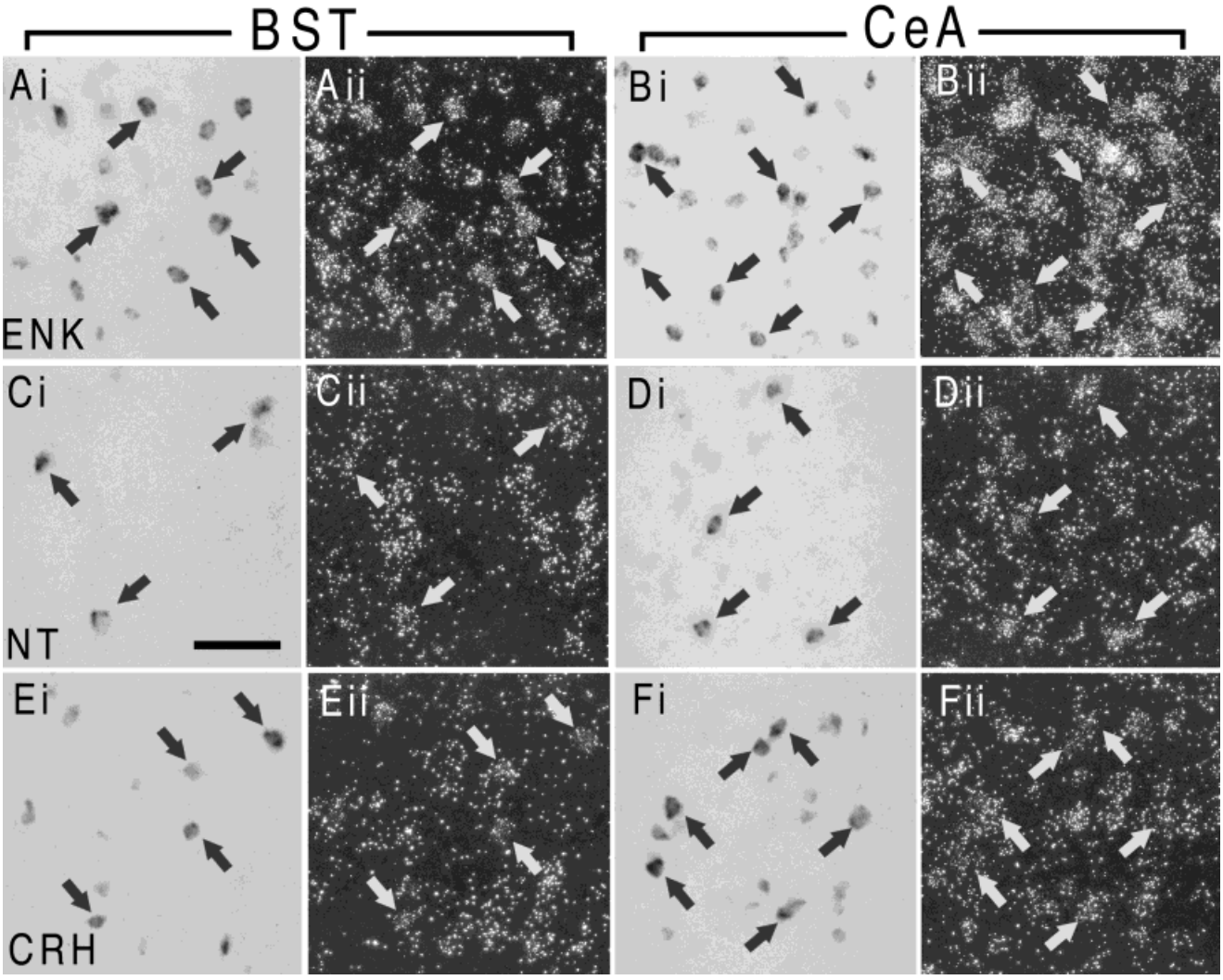

Fig. 4. **A–F:** Photomicrographs (single exposures) of double in situ hybridization experiments showing the colocalization of GAD65 and GAD67 mRNA ([^{35}S]-labeled probe; Aii–Fii) with various peptides (Dig-labeled probe; Ai–Fi) in the BSTov (Ai,Aii; Ci,Cii; Ei,Eii) and the CEAl (Bi,Bii; Di,Dii; Fi,Fii). A: Dig-ENK + [^{35}S]-GAD65/67 in the BSTov. B: Dig-ENK + [^{35}S]-GAD65/67 in the CEAl. C: Dig-NT + [^{35}S]-GAD65/67 in the BSTov. D: Dig-NT + [^{35}S]-GAD65/67 in the CEAl. E: Dig-CRH + [^{35}S]-GAD65/67 in the BSTov. F: Dig-CRH + [^{35}S]-GAD65/67 in the CEAl. Dark arrows indicate cells expressing peptide mRNA (Dig-labeled probe). Light arrows indicate the same cells labeled for [^{35}S]-GAD65/67 mRNA. Every cell that expressed either ENK, CRH, or NT also expressed GAD65/67 mRNA. Scale bar = 50 μm.

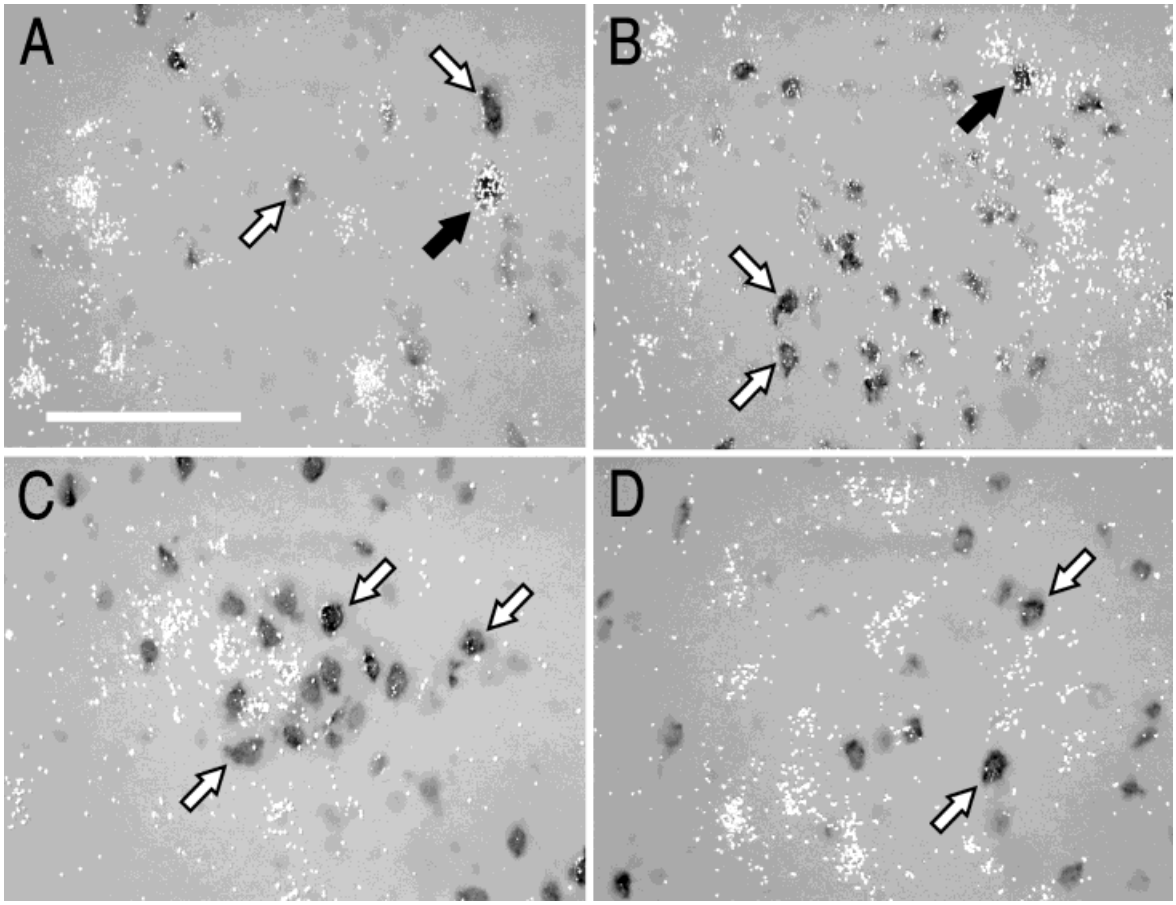

Fig. 5. Photomicrographs (double exposure) of double in situ hybridization experiments showing the extent of colocalization of ENK mRNA (Dig-labeled probe) with NT (**A,B**) or CRH (**C,D**) mRNAs ([^{35}S]-labeled probes) in the BSTov (A,C) and the CEAl (B,D). White arrows indicate cells hybridized with the Dig-labeled probe only. Black arrows indicate double-labeled cells. Scale bar = 100 µm.

expressed CRH mRNA (BSTov: 1.2% ± 0.4%; n = 3; Fig. 5C; CEAl: 0.5% ± 0.1%; n = 4; Fig. 5D).

Expression of c-fos mRNA in neurochemically defined neurons of the BSTov and CEAl

A marked increase in c-fos mRNA expression was observed in the BST (Fig. 6) and the CEA (Fig. 7) 30 minutes after administration of IL-1β (5 µg/kg, i.p.) compared with saline-injected controls, consistent with results from previous studies (Brady et al., 1994; Day and Akil, 1996). Within the BST, expression was highly discrete, with the majority of labeled cells observed within the BSTov. Furthermore, expression of c-fos mRNA within the BSTov was most abundant toward the lateral extent of this nucleus. Expression of c-fos mRNA within the CEA was localized essentially to the CEAl, with very few labeled cells observed in the CEAm or CEAc. Expression of c-fos mRNA was observed throughout the CEAl but was strongest in the ventrolateral region, caudal to the PVH.

The IL-1β-induced expression of c-fos mRNA in cells of the BSTov and CEAl was determined with respect to colocalization in GABAergic (as defined by the expression of GAD65 and/or GAD67 mRNA) or peptidergic (CRH, ENK, or NT mRNA-containing) cells. A striking parallel was noted between the neurochemical identity of Fos-positive cells in the BSTov and CEAl, as shown in Figures 8 and 9. Cells that expressed c-fos mRNA in either the BSTov (Figs. 8A, 9) or the CEAl (Figs. 8B, 9) invariably expressed GAD65/67 mRNA (BSTov, n = 4; CEAl, n = 6). The 100% colocalization of c-fos mRNA in GABAergic cells was specific for these nuclei. Other areas that expressed GAD65/67 mRNA, for example, the reticular thalamic nucleus, did not express c-fos mRNA (data not shown), and c-fos mRNA-containing cells of the PVH did not express GAD65/67 mRNA (Fig. 1C). In addition, in both the BSTov (Figs. 8C, 9) and the CEAl (Figs. 8D, 9), the majority of cells expressing c-fos mRNA in the BSTov (81% ± 1.4%; n = 4) and CEAl (94% ± 1.3%; n = 4) also expressed ENK mRNA. A smaller population of cells positive for c-fos mRNA in the BSTov (Figs. 8E, 9) and CEAl (Figs. 8F, 9) expressed NT mRNA (BSTov: 23% ± 0.5%; n = 4; CEA: 22% ± 1.8%; n = 5). Very few cells that expressed c-fos mRNA in the BSTov (Figs. 8G, 9) and CEAl (Figs. 8H, 9) also expressed CRH mRNA (BSTov: 4% ± 1.4%; n = 4; CEA: 1% ± 0.5%; n = 3). This result is in contrast to that observed for CRH and c-fos mRNAs in the PVH, where there is substantial overlap of expression (Fig. 1D), in agreement with previous observations after intravenous infusion of IL-1β (Veening et al., 1993; Ericsson et al., 1994).

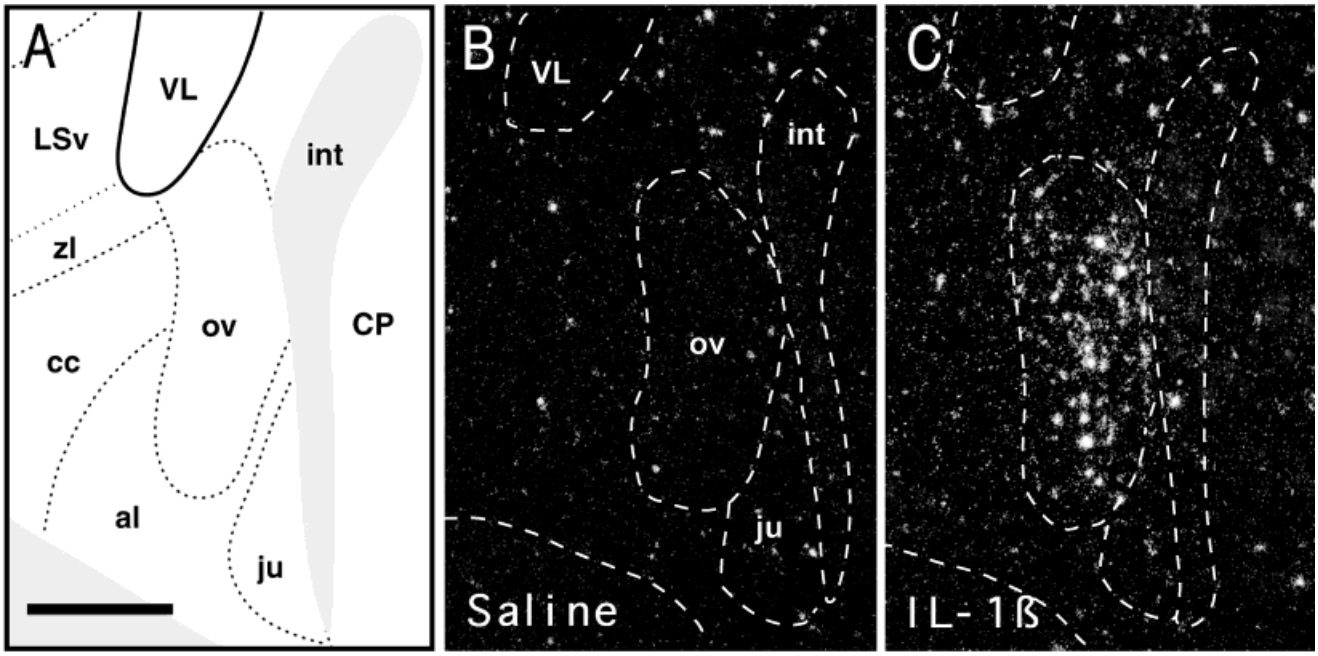

Fig. 6. **A:** Schematic diagram of the BSTov. **B,C:** Distribution of c-fos mRNA in the BSTov 30 minutes after administration of 0.9% saline, i.p.(B), or IL-1β (5 μg/ml/kg i.p.; C). Dashed lines delineate anatomical subdivisions as indicated in B. Divisions of the anterior BST: al, anterolateral area; cc, central core, anterodorsal area; ju, juxtacapsular nucleus; ov, oval nucleus; CP, caudate putamen; int, internal capsule; LSv, lateral septal nucleus, ventral part; VL, lateral ventricle; zl, zona limitans. Scale bar = 200 μm.

DISCUSSION

The data presented in the current study provide evidence for neurochemically distinct subsets of cells in the BSTov and CEAl and suggest that these subpopulations may be activated differentially by systemic IL-1β. The mRNA encoding for the peptides ENK, NT, and CRH invariably is expressed in GABAergic cells in these regions. Although there is some colocalization of ENK and NT mRNAs, enkephalinergic cells rarely express CRH mRNA in either the BSTov or the CEAl. The demonstration that systemic administration of IL-1β causes the robust activation of enkephalinergic cells but extremely sparse activation of CRH-containing cells (as determined by c-fos mRNA expression) suggests that these neurochemically defined neuronal subsets of the BSTov and CEAl may have differential functions. Furthermore, the proportion of neurochemically similar and functionally activated cells showed remarkable similarities between the BSTov and the CEAl, consistent with the concept of an extended amygdala.

Technical considerations

In general, the dual-ISH method is a powerful tool for studying the colocalization of two molecules in a single neuron. This technique has the advantage over some dual-immunocytochemistry methods, because it can be used in animals without the severe stress of colchicine pretreatment. However, for each combination of probes, a number of considerations must be taken into account before results can be validated. For example, tissue with different levels of expression should be used to control for nonspecific probe interactions in cases in which a high degree of colocalization is observed and to control for quenching when a low degree of colocalization is observed. Specific examples are described in the Results (above) and are illustrated in Figure 1. Furthermore, a particular consideration lies in the fact that the nonradioactive ISH technique is less sensitive than the radioactive ISH technique, so that the numbers of cells observed to be labeled after hybridization with a Dig-labeled probe are less than those observed with a similar [^{35}S]-labeled probe. Because of this technical limitation, the results expressed relative to the [^{35}S]-labeled probe are misleading, because they underestimate the proportion of double-labeled cells. Hence, data from the current study were expressed with respect to the Dig-labeled probe. It also should be noted that a cell profile count methodology was used to generate these data, which inherently leads to a biased count of absolute cell numbers (Coggeshall and Lekan, 1996). However, we did not attempt to determine the absolute numbers of cells, and this counting method is suitable to estimate the approximate proportion of cells expressing a particular mRNA (Saper, 1996). Caution also must be exercised when interpreting c-fos mRNA expression and, more specifically, the lack of expression of this gene in different cell populations. In particular, the lack of expression in CRH mRNA-containing cells is discussed in detail below. Finally, it should be kept in mind that a single dose of IL-1β, and a single time point was used in the present study. The experimental conditions, as discussed in more detail below, were chosen to elicit a robust increase in c-fos mRNA expression at the time of peak expression. Although a dose-response curve was not run in the present experiment, the dose used was comparable to that used in several other studies. For example, studies to establish the role of vagal afferents after intraperitoneal administration of IL-1β have used doses of 2 μg/kg i.p. IL-1β (Watkins et

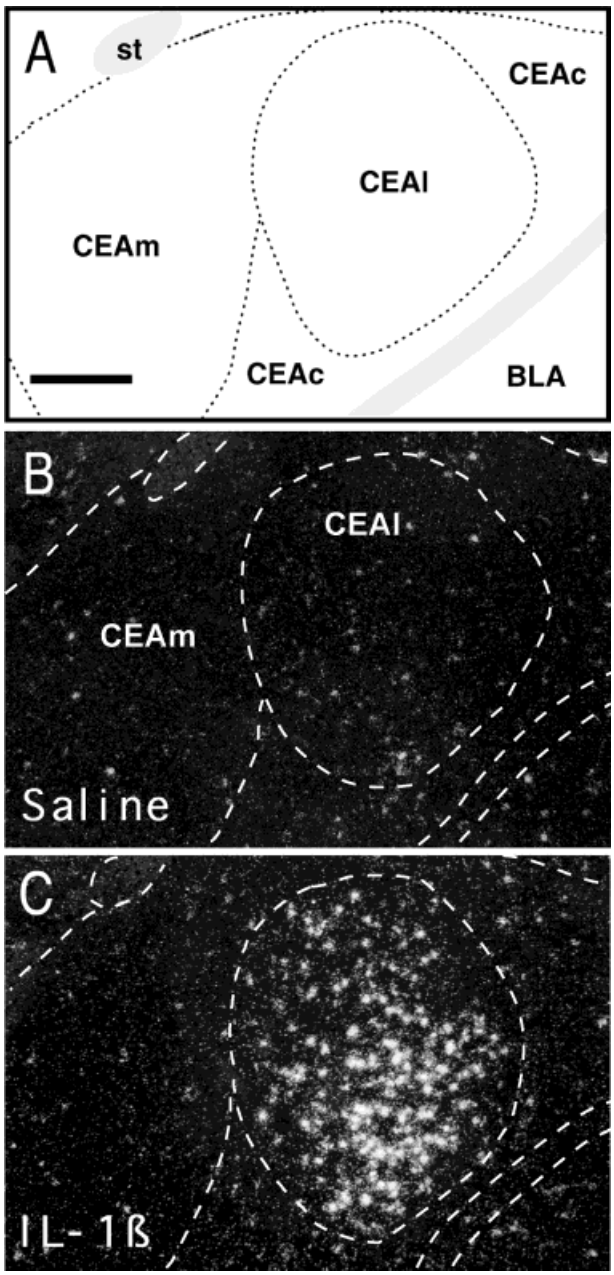

Fig. 7. **A:** Schematic diagram of the central nucleus of the amygdala (CEA). **B,C:** The distribution of c-fos mRNA 30 minutes after administration of 0.9% saline, i.p. (B) or IL-1β (5 μg/ml/kg, i.p.; C). Dashed lines delineate anatomical subdivisions as indicated in A. For abbreviations, see Figure 3. Scale bar = 200 μm.

al., 1995), 10 μg/kg i.p. IL-1β (Fleshner et al., 1995), and 4 μg/kg or 20 μg/kg i.p. IL-1β (Kapcala et al., 1996).

Neurochemical determination of subpopulations of neurons of the BSTov and CEAl

It has long been known that the BSTov and CEAl are highly GABAergic (Sun and Cassell, 1993) and that opioid peptide-like immunoreactivity is localized in GABAergic neurons of the rat CEA (Oertel et al., 1983). More recently, a study using immunocytochemistry on adjacent sections suggested that GABAergic neurons of the central extended amygdala synthesize either CRH or ENK (Veinante et al., 1997). However, the extent of colocalization of different peptides in GABAergic cells has not been demonstrated previously. Data presented in this paper suggest that every cell that expresses ENK, NT, or CRH mRNA in the BSTov and CEAl is GABAergic. However, as expected, not all GABAergic cells in these regions express the mRNA encoding these peptides. In addition, it is important to note the widespread expression of GAD65/67 mRNA observed in the medial division of the CEA, the major source of CEA efferent projections. In keeping with this, it has been demonstrated recently that efferents from the CEA form inhibitory type synapses with neurons in the rat NTS (Pickel et al., 1995) and that the CEA projection to the medulla is mostly GABAergic (Jia et al., 1997). Together, these data suggest that the major output of the CEA may be inhibitory rather than excitatory. Within the BSTov and CEAl, ≈ 10% enkephalinergic neurons were found to coexpress NT mRNA, whereas enkephalinergic neurons rarely expressed CRH mRNA. Although we did not investigate the overlap between NT and CRH mRNAs in the present study, a previous study has shown that 80% of NT cells in the BSTov and 80–90% of NT cells in the CEAl were immunoreactive for CRH (Shimada et al., 1989). Hence, there appear to be two major subpopulations of GABAergic neurons: one containing CRH and one containing ENK, with NT expressed to differing extents within these neurons.

The potential activation of these distinctive neuronal subpopulations was investigated after i.p. administration of IL-1β, because our group and others have demonstrated that this stimulus results in activation of both the BSTov and the CEAl, as determined by the expression of c-fos mRNA. Although it was not demonstrated directly, the data presented in the current study suggest that the majority of the IL-1β-responsive neurons of the BSTov and CEAl contain both GAD65/67 and ENK mRNAs. Smaller proportions of activated neurons appear to contain both GAD65/67 and NT mRNAs. It is not clear whether these represent distinct populations or whether some of the activated GABAergic neurons express both NT and ENK. However, despite the generally high degree of colocalization between CRH and NT (Shimada et al., 1989), it seems likely that, given the lack of colocalization of CRH and c-fos mRNAs, the NT mRNA-containing cells that were activated by IL-1β represent a specific subpopulation of NT cells that do not contain CRH.

The lack of c-fos mRNA in CRH cells is difficult to interpret definitively. It is unlikely that CRH-containing cells are unable to express c-fos mRNA, because they have been shown to express Fos protein after nicotine administration (Matta et al., 1997). It also is possible that a different dose of IL-1β or a different time after injection would elicit c-fos mRNA expression in CRH cells. However, we believe that this is unlikely, because the dose employed in this study was relatively high, and c-fos mRNA peaks early in these areas after i.p. injection and returns to basal levels after 3 hours (Brady et al., 1994). It is not clear from the present data whether or not i.p. IL-1β increases expression of other transcription factors in CRH mRNA-containing cells, and the lack of expression of c-fos mRNA certainly does not preclude these cells from being involved in the coordinated response of the animal to this stimulus.

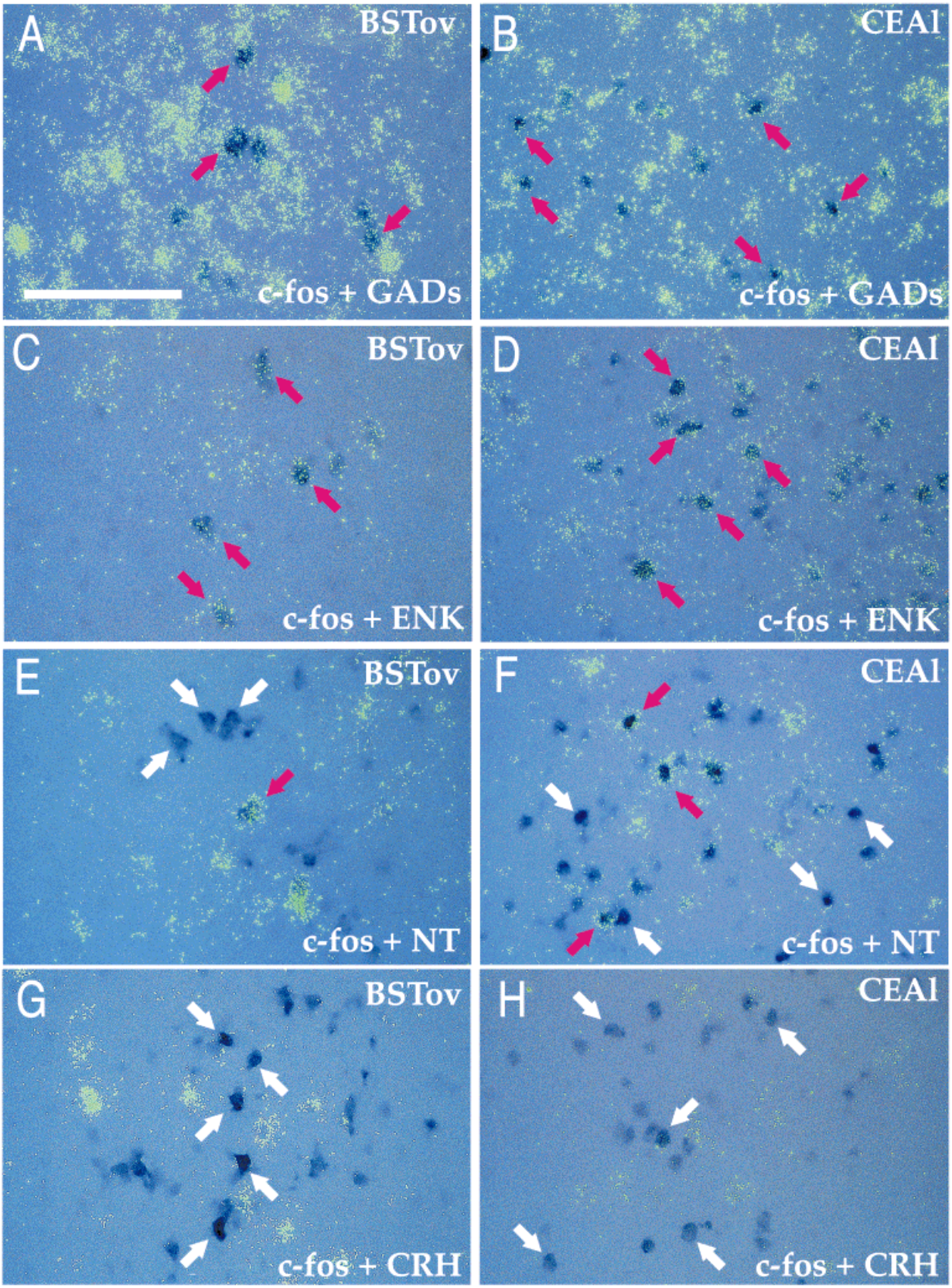

Fig. 8. Photomicrographs (double exposure) of double in situ hybridization experiments showing the extent of colocalization of c-fos mRNA (Dig-labeled probe) 30 minutes after IL-1β administration (5μg/ml/kg, i.p.) with GAD65 and GAD67 (**A,B**), ENK (**C,D**), NT (**E,F**), and CRH (**G,H**) mRNAs ([^{35}S]-labeled probes) in the BSTov (A,C,E,G) and the CEAl (B,D,F,H). White arrows indicate cells that contain c-fos mRNA alone. Magenta arrows indicate double-labeled cells that contain c-fos mRNA with the appropriate radioactive-labeled marker. Scale bar = 100 μm

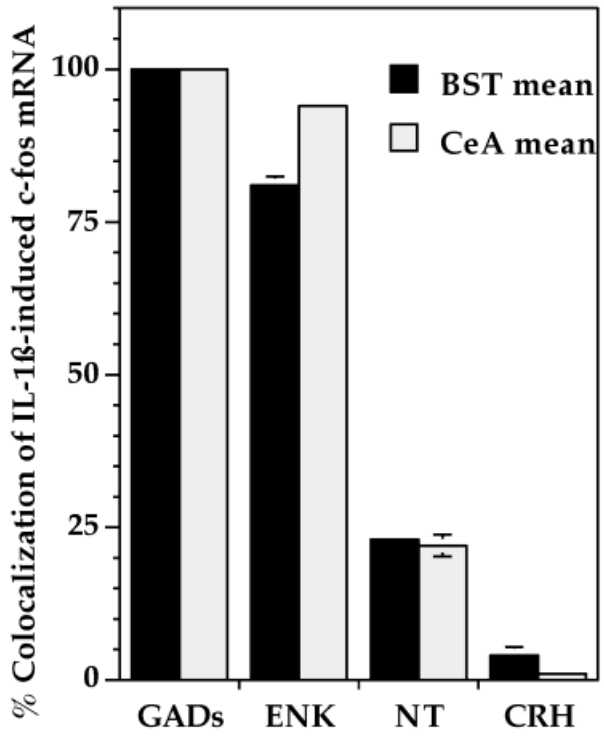

Fig. 9. Graph illustrating the relative proportions of c-fos mRNA expressing cells in the BSTov and the CEAl that expressed either GAD65/67, ENK, NT or CRH mRNA, 30 minutes after administration of IL-1β (5 µg/kg, i.p.).

However, we believe it is most likely that these cells are under active inhibition, as is discussed below. Nevertheless, the present data indicate the existence of neurochemically distinctive subpopulations within the BSTov and CEAl and suggest that they may represent functionally discrete neural circuits that may be activated differentially by a specific stimulus.

Potential functional implications of activation of the BSTov and CEAl by IL-1β

Although, currently, there are no data regarding specific functional roles for CRH- versus ENK-containing cells of the BSTov and CEAl, we hypothesize that differential activation of these neurochemically distinct neuronal populations allows the BSTov and CEAl to subserve different functional roles. The similarities in c-fos mRNA expression between the two nuclei are consistent with the anatomical concept of an extended amygdala (for review, see Alheid et al., 1995), and, broadly, these two nuclei have been linked functionally. Furthermore, it has been argued recently that the terms "amygdala"; or "amygdalar complex"; combine cell groups arbitrarily (Swanson and Petrovich, 1998); indeed, it is possible that, functionally, the CEAl would be better classified with the BSTov than within the amygdalar complex.

Of particular interest to our laboratory is the regulation of the hypothalamic-pituitary-adrenal (HPA) axis, the activation of which is a defining feature of a stress response. Several studies have indicated that both the CEA and the BST play a role in the regulation of stress-induced adrenocorticotropin hormone (ACTH) and corticosterone release, with the suggestion that this regulation is effected directly at the level of the PVH (Dunn, 1987; Gray et al., 1989, 1993). Although administration of IL-1β results in activation of the HPA axis (Besedovsky et al., 1986), the BSTov and CEAl express very low levels of c-fos mRNA after a variety of other stressors, including restraint, swim stress (Cullinan et al., 1995), audiogenic stress (Campeau and Watson, 1997), and fear conditioning (Campeau et al., 1997). Hence, c-fos mRNA expression within the BSTov and CEAl is not a feature common to the central response to acute stress. Furthermore, although early anatomical evidence suggested that the CEA provides direct input to the PVH (Gray et al., 1989), more recent studies using highly discrete injections of the retrograde tracer FluoroGold into the parvocellular (CRH-containing) regions of the PVH indicate that neither the BSTov nor the CEA provide direct input to the parvocellular PVH (Cullinan et al., 1996; Prewitt and Herman, 1998). Of particular relevance is the recent study of Lee et al. (1998), who described an anatomical dissociation of the c-fos mRNA response to intravenous IL-1β. Although lesions of the area postrema inhibited the IL-1β-induced ACTH response and c-fos mRNA response in the NTS and PVH, these lesions did not affect the IL-1β-induced c-fos mRNA expression in the BST or CEA. Taken together, we propose that the c-fos mRNA expression in the BSTov and CEAl after the administration of IL-1β does not reflect a primary role for these nuclei in HPA axis regulation. However, it is noteworthy that the majority of ENK-, NT-, and CRH-containing neurons of the CEA also contain glucocorticoid receptor immunoreactivity (Honkaniemi et al., 1992): Thus, these neurons probably are influenced by glucocorticoids released by IL-1β.

Many studies have suggested that the BSTov and CEA are involved in central autonomic regulation (for review, see Saper, 1995). The visceral nature of a systemic immune challenge suggests that the activation of these areas by IL-1β indeed may reflect an autonomic role. Administration of IL-1β results in a number of autonomic responses, such as inhibition of gastric acid secretion (Saperas et al., 1992), and the CEA in particular has been linked with this function (Henke et al., 1991). Administration of IL-1β also results in complex cardiovascular effects (Weinberg et al., 1988; Bataillard and Sassard, 1994), and many studies have demonstrated the roles that the BST and CEA play in cardiovascular responses (Loewy and McKeller, 1980; Iwata et al., 1987; Gelsema et al., 1989; Lewis et al., 1989; Roder and Ciriello, 1993). It is noteworthy that Fos protein expression has been demonstrated in the CEA and may be associated with various cardiovascular parameters, including hypotension with bradycardia and hypertension with tachycardia (Graham et al., 1995). Furthermore, in another model of immune challenge, systemic administration of endotoxin at a dose that did not alter blood pressure but caused tachycardia, also elicited robust Fos protein expression in the CEA (Tkacs et al., 1997). Hence, it appears that Fos may be induced in the CEA under a variety of different cardiovascular conditions, and it would be interesting to determine whether these different conditions elicit Fos in different cell types of the CEA. In addition, administration of IL-1β decreases feeding, and several other anorexic agents also increase the expression of c-fos in the CEAl and BSTov, although the neurochemical phenotype is not known (Bonaz et al., 1993; Li and Rowland, 1995; Rowland et al., 1996). Indeed, we believe it is unlikely that the activation of ENK- versus CRH-containing cells is specific

to an immune stimulus, because preliminary data suggest that an i.p. injection of amphetamine elicits a broadly similar neurochemical pattern of c-fos mRNA distribution in these areas.

Neural circuits potentially involved in IL-1β activation in the BSTov and CEAl

Although projections to and from IL-1β-activated cells of the BSTov and CEAl were not defined in the present study, it is important to think about the activated cells in terms of neural circuitry rather than in isolation. A number of elegant studies have been published that, together with the current data, suggest specific neural circuitry is engaged by i.p. IL-1β. In terms of activational pathways, although the major afferent input to the BST is from the amygdala (Weller and Smith, 1982; Alheid et al., 1995), the GABAergic nature of the activated cells of the CEAl likely precludes this cell group from directly activating the BSTov. However, i.p. administration of IL-1β also results in the expression of c-fos mRNA in the medial division of the NTS and the lateral parabrachial nucleus (PBl; Brady et al., 1994; Day and Akil, 1996). The NTS projects heavily to the PB (Herbert et al., 1990) but also projects directly to both the CEA and the BST (Ricardo and Koh, 1978). Similarly, both the BSTov and the CEAl receive projections from the PBl (Bernard et al., 1993; Krukoff et al., 1993; Alden et al., 1994). Studies to lesion the PBl and NTSm or to combine neuroanatomical tracers with Fos protein or c-fos mRNA localization may help to ascertain whether or not these nuclei are critical in the IL-1β activation of the BSTov and CEAl. It is noteworthy that, in addition to receiving afferents from the NTS, the PBl receives input from the spinal cord (Slugg and Light, 1994), and a transneuronal labeling technique has been used to demonstrate a pathway from the spinal cord to the CEA through the PB (Jasmin et al., 1997). Although the involvement of spinal projections has not been demonstrated after systemic administration of IL-1β, the possibility exists that a spinal-parabrachial pathway may be involved in the activation of the BSTov and CEAl after systemic administration of IL-1β.

Although efferent projections from the BSTov and CEAl have been demonstrated to the PB (Moga and Gray, 1985; Moga et al., 1989), the dorsal vagal complex (Gray and Magnuson, 1987), and the midbrain central gray (Gray and Magnuson, 1992), current evidence suggests that these projections do not arise from ENK-containing neurons. Because the majority of c-fos mRNA-containing neurons in the BSTov and CEAl also contain ENK, it seems unlikely that the major projection pathway of the activated cells is back to these areas. Two elegant studies using the anterograde tracer *Phaseolus vulgaris* leucoagglutinin injected exclusively into subdivisions of the CEA have demonstrated a relatively discrete population of efferent projections from the CEAl (Petrovich and Swanson, 1997; Jolkkonen and Pitkänen, 1998). Cells within the CEAl were shown to project intrinsically to other cells within the CEAl and CEAm in addition to projections to the BST and PB. ENK neurons of the CEAl, as discussed above, do not appear to project to the PB (Moga and Gray, 1985), but an enkephalinergic projection from the CEA to the BST has been demonstrated (Rao et al., 1987). Hence, it seems likely that the activated neurons of the CEAl observed in the present study project to the BST and/or

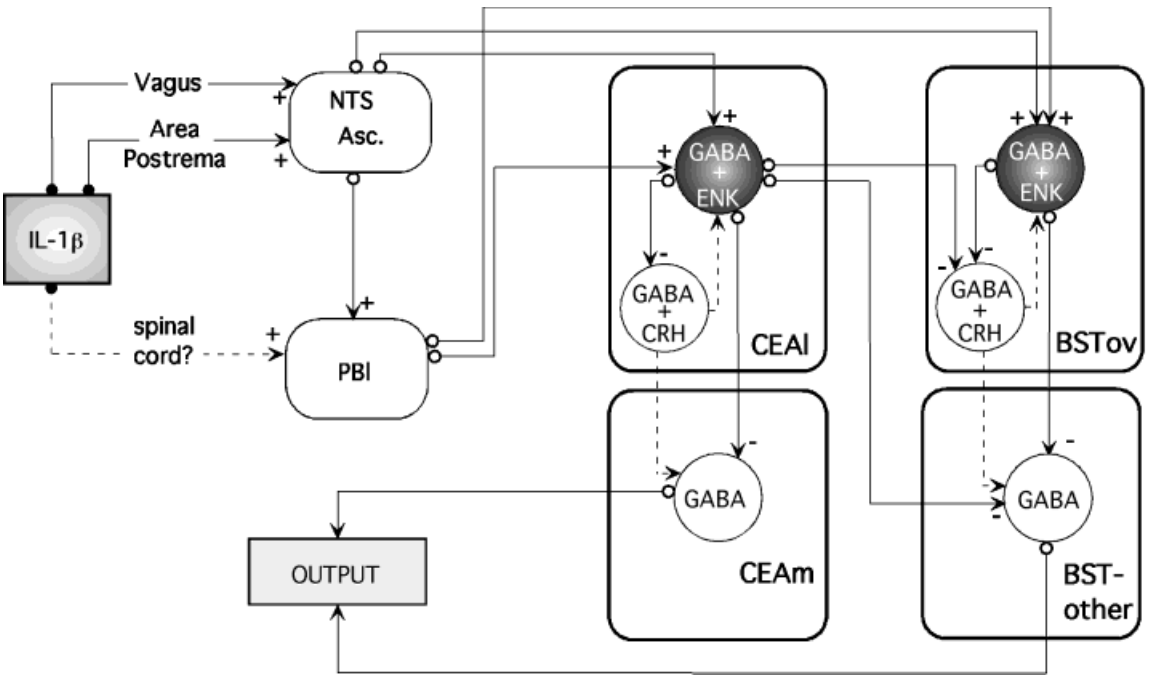

Fig. 10. Schematic diagram showing some of the pathways that may be involved in the activation of enkephalinergic neurons of the BSTov and CEAl, and some of the putative efferent projections from these neurons. It should be noted that the involvement of the spinal cord projection in activation of the parabrachial nucleus in this model is speculative at this time. In addition, although this figure highlights possible pathways involved in the c-fos mRNA response to the intraperitoneal administration of IL-1β, it does not attempt to show all possible projections to and from the illustrated nuclei. Asc, ascending; NTS, nucleus tractus solitarius; PBl, parabrachial nucleus, lateral division; CEAm, central nucleus, Amygdala, medical devision. For other abbreviations, see Figure 1.

intrinsically within the CEAl/m. Given the relatively sparse activation of other regions of the BST, the medial aspect of the CEA or the CRH mRNA-containing cells in the CEAl and BSTov, it is conceivable that some or all of these cell populations are under active inhibition by the Fos-positive cells of the CEAl. The efferent projections of the BSTov are less well defined. However, there is some evidence to suggest that GABAergic cells of the BSTov project intrinsically to posterior subdivisions of the BST, whereas there does not seem to be a major GABAergic projection from the BSTov to the CEA (Sun and Cassell, 1993). Hence, our working hypothesis is that the BSTov and CEAl are activated in parallel by ascending projections from the brainstem, and the activated neurons most likely project intrinsically within the BST or CEA. It should be noted that, although the activated cell types (as determined by c-fos mRNA expression) are GABAergic, this does not imply an inhibitory output. Indeed, there is the potential for significant complexity of response, depending on the degree of activation of intrinsic GABAergic connections within and between the CEA and the BST that will have a profound impact on whether the overall output of these nuclei is inhibitory or excitatory. A simplified schematic diagram of this hypothesis indicating some of the potential connections of specific cells groups within the BSTov and CEAl is shown in Figure 10.

In summary, cells of the BSTov and the CEAl which express ENK, NT, or CRH mRNA, invariably are GABAergic. Two distinct subpopulations of GABAergic neurons exist within these areas: one containing ENK and the other containing CRH. Both populations exhibit partial overlap with NT (Shimada et al., 1989; current study). The intraperitoneal administration of the cytokine IL-1β resulted in the activation of a proportionally similar population of neurochemically identified neurons in the BSTov and CEAl. All neurons in these regions that expressed c-fos mRNA were found to be GABAergic. The majority of these neurons also contained ENK mRNA, and a smaller population contained NT mRNA. However, very few neurons in the BSTov or CEAl that expressed c-fos mRNA contained CRH mRNA. The specificity of the response strongly suggests that discrete neural circuits may be targeted differentially within the BSTov and CEAl. In addition, the present results are consistent with the concept of an extended amygdala and demonstrate a specific coupling between the CEAl and the BSTov through the activation of distinct neurochemical subpopulations. Although the function of the activated neurons is not clear at present, it seems probable that the c-fos mRNA expression observed in the enkephalinergic cells of the CEAl and BSTov after the administration of IL-1β reflects autonomic or somatic regulation. Identification of the efferent targets of these neurons, together with the neurochemical identification of cell populations in the BSTov and CEAl responsive to other stimuli, may help to elucidate the functional significance of their activation.

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