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Proenkephalin Transgene Regulation in the Paraventricular Nucleus of the Hypothalamus by Lipopolysaccharide and Interleukin-1β

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ABSTRACT

Immunologic challenge with lipopolysaccharide (LPS) or interleukin-1β (IL-1β) produces a functional response within the paraventricular nucleus of the hypothalamus (PVN) and leads to changes in gene expression within PVN neurons. Regulated expression of neuropeptide genes within neurons of the PVN is a potential mechanism by which an organism can adapt to stressful challenges. Here, the authors used a transgenic mouse model in which expression of a readily measurable β-galactosidase reporter was driven in PVN neurons by human proenkephalin regulatory sequences. This proenkephalin-β-galactosidase transgene has been demonstrated previously to respond appropriately to a variety of stressors. It is demonstrated that expression of the proenkephalin transgene product was up-regulated significantly in a subset of PVN neurons 6 hours following intraperitoneal LPS (16–400 μg/kg) administration, remained elevated at 12 hours, and fell below basal levels by 24 hours. A more rapid and transient pattern of transgene up-regulation in the PVN followed administration of intraperitoneal IL-1β (10 μg/kg) with significant induction by 2 hours, peak levels reached by 4 hours, and a return toward basal levels by 6 hours. IL-1β (10–50 ng/mouse) administered intracerebroventricularly also led to up-regulation of the transgene 6 hours following infusion. Transgene expression was not up-regulated in hypothalamic slice cultures treated directly with IL-1β (5–10 ng/ml media). Up-regulation of transgene expression does not appear to result from local action of IL-1β at the level of the PVN but, rather, through as yet unidentified intermediates. The authors demonstrate phosphorylation of the cyclic amino-3-hydroxy-5-methyl-4-isoxazole-propionate response element binding protein, a transcription factor known to interact with proenkephalin regulatory sequences within the transgene, in the PVN following LPS administration. LPS induced up-regulation of the transgene was blocked by pretreatment with naltrexone, indicating an additional role for endogenous opioid systems in regulation of the PVN response to immune challenge. J. Comp. Neurol. 405:199–215, 1999.

Indexing terms: phosphocyclic amino-3-hydroxy-5-methyl-4-isoxazole-propionate response element-binding protein; naltrexone; stress; mice

Neurons of the paraventricular nucleus of the hypothalamus (PVN) are particularly well poised to mediate the central nervous system's response to stressful stimuli, including immunologic challenge (Swanson and Sawchenko, 1980). The PVN receives a diverse array of inputs. These include afferent connections from brainstem nuclei,

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a source of visceral sensory information; from the bed nucleus of the stria terminalis, a potential conduit for signals of limbic origin; from the subfornical organ, a relay for blood-borne signals; and from surrounding hypothalamic nuclei. In turn, distinct components of the PVN control release of neuroendocrine signals to the anterior and posterior pituitary, and still other components provide output to the autonomic nervous system through efferent connections to brainstem and spinal cord nuclei (Swanson and Sawchenko, 1983; Cechetto and Saper, 1988; Cunningham and Sawchenko, 1988; Cunningham et al., 1990; Roland and Sawchenko, 1993; Larsen et al., 1994; Herman and Cullinan, 1997).

Immunologic challenge produces a coordinated set of homeostatic neuroendocrine, autonomic, and behavioral responses. Administration of lipopolysaccharide (LPS), a potent secretagogue for interleukin 1β (IL-1β), or direct administration of recombinant IL-1β both produce increases in circulating levels of corticotropic hormone (ACTH) and glucocorticoids as well as induction of Fos in PVN neurons. These responses are similar to responses observed to natural pathogens (Sapolsky et al., 1987; Rivier et al., 1989a,b; Chang et al., 1993; Brady et al., 1994; Ericsson et al., 1994; Wan et al., 1994; Elmquist et al., 1996). Like what is seen in the PVN in response to other stressors, there also are significant changes in the expression of neuropeptide genes following LPS or IL-1β administration (Sternberg et al., 1989; Suda et al., 1990; Young, 1992; Kakucska et al., 1993; Ericsson et al., 1994; Rivest and Rivier, 1994). Transcription of neuropeptide genes, including preproenkephalin, is responsive to neural and humoral signals; modulation of neuropeptide gene expression provides one mechanism by which an organism's response to stressful challenge may be adjusted over time and with respect to current conditions (Goodman, 1990; Tilders et al., 1993; Hökfelt et al., 1994; Watts, 1996).

Of the many neuropeptides expressed in PVN neurons, physiologic roles have been well established for only a few, such as corticotropin-releasing hormone (CRH), vasopressin, and oxytocin. For other neuropeptides, such as the enkephalins, it is not yet possible to specify fully their functional roles (Bondy et al., 1989; Palkovits, 1992). However, evidence exists to suggest a role for endogenous opioids in the regulation of neuroendocrine and autonomic function (Holaday, 1983; Pfieffer and Herz, 1984; Szekely, 1990; McCubbin, 1993; Hammer and Cheung, 1995). Opiates have been shown to regulate a variety of neuroendocrine outputs, including promotion of growth hormone, prolactin, and CRH secretion and inhibition of luteinizing hormone and follicle-stimulating hormone secretion (Buckingham and Cooper, 1984, 1986; Howlett and Rees, 1986). Opiates acting at or near the PVN can regulate cardiovascular function, synthesis and release of ACTH, and feeding behavior, each of which is altered in response to immune challenge (Pfeiffer and Herz, 1984; Tsagarakis et al., 1990; Koch et al., 1995; Kotz et al., 1995). Opioids also have been demonstrated to influence other aspects of the central nervous system response to immune challenge. For example, chronic morphine treatment has been shown to attenuate expression of IL-1β within the central nervous system (Patel et al., 1996) and to inhibit IL-1β-induced immediate-early gene expression in the PVN, suggesting a possible role for opiates in modulating the transcriptional response of PVN neurons to immune challenge (Chang et al., 1996). These multiple effects of opioids are likely to occur endogenously through synthesis and release of one or more opioid peptides at a number of anatomically specific sites (both peripheral and central) under distinct physiologic circumstances. Regulated expression of the proenkephalin gene within PVN neurons represents one aspect of the opioid response to stress, including immunologic stress.

Within PVN neurons, proenkephalin gene expression is highly responsive to a variety of stressors (Lightman and Young, 1987; Sternberg et al., 1989; Watts, 1991; Young and Lightman, 1992; Borsook et al., 1994a,b; Mansi et al., 1998). The proenkephalin gene is expressed in magnocellular, parvocellular, and autonomic components of the PVN and is coexpressed in distinct neuronal subsets with other neuropeptides, including CRH, vasopressin, and neurotensin, which also display considerable alterations in expression in response to specific stressors (Sawchenko and Swanson, 1982; Fallon and Leslie, 1986; Merchenthaler et al., 1986; Swanson et al., 1986; Harlan et al., 1987; Pretel and Piekut, 1990; Sakanaka et al., 1990; Watts and Sanchez-Watts, 1995; Watts, 1996). Although the direct physiologic actions of opioid peptides synthesized in the PVN remain unknown, they may be involved in paracrine regulation of other hypothalamic neurohormonal peptides (Lightman and Young, 1987).

The proenkephalin gene has served as an important model of stimulus-coupled transcriptional activation in cell lines (Comb et al., 1986, 1988; Hyman et al., 1988, 1989; Nguyen et al., 1990) and within the nervous system (Konradi et al., 1993; Borsook et al., 1994c). To investigate the molecular mechanisms by which the proenkephalin gene is regulated within the nervous system in response to specific neuronal activation in vivo, we have established a transgenic mouse model system in which the regulatory sequences of the human proenkephalin gene (3 kb of 5′′-flanking sequence, the first exon and intron; 1.2 kb of 3′-flanking sequence) direct expression of the reporter *Escherichia coli* β-galactosidase (Borsook et al., 1992; Borsook and Hyman, 1995). We previously demonstrated that this construct contains sufficient information to replicate expression of the endogenous proenkephalin gene within the PVN both basally and in response to a variety of stressors, including hypertonic saline stress, cold swim stress, hypovolemia, and naloxone-precipitated opiate withdrawal (Borsook et al., 1994a–c). Our transgenic model provides a highly sensitive assay of proenkephalin gene regulation with cellular specificity. In addition, the genomic sequences that confer this regulation must be contained within the well-defined transgene sequences, thus permitting integration of in vivo and in vitro investigations of the signal-transduction mechanisms underlying gene regulation in the PVN.

The present study was carried out to determine whether proenkephalin gene expression and, more specifically, expression of our defined transgene is up-regulated in the PVN in response to immunologic challenge with LPS or IL-1β and to begin to analyze the pathways by which this occurs. With respect to IL-1β effects within the central nervous system, controversy remains about the primary site of IL-1β action. We therefore examined the effects of different routes of administration in eliciting proenkephalin transgene up-regulation following IL-1β treatment. In addition, by using in vitro slice cultures, we examined the

importance of intact afferent input to the PVN in eliciting this response. Finally, because it seems likely that modulatory effects of endogenous opioids may occur simultaneously at multiple levels within the system, we thought it would be of interest to determine whether proenkephalin transgene expression within the PVN itself is under the control of endogenous opioid systems.

MATERIALS AND METHODS

Animals

Production and characterization of the proenkephalin-β-galactosidase transgenic line of mice have been described elsewhere (Borsook et al., 1992, 1994a,b). Adult male ENK 1.1 transgenic mice (25–30 g) homozygous for the transgene were used in all experiments, with the exception of experiments in which transgene expression was not assayed. For those experiments [phospho-cyclic amino-3-hydroxy-5-methyl-4-isoxazole-propionate (cAMP) response element-binding protein (CREB) immunoreactivity (CREB-IR) experiments; see below], adult male Swiss Webster mice (25–30 g; Taconic, Germantown, NY) were used. Animals were housed three to five per cage under a standard 12 hour light-dark cycle with free access to food and water. All experiments were carried out in accordance with both Massachusetts General Hospital and National Institutes of Health guidelines for animal experimentation. Except where noted, each experimental group included four animals. Efforts were made to minimize animal suffering and to limit the number of animals used.

Immunologic stressors

LPS injections were made intraperitoneally (i.p.) in a volume of 0.4 ml and at doses of 3.2 μg/kg, 16 μg/kg, 80 μg/kg, or 400 μg/kg. A 1 mg/ml stock solution of LPS (Difco Laboratories, Detroit, MI) was diluted in sterile saline as required. For i.p. injections, IL-1β (mouse recombinant; R&D Systems, Minneapolis, MN) was freshly reconstituted at 25 μg/ml in sterile saline. Injections of IL-1β were made in a volume of 0.2 ml and at doses of 0.5 μg/mouse, 1.0 μg/mouse, or 5,0 μg/mouse (in the initial experiment) or 10 μg/kg (time course), with dilutions of the stock solution in sterile saline made as required. For intracerebroventricular (i.c.v.) injections, IL-1β (mouse recombinant; Sigma, St. Louis, MO) was reconstituted at 25 ng/μl in sterile saline with 0.1% bovine serum albumin (BSA). Aliquots were stored at −70°C until required. Intracerebroventricular injections were made in a total volume of 2 μl at doses of 2.5 ng/mouse, 5 ng/mouse, 10 ng/mouse, or 50 ng/mouse, with dilutions of the stock solution in sterile saline made as required. Sterile saline with 0.1% BSA served as a vehicle control.

Intracerebroventricular injection

For i.c.v. administration of IL-1β, a stainless-steel guide cannula (26 gauge, cut 2.5 mm below the pedestal; Plastics One, Inc., Roanoke, VA) was implanted into the right lateral ventricle. Mice were anesthetized with 2.5% avertin (0.1 ml/10 g body weight) and secured in a Kopf stereotaxic frame (Tujunga, CA). The skull was cleaned and dried thoroughly, a small hole was drilled, and the cannula was lowered slowly into place. The coordinates for placement were 0.5 mm posterior to Bregma, 1.6 mm lateral, and 2.5 mm below skull surface. The guide was secured in place with cranioplastic cement (Plastics One, Inc.), and a dummy cannula was inserted to maintain patency and sterility of the guide cannula. On postsurgical days 1–7, animals were handled daily. On days 8 and 9, animals were exposed (for acclimation) to the infusion cage (Instech Laboratories, Plymouth Meeting, PA). On postsurgical day 10, the dummy cannula was removed, and an internal cannula (33 gauge) was inserted. The internal cannula was attached with PE 20 tubing to a low-torque fluid swivel mounted on a counterbalancing arm (Instech Laboratories) and subsequently to a 10 μl Hamilton syringe mounted in a mechanized infusion pump (KD Scientific, Inc., Boston, MA). Two microliters of vehicle or IL-1β solution were infused at a rate of 0.25 μl/minute. The internal cannula was left in place for 10 minutes following the infusion. It was then removed and replaced with the dummy cannula, and the animal was returned to its home cage until it was killed. Because of the daily handling sessions, in which each mouse was allowed to explore a cotton-gloved hand for several minutes, the animals adapted behaviorally, such that they would sit passively for periods long enough to accomplish insertion and removal of the internal cannula with no need for restraint. This extensive previous handling allowed insertion and removal of the internal cannula to be accomplished with no apparent stress to the animal (e.g., no defecation or urination). Animals were killed 6 hours from the end of the infusion period, then correct placement of the cannula was verified by sectioning through the guide path. Only data obtained from animals with cannula clearly placed in the ventricle were included in analysis.

Naltrexone treatment

For naltrexone pretreatment, mice were anesthetized lightly with ether (Sigma), a small incision was made in the skin of the back, a small pouch was made under the skin with sterile forceps, and either a naltrexone pellet (30 mg) or a placebo pellet was implanted. The incision was sutured with 2/0 silk. The procedure lasted less than 2 minutes, and the mice recovered rapidly. Pellets were inserted subcutaneously 24 hours prior to LPS injection. Animals were killed 6 hours after LPS administration. Pellets were provided courtesy of Dr. Robert Walsh at the National Institute of Drug Abuse.

Hypothalamic slice cultures

We produced hypothalamic slice cultures as previously described (Borsook et al., 1998). Briefly, under sterile conditions, postnatal day 6 (P6) through P8 mouse pups were decapitated rapidly, and their brains were removed and placed in a Petri dish. A block of tissue containing the hypothalamus was dissected and placed on a McIllwain tissue chopper (Gomshall, Surrey, United Kingdom). Four hundred-micrometer sections were cut, and the hypothalamic slices were placed in a small Petri dish containing minimal essential medium (MEM) with 10 mM Tris (200 ml MEM, 0.24 g Tris, pH 7.2). Five or six hypothalamic slices were then transferred carefully onto a millicell insert set in a six-well Falcon plate (Millipore, Bedford, MA) with 1 ml of culture media. The slices were not covered by the media. The Falcon plate was covered and

incubated at 37°C with 5% CO^2. The media was changed every 3–4 days. Culture media was made as follows: 200 ml MEM, 100 ml Hank's balanced salt solution (HBSS), 100 ml heat-inactivated horse serum, 2.3 g glucose, 120 mg glutamine, 4 ml penicillin-streptomycin, adjusted to pH 7.2 and filtered under sterile conditions. Prior to IL-1β application, serum-free media was placed in the culture wells 24 hours prior to drug application. On the day of drug application, the serum-free media was removed from the culture well, 1 ml of culture media containing IL-1β was placed over the slice for 10 minutes, and 1 ml of the same (drug-containing) media was placed in the well. The media covering the slice was discarded after 10 minutes. Control slices were subjected to the same manipulation, except that no drug was present in the culture media.

Galactopyranoside staining

X-gal staining using the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal; Boehringer Mannheim, NJ) was used to detect expression of β-galactosidase. The method for X-gal staining was adapted from Price et al. (1987) and was used exactly as described previously (Borsook et al., 1994a,b). Mice were anesthetized with an i.p. injection of avertin (0.4 ml, 2.5%) and were perfused transcardially with 4% paraformaldehyde in phosphate-buffered saline (PBS). Brains were removed, postfixed for 1 hour in 4% paraformaldehyde, and placed in 30% sucrose solution overnight at 4°C. Brains were frozen and 40 μm sections cut on a freezing microtome. Serial sections were mounted onto Superfrost Plus slides (VWR Scientific, Boston, MA) and allowed to air dry. Slides were then incubated overnight at 37°C in the X-gal staining solution. X-gal staining solution was made as follows: 0.125 ml 240 mM $KFe^{2+}CN$, 0.125 ml 240 mM $KFe^{3+}CN$, 2.5 ml 0.2 M Na PO_4 buffer, 1.0 ml 5 M NaCl, 12 μl 4.9 M $MgCl_2$ (Sigma), 3 μl 10% stock NP-40, 6 μl 1 N NaOH, 3 mg deoxycholic acid (sodium salt), 24.75 ml dH_2O, and 1.43 ml X-gal solution (2% in dimethylformamide). Slides were rinsed twice in PBS and once in dH_2O before being allowed to air dry. Slides were then dipped in xylene and coverslipped with mounting medium. For each experimental set, all groups were processed in parallel to minimize the impact of any potential minor variation across batches of staining solution.

Phospho-CREB immunocytochemistry

We have described previously the phosphorylation of CREB in the PVN following a hypertonic saline stress (Borsook et al., 1994c). Immunostaining procedures for phospho-CREB were carried out exactly as described in the previously published study (Borsook et al., 1994c). Animals were handled daily for 5 days prior to LPS administration to minimize the effects of handling stress on phospho-CREB induction. Animals were killed at either 15 minutes or 30 minutes following LPS (400 μg/kg) injection. The phospho-CREB antibody was the generous gift of David Ginty (Ginty et al., 1993) and was used at a dilution of 1:1,000.

Quantitation of transgene expression as determined by X-gal histochemistry

Quantitation of transgene expression was carried out by using a computer-assisted method that has been described in detail (Borsook et al., 1994b). Briefly, a single section was selected from each experimental animal for quantitation. Standardized sections were defined by the following criteria: the shape of the PVN in brightfield; a clearly defined suprachiasmatic nucleus at the base of the third ventricle (identified by its typical nuclear density); and the distance of the supraoptic nucleus (SON) from the midline, defined as just lateral to a hypothetical line drawn through the fornix. Basal expression of the proenkephalin-β-galactosidase transgene is apparent following X-gal staining as small, blue puncta within cells. Upon up-regulation of transgene expression, X-gal staining demonstrates filling of cells with blue X-gal reaction product. This feature of transgene expression allows for a simple filled-area based method of quantitation. The selected section was viewed with a Leitz microscope (×20; Leitz, Wetzlar, Germany) and an image transferred with an MTI 72 Dage camera (Michigan City, IN) onto a computer [note: for the data shown in Fig. 3, sections were viewed with a Zeiss Axioskop microscope (×20; Thornwood, NY), and images were captured by using NIH Image software (W. Rasband; NIMH, Bethesda, MD) with an MTI VE1000 Dage camera]. Each image was then analyzed using MCID M4 version 1.2 software. The region of interest was defined by drawing a line around the PVN, as shown in Figure 3.

X-gal reaction product was quantified as total area (μm^2) within the defined region that was X-gal-positive based on a standard, preset cut off. Data were then subjected to statistical analysis (Statview version 4.5). This analysis provides for objective relative comparison between animals. We determined previously that blinded, manual counting of filled neurons also can demonstrate their differences (Borsook et al., 1994a).

Figure production

The images depicted in Figures 1, 2, 4, 6, and 8 were produced as follows: Color photomicrographs were taken of individual images by using a camera attached to a Leitz microscope (×20 with the exception of Fig. 6, at ×5). Individual color prints were labeled and aligned in the appropriate montage along with a printed copy of any accompanying graph (CA-Cricket Graph III, version 1.5.3; Cricket Software, Philadelphia, PA). The assembled montages were then photographed in color (Figs. 2, 6) or in black and white (Figs. 1, 4, 8), and the photomicrographs were printed at either 70% (Figs. 1, 2, 4, 8) or 55% (Fig. 6) of the original montage size. Figure 7 was produced in essentially the same manner, with the exception that the montage was scanned electronically rather than photographed, and the image was printed at 55% of the original montage size without further alterations.

RESULTS

Time course of proenkephalin transgene induction in the PVN by LPS

Basal levels of the proenkephalin-β-galactosidase transgene are expressed as small, blue puncta within cells following β-galactosidase histochemistry. Stimulation of transgene expression results in a progressive filling of cells with the blue β-galactosidase reaction product. The total area within a defined border around the PVN staining positive for β-galactosidase was taken as our measure of transgene expression (see Materials and Methods). Expres-

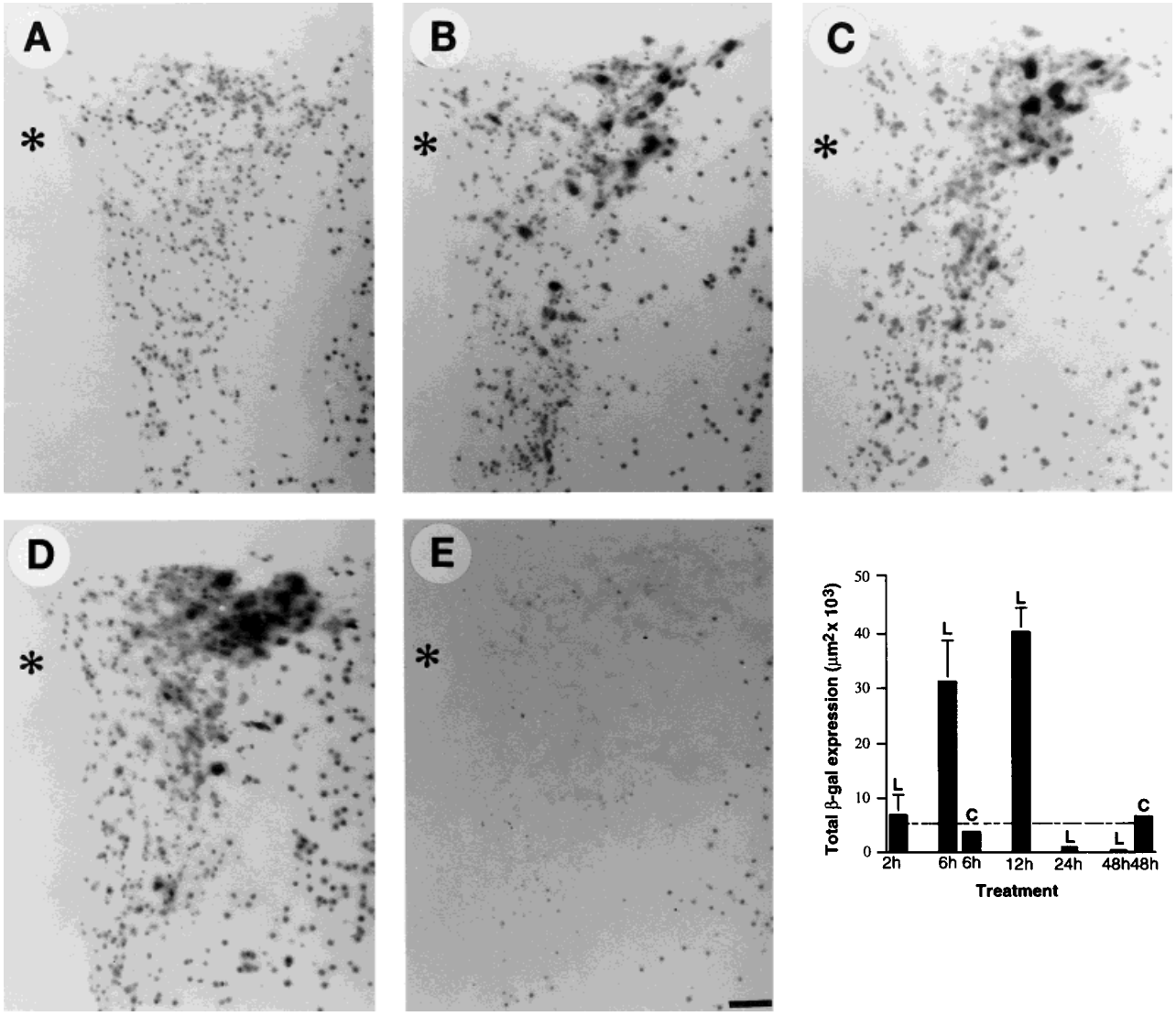


Fig. 1. Induction of transgene in the paraventricular nucleus of the hypothalamus (PVN) by lipopolysaccharide (LPS): time course. Photomicrographs of representative coronal sections through the PVN stained for β-galactosidase (β-gal) activity. **A:** Transgene expression levels 6 hours following intraperitoneal (i.p.) administration of normal saline. B–E demonstrate the time course of transgene induction following a single i.p. injection of LPS (400 μg/kg) at 2 hours (**B**), 6 hours (**C**), 12 hours (**D**), and 24 hours (**E**). Expression of the transgene at 48 hours was similar to that seen at 24 hours. Asterisks indicate the third ventricle. **Graph:** Quantitation of total β-galactosidase expression (μm^2) in the PVN at 2, 6, 12, 24, and 48 hours following a single injection of LPS (L; 400 μg/kg;. Control mice (C) received a single injection of normal saline and were killed at 6 hours or 12 hours postinjection. There is a significant increase in total β-galactosidase expression by 6 hours following LPS. Transgene expression levels reach a maximum at 12 hours and decrease to below control levels by 24 hours and 48 hours. The dashed line represents the mean control value. Because transgene expression in saline-treated mice does not differ from basal levels, the two saline groups were pooled and treated as basal state controls for statistical purposes. The 6-hour and 12-hour time points demonstrate statistically significant transgene induction [vs. saline control; $P < 0.0001$, one-way analysis of variance (ANOVA); Fisher's protected least significant difference (PLSD)]. Scale bar = 50 μm.

sion of the transgene in the PVN of mice treated with normal saline does not differ from the level of expression detected in naive animals. Within 2 hours following an injection of LPS (400 μg/kg, i.p.), transgene expression is detectable in the PVN compared with saline-treated controls, although, across the treatment group, such induction does not reach quantitative significance (Fig. 1A,B). The induction reaches statistical significance by 6 hours compared with saline-treated controls (Fig. 1A,C); transgene expression remains elevated 12 hours after injection (Fig. 1D). By 24 hours and 48 hours following LPS administration, transgene expression falls to levels below those of saline-treated controls (Fig. 1A,E). Based on this time course, the 6-hour time point was chosen as the end point for use in further experiments. It should be noted that it is a subset of the proenkephalin-β-galactosidase transgene-expressing neurons in the PVN that show induction in response to LPS.

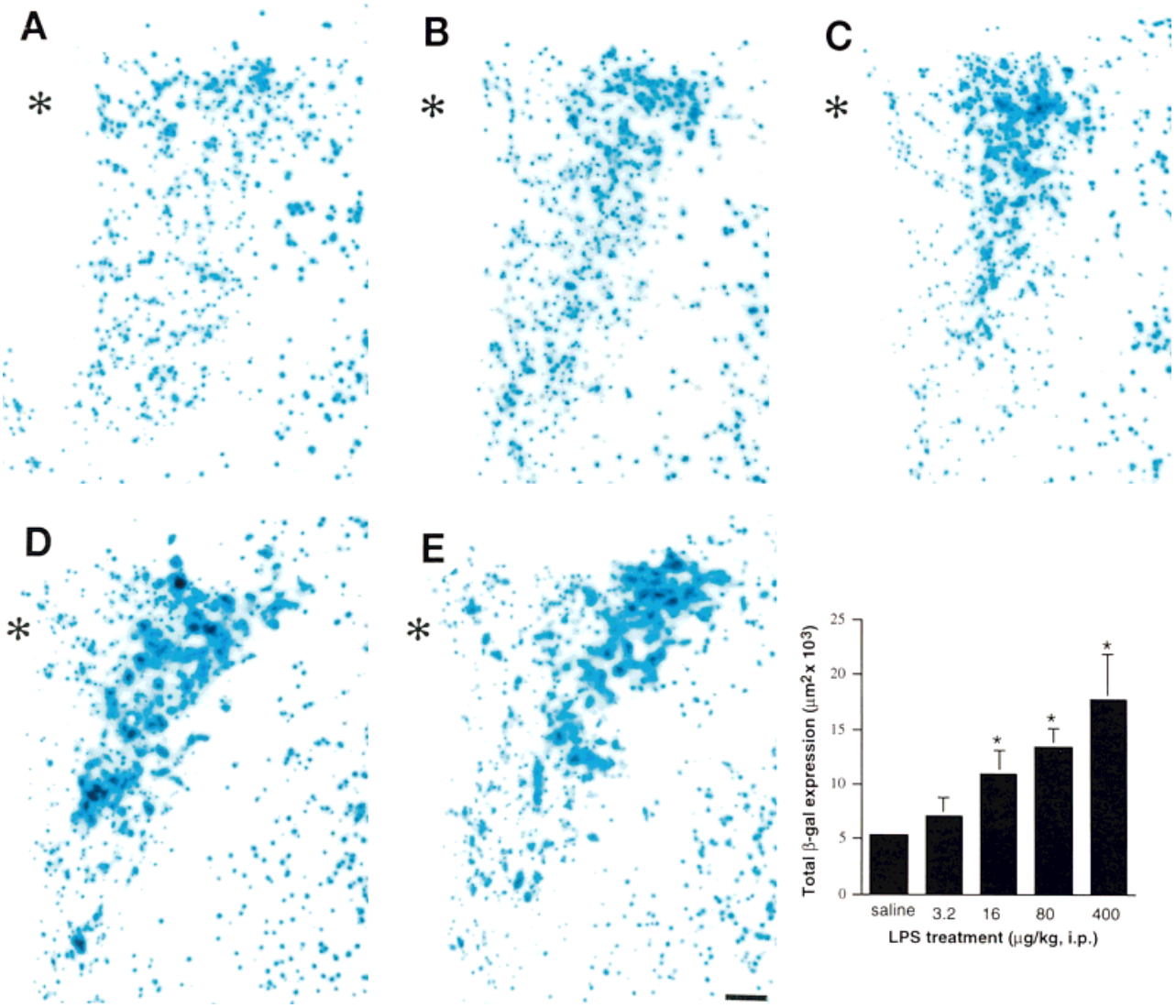


Fig. 2. Induction of transgene in the PVN by LPS: dose response. Photomicrographs of representative coronal sections through the PVN stained for β-galactosidase activity. **A:** Transgene expression levels 6 hours following administration of normal saline. B–E demonstrate the dose response for transgene induction 6 hours following administration of 3.2 μg/kg LPS (**B**), 16 μg/kg LPS (**C**), 80 μg/kg LPS (**D**), or 400 μg/kg (**E**). Asterisks indicate the third ventricle. **Graph:** Quantitation of total β-galactosidase expression (μm²) in the PVN 6 hours following the administration of normal saline or increasing doses of LPS. There is significant induction of the transgene following 16 μg/kg LPS ($P < 0.05$), 80 μg/kg LPS ($P < 0.009$), and 400 μg/kg LPS ($P < 0.0004$; note that n = 3 for this group) compared with saline-treated controls (one-way ANOVA; Fisher's PLSD). Asterisks denotes statistically significant transgene induction. Scale bar = 50 μm.

Dose response for proenkephalin transgene induction in the PVN by LPS

We administered LPS by i.p. injection at doses ranging from 3.2 μg/kg to 400 μg/kg and examined transgene expression 6 hours later. At a dose of 3.2 μg/kg, only a few individual neurons within the PVN demonstrate transgene induction (Fig. 2B). There is significant, quantitative up-regulation of transgene expression 6 hours following 16 μg/kg LPS (Fig. 2C) and increasing levels of expression following doses of 80 μg/kg and 400 μg/kg (Fig. 2D,E). The highest dose of LPS (400 μg/kg) is associated with significant signs of "sickness behavior" in our mice, including reduced exploration, piloerection, huddled posture, and reduced reactivity to handling. In contrast, doses at the low end of this range (3.2 μg/kg, 16 μg/kg) are accompanied by minimal visible signs of distress. It is notable that significant, quantitative up-regulation of transgene expression occurs following 16 μg/kg LPS in the absence of typical signs of "sickness behavior" (Fig. 2C).

Peripheral (i.p.) or central (i.c.v.) IL-1β administration induces up-regulation of proenkephalin transgene expression in the PVN

IL-1β is thought to be one potential mediator of the effects of LPS on PVN activation. However, controversy remains regarding the site of IL-1β receptor stimulation that eventually leads to activation of neurons within the

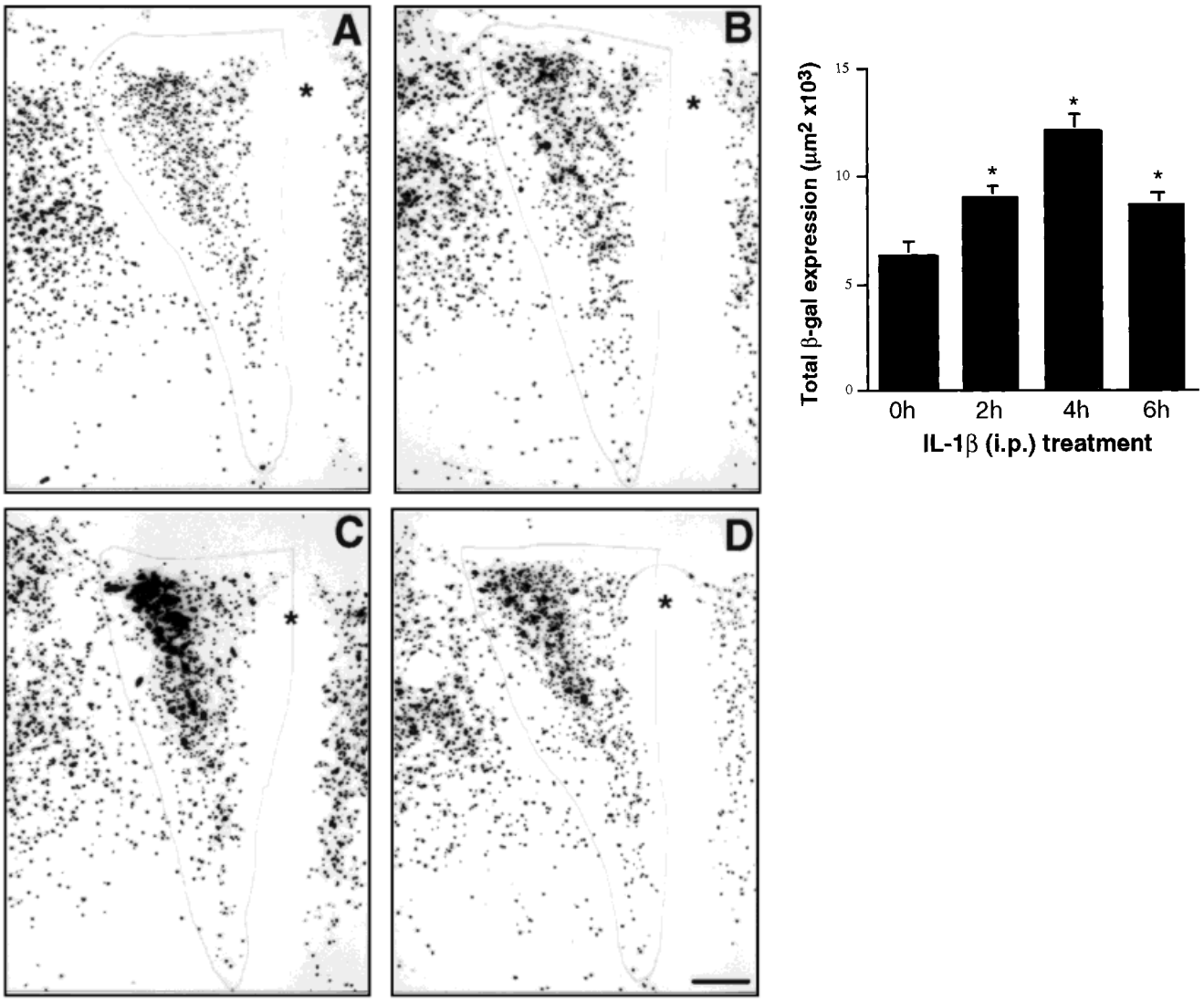


Fig. 3. Induction of transgene in the PVN by i.p. administration of interleukin 1β (IL-1β). Digitally captured images of representative coronal sections through the PVN stained for β-galactosidase activity. **A:** Basal transgene expression. B–D demonstrate the time course for transgene induction following a single i.p. dose of IL-1β (10 μg/kg) at 2 hours (**B**), 4 hours (**C**), and 6 hours (**D**). Sections were viewed with a Zeiss Axioskop microscope (×20), and images were captured by using NIH Image software (W. Rasband; NIMH) with an MTI VE1000 Dage camera. Images were cropped, labelled (Adobe Photoshop, Illustrator; Adobe Systems, Mountain View, CA) and printed without further alterations (phaser 450; Tektronix, Beaverton, OR). The line encircling the PVN encompasses the area measured for positive β-galactosidase signal. Asterisks indicate the third ventricle. **Graph:** Quantitation of total β-galactosidase expression (μm^2) in the PVN in the basal state or 2, 4, and 6 hours following a single injection of IL-1β (10 μg/kg). There is significant induction of the transgene in the PVN by 2 hours ($P < 0.05$), peak levels of induction by 4 hours ($P < 0.0001$), and a return toward control levels by 6 hours ($P < 0.05$; vs. basal levels; one-way ANOVA; Fisher's PLSD). Asterisks denote statistically significant transgene induction. Scale bar = 100 μm.

PVN. Therefore, we examined the effects of IL-1β administered either within or outside of the central nervous system. A dose-finding experiment demonstrated up-regulation of the transgene 6 hours following i.p. IL-1β administration only with doses in the supraphysiologic range (5 μg/mouse; data not shown). A subsequent time course study demonstrates that, following i.p. administration of IL-1β at a dose of 10 μg/kg, there is a rapid and transient profile of transgene up-regulation, with significant induction reached by 2 hours (Fig. 3B), peak levels obtained by 4 hours (Fig. 3C), and a return toward control levels by 6 hours (Fig. 3D). It is seen following LPS treatment that it is a subset of the proenkephalin-β-galactosidase transgene-expressing neurons in the PVN that show induction of the transgene following IL-1β administration.

It has been demonstrated that administration of IL-1β directly into the cerebroventricular system can replicate the effects of peripherally applied IL-1β, such as fever, slow-wave sleep, hyperalgesia, reduced food and water intake, and reduced social interaction, with a lower dose requirement (Rothwell and Hopkins, 1995). We administered IL-1β (2.5–50 ng/mouse) into the right lateral ventricle of awake, freely moving mice fitted at least 7 days before with a stereotaxically placed guide cannula. No difference from basal levels of transgene expression is seen

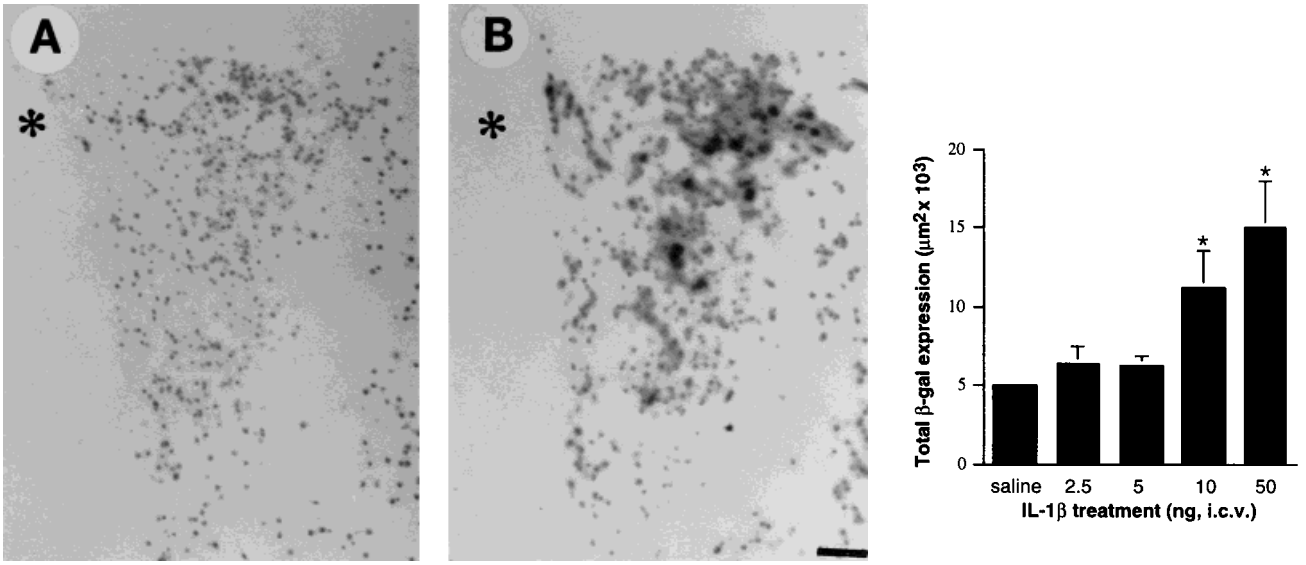


Fig. 4. Induction of transgene in the PVN by intracerebroventricular administration of IL-1β. Photomicrographs of representative coronal sections through the PVN stained for β-galactosidase activity. Paraventricular transgene expression 6 hours following intracerebroventricular (i.c.v.) administration of vehicle (sterile saline, 0.1% bovine serum albumin; **A**) or 50 ng IL-1β (**B**). Asterisks indicate the third ventricle. **Graph:** Quantitation of total β-galactosidase expression (μm^2) in the PVN 6 hours following the i.c.v. administration of vehicle or increasing doses of IL-1β. There is significant induction of the transgene following 10 ng IL-1β ($P < 0.005$) and 50 ng IL-1β ($P < 0.0003$) compared with vehicle-treated controls (one-way ANOVA; Fisher's PLSD). Transgene expression following 2.5 ng and 5 ng doses of IL-1β does not differ statistically from controls. Asterisks denote statistically significant transgene induction. Scale bar = 50 μm.

following placement of the chronic cannula (compare Fig. 4A with Fig. 1A). Intracerebroventricular treatment with vehicle (sterile saline with 0.1% BSA) does not result in detectable up-regulation of transgene expression (Fig. 4A). Intracerebroventricular administration of either 10 ng or 50 ng recombinant mouse IL-1β induces significant up-regulation of transgene expression in the PVN compared with vehicle-treated controls (Fig. 4A,B). The average response to lower doses of IL-1β (2.5 ng, 5.0 ng) did not differ significantly from saline-treated controls; however, a few individual animals displayed visible induction of transgene expression (not shown). Transgene expression was assayed only at the 6-hour time point following i.c.v. IL-1β. It should be noted that, given the rapid and transient induction observed following i.p. IL-1β, it is possible that assaying transgene expression at an earlier time point may be necessary to determine true peak induction levels following i.c.v. IL-1β.

LPS and IL-1β do not induce up-regulation of transgene expression in the SON

LPS- and IL-1β-induced up-regulation of transgene expression is highly specific anatomically. Despite significant levels of transgene up-regulation in the PVN in response to LPS and IL-1β (administered either i.p. or i.c.v.), there is no up-regulation of transgene expression observed in any other hypothalamic nucleus, including the SON (Fig. 5B). This is a contrast to our studies utilizing other stressors (Borsook et al., 1994a–c), which demonstrated up-regulation of transgene expression in both the PVN and the SON in response to hypertonic saline stress (Fig. 5C) and naloxone-precipitated opiate withdrawal.

IL-1β treatment of hypothalamic slice cultures does not induce up-regulation of the transgene

Hypothalamic slice cultures prepared from newborn transgenic mice express the transgene in an anatomic pattern matching that seen in vivo (Borsook et al., 1998); the integrity of hypothalamic anatomy is maintained under slice culture conditions (Fig. 6). Isolated coronal slices of the hypothalamus derived from P6–P8 transgenic mice and maintained in culture for 7 days were treated by direct application of 5–10 ng IL-1β/ml culture media for 1 hour, 2 hours, 4 hours, or 12 hours. No change in transgene expression is observed in the PVN or in any other hypothalamic nuclei in response to this treatment compared with controls (compare Fig. 6A,B with Fig. 6C,D). The experiment was repeated four times for each time point. Figure 6 demonstrates the results from a 4-hour treatment with IL-1β (10 ng/ml). Transgene expression in these slice cultures is inducible by forskolin and other pharmacological manipulations (Borsook et al., 1998).

LPS administration induces phospho-CREB-like immunoreactivity in the PVN

We sought to determine whether CREB was phosphorylated in the PVN of mice in response to LPS administration. By using an antiserum that specifically detects CREB phosphorylated on Ser^{133}, an induction of CREB phosphorylation was observed at 15 minutes and 30 minutes following LPS administration (400 μg/kg; Fig. 7B,C).

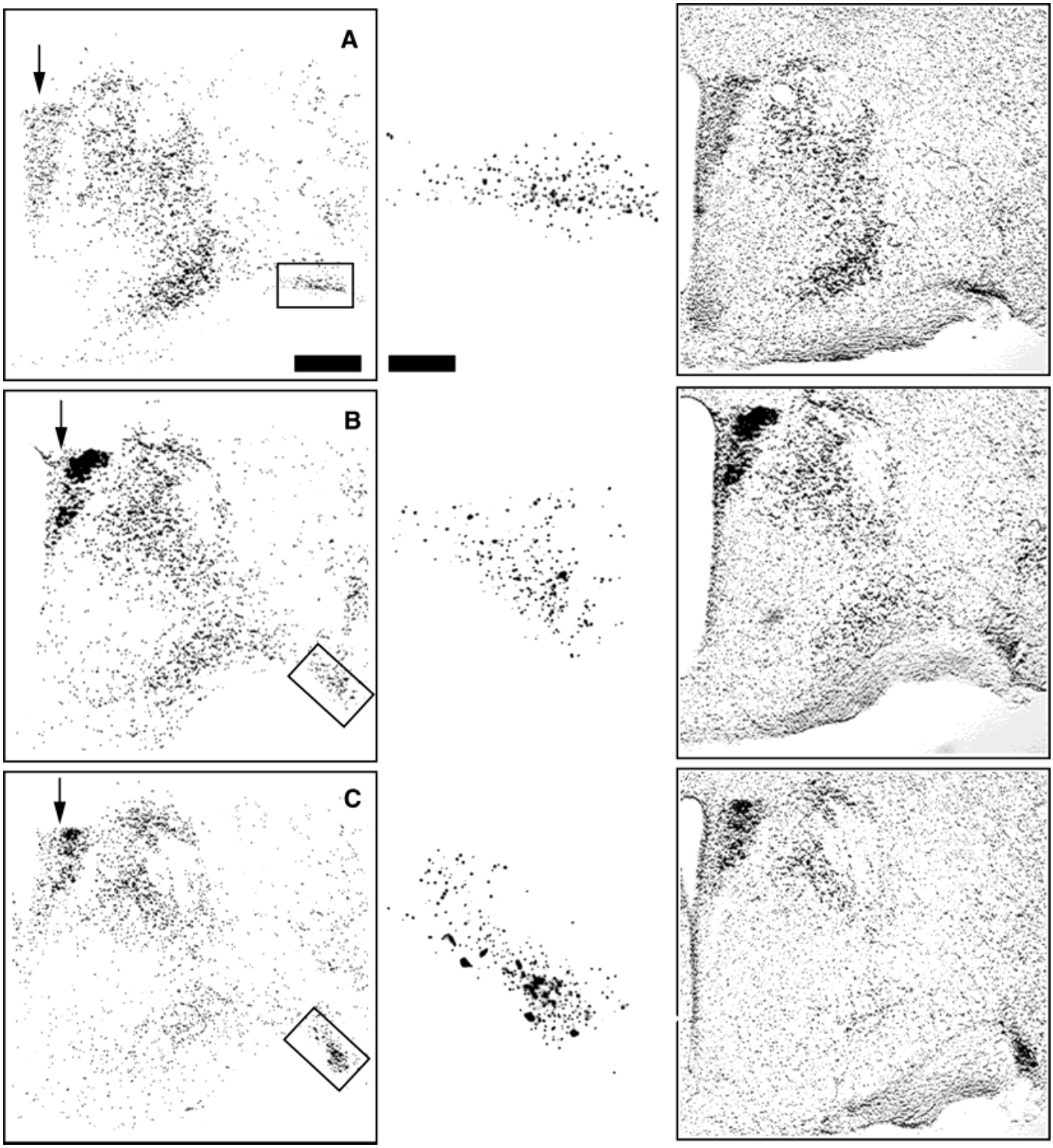


Fig. 5. Transgene induction by LPS is specific to the PVN. Representative coronal sections through the hypothalamus at the level of the PVN and caudal supraoptic nucleus (SON) stained for β-galactosidase activity (left). The same section subsequently counterstained with thionin is shown on the right. Transgene expression 6 hours following i.p. administration of saline (**A**), 400 μg/kg LPS (**B**), or hypertonic saline (0.4 ml, 1.5 M; **C**) are shown. Note that, although there is significant induction of the transgene in the PVN following LPS (B, arrow), there is no detectable up-regulation of transgene expression in the SON (B, box) or elsewhere in the hypothalamus. IL-1β, whether it is administered i.p. or i.c.v., results in an equivalent, PVN-restricted pattern of transgene induction (not shown). In contrast, there is detectable up-regulation of transgene expression in the SON following hypertonic saline treatment (C, box). Boxed regions are shown at higher magnification in the middle. Although only the caudal SON is shown here, the presence or absence of transgene up-regulation in the SON is consistent across the rostral-caudal extent of this nucleus. Sections were viewed with a Zeiss Axioskop microscope (×5 or ×20), and images were captured by using NIH Image software (W. Rasband; NIMH) with an MTI VE1000 Dage camera. Images were cropped, labelled (Adobe Photoshop, Illustrator), and printed without further alterations (phaser 450; Tektronix). Scale bars = 300 μm on the left (also applies to the right), 75 μm in the middle.

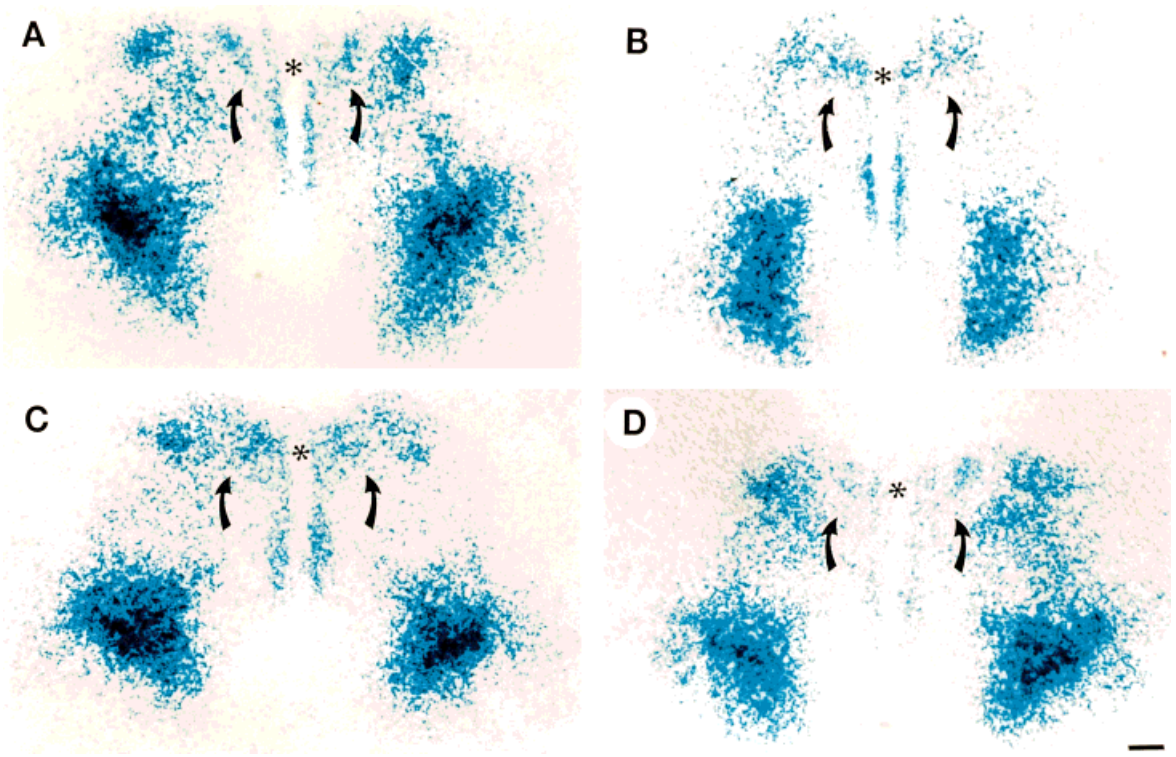


Fig. 6. **A–D:** IL-1β does not induce transgene expression in hypothalamic slice cultures. Photomicrographs of representative hypothalamic slice cultures stained for β-galactosidase activity. Hypothalamic slice cultures maintain their anatomical integrity over 7 days in culture. Transgene expression in slice cultures treated with IL-1β (10 ng/ml) for 4 hours (C,D) did not differ from expression in untreated control slices (A,B). Treatment of shorter (1 or 2 hours) or longer (12 hours) duration also had no effect on transgene expression. Arrows indicate the PVN, and asterisks indicate the third ventricle.: Scale bar = 200 mm.

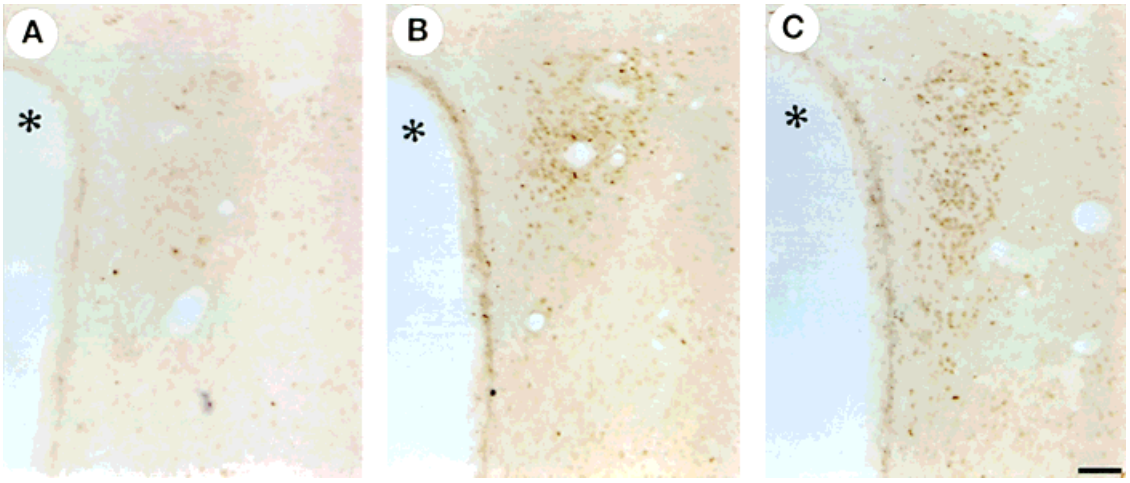
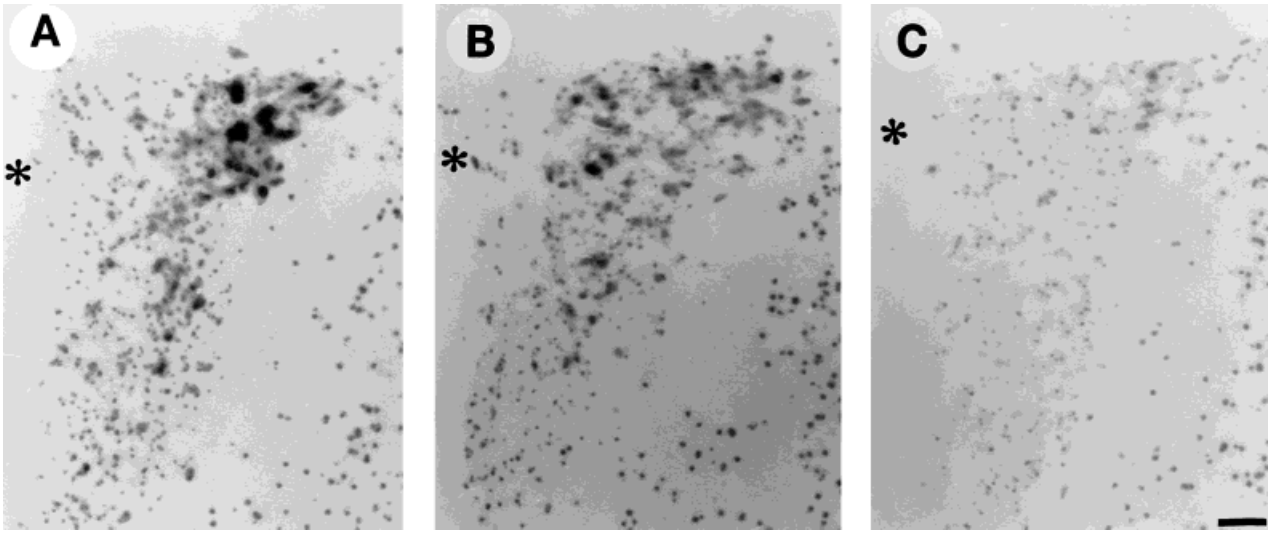


Fig. 7. LPS administration induces phosphocyclic amino-3-hydroxy-5-methyl-4-isoxazole-propionate response element binding protein (phospho-CREB)-like immunoreactivity in the PVN. Photomicrographs of representative coronal sections through the PVN stained for phospho-CREB immunoreactivity. A–C demonstrate phospho-CREB immunoreactivity following no treatment (**A**), 15 minutes following administration of LPS (400 µg/kg, i.p.; **B**), and 30 minutes following administration of LPS (400 µg/kg, i.p.; **C**). Asterisks indicate the third ventricle. Scale bar = 50 µm.

Naltrexone pretreatment inhibits LPS induced up-regulation of transgene expression

We previously demonstrated that opiate administration can modulate response of the transgene to a hypertonic saline stressor (Borsook et al., 1994b). To determine whether endogenous opioid systems may have a role in modulating the response of enkephalinergic neurons in the PVN to an immunologic stressor, we pretreated transgenic mice for 24 hours with the opioid antagonist naltrexone (30 mg; delivered by subcutaneous pellet) prior to administra-



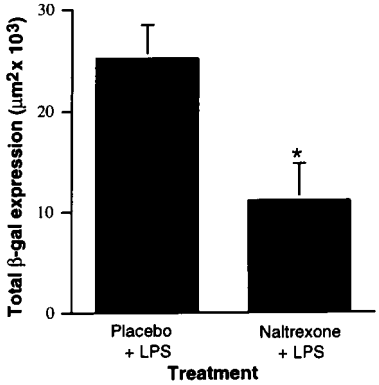


Fig. 8. Naltrexone pretreatment inhibits transgene induction by LPS. Photomicrographs of representative coronal sections through the PVN stained for β-galactosidase activity. A–C demonstrate transgene expression levels 6 hours following administration of LPS (400 μg/kg) with no pretreatment (**A**), administration of LPS following pretreatment with a placebo pellet (**B**), and administration of LPS following pretreatment with the opioid antagonist naltrexone (30 mg; subcutaneous pellet) for 24 hours (**C**). Naltrexone treatment alone does not alter basal levels of transgene expression significantly. Asterisks indicate the third ventricle. **Graph:** Quantitation of total β-galactosidase expression (μm^2) in the PVN 6 hours following the administration of LPS (400 μg/kg, i.p.) to mice pretreated for 24 hours with either placebo or naltrexone (30 mg; subcutaneous pellet). Naltrexone pretreatment significantly inhibits LPS-induced up-regulation of transgene expression compared with placebo pretreatment ($P < 0.01$; Student's t test). Scale bar = 50μ m.

tion of LPS. Naltrexone treatment alone does not alter basal levels of transgene expression significantly (data not shown). LPS (400 μg/kg) administration in untreated (Fig. 8A) or placebo-treated (Fig. 8B) mice induces the expected up-regulation of transgene expression. This up-regulation is blocked by naltrexone pretreatment (Fig. 8C).

DISCUSSION

Endogenous opioids within the hypothalamus have been hypothesized to regulate both neuroendocrine and autonomic function; however, relevant anatomic substrates underlying such modulatory influence remain largely unidentified (Holaday, 1983; Pfieffer and Herz, 1984; Szekely, 1990; McCubbin, 1993; Hammer and Cheung, 1995). The PVN, which is an important site for integration of neuroendocrine, autonomic, and behavioral responses, is an obvious candidate. The fact that proenkephalin gene expression demonstrates transcriptional plasticity within neurons of the PVN in response to a variety of stressful challenges lends support to the idea that enkephalin expressed within neurons of the PVN has a role to play in the response of this nucleus to stress (Lightman and Young, 1987; Sternberg it al., 1989; Watts, 1991; Young and Lightman, 1992; Borsook at al., 1994a,b; Mansi et al., 1998). Regulation of the expression of gene products with a direct role in modulation of neuronal or neuroendocrine signaling is one means by which the nervous system's response to stressful challenge may be controlled over time. Such control is particularly important in response to challenges to bodily homeostasis, as in activation of an immune response. Investigations into the intercellular and intracellular signaling mechanisms of transcriptional control within PVN neurons, thus far, have focused on the regulation of immediate-early genes, such as c-fos and nerve growth factor-induced gene-B (NGFI-B), and of neuropeptide genes

whose products have clearly defined roles in the neuroendocrine response to stress (Lightman and Young, 1988; Bartanusz et al., 1993; Chan et al., 1993; Chang et al., 1993; Kakucska et al., 1993; Brady et al., 1994; Honkaniemi et al., 1994; Rivest and Rivier, 1994; Herman, 1995; Sagar et al., 1995; Elmquist et al., 1996; Kovacs and Sawchenko, 1996). In particular, the CRH and vasopressin genes demonstrate up-regulation in response to stressful challenge, each with its own time course following stimulation, suggesting that differing regulatory mechanisms are at work in each case (Lightman and Young, 1988; Bartanusz et al., 1993; Kakucska et al., 1993; Rivest and Rivier, 1994; Herman, 1995; Kovacs and Sawchenko, 1996).

In this study, by using a transgenic model system, we demonstrate that administration of an immunologic stressor, in the form of LPS or IL-1β, leads to up-regulated expression of a proenkephalin-β-galactosidase transgene in a circumscribed population of neurons within the PVN. The proenkephalin-β-galactosidase transgene has been shown previously to be regulated in parallel with the endogenous proenkephalin gene (Borsook et al., 1992, 1994a,c). Although the exact role of enkephalin expression in the PVN is not yet clear, its induction within the PVN in response to challenge with LPS or IL-1β suggests that it plays a role in one or more of the effector functions of the PVN in response to immunologic activation. The proenkephalin gene is expressed in magnocellular, parvocellular, and autonomic components of the PVN and is coexpressed in distinct neuronal subsets with other neuropeptides, including CRH, vasopressin, and neurotensin (Sawchenko and Swanson, 1982; Fallon and Leslie, 1986; Merchenthaler et al., 1986; Harlan et al., 1987; Pretel and Piekut, 1990; Sakanaka et al., 1990; Watts and Sanchez-Watts, 1995). Enkephalin-immunoreactive neurons in the parvocellular PVN are known to extend axons to the external zone of the median eminence, where enkephalin peptides are likely coreleased along with CRH and vasopressin (Merchenthaler, 1992). A small subset of enkephalin-immunoreactive neurons in the PVN have been shown by retrograde labelling to extend axons to the dorsal vagal complex and the spinal cord, where they may influence autonomic responses (Sawchenko and Swanson, 1982). It is a subset of the proenkephalin-β-galactosidase transgene-expressing neurons in the PVN that show induction following either LPS or IL-1β (i.p. or i.c.v.). At present, we do not possess sufficient information to state precisely which subpopulation of enkephalinergic cells may be responsive. Further definition of the neurochemical phenotype and connectivity of these cells should contribute to an understanding of the physiologic response to an immunologic challenge and what role these proenkephalin-expressing neurons might play in that response.

Technical considerations—a transgenic model

These studies were carried out by using a transgenic mouse model system in which the regulatory sequences of the human proenkephalin gene (3 kb of 5′-flanking sequence, the first exon and intron, and 1.2 kb of 3′-flanking sequence) direct expression of the reporter *Escherichia coli* β-galactosidase (Borsook et al., 1992; Borsook and Hyman, 1995). We demonstrated previously that this construct contains sufficient information to replicate expression of the endogenous proenkephalin gene within the PVN both basally and in response to a variety of stressors, including hypertonic saline stress, cold swim stress, hypovolemia, and naloxone-precipitated opiate withdrawal (Borsook et al., 1994a–c). The model provides a highly sensitive assay of proenkephalin gene expression with cellular specificity. The transgenic model system shares the attributes of high sensitivity and ready quantitation compared with standard, semiquantiative in situ hybridization methods and provides the added advantage of a rapid (overnight), single-step assay of gene induction. More important is the fact that the complete genomic sequences responsible for the observed regulation are known to be contained within the defined transgene. This knowledge will aid in future analyses of in vivo transcriptional mechanisms, particularly as further transgenic models are developed, including knock-outs directed at potentially relevant signaling systems or those allowing in vivo manipulation of signal-transduction mechanisms. The extensive knowledge of preproenkephalin gene regulation in other systems provides important background for investigation of the mechanism of transcriptional control of proenkephalin gene expression in the PVN in vivo. Another advantage is that the transgenic model allows us to overcome the difficulty of observing enkephalin peptide expression in PVN neurons in the basal state. Colchicine pretreatment generally is required to allow sufficient accumulation of enkephalin peptide in PVN neurons to allow substantial visualization of immunoreactivity (Sawchenko and Swanson, 1982). The ability to identify enkephalin/transgene-expressing cells in both the basal state and following induction becomes increasingly important in light of the fact that there are potentially identifiable subpopulations within the enkephalin/transgene-positive population that may respond differentially to a particular stimulus. One major disadvantage of the transgenic model compared with standard in situ hybridization methods, particularly those that use intronic probes, is a loss of resolution with respect to the timing of transcriptional events, because it is translated protein levels that are assayed rather than messenger RNA levels.

Transgene quantitation

Our method of transgene quantitation reflects a changing intracellular pattern of transgene product localization as increasing quantities accumulate within the cell. Basal expression of the proenkephalin-β-galactosidase transgene is apparent following X-gal staining as small, blue puncta within cells. Upon up-regulation of transgene expression, X-gal staining demonstrates filling of cells with blue X-gal reaction product. This feature of transgene expression allows for a simple, filled area-based method of quantitation in which the total area within a defined border around the PVN (see Fig. 3) that stains positive for β-galactosidase is taken as the measure of transgene expression. This method permits a semiquantitative measure of the underlying pattern and degree of proenkephalin gene induction. Dose and time course experiments (Figs. 1–3) indicate a substantial range of expression levels based on this filled-area measure; however, the linearity of this measure with respect to underlying β-galactosidase protein levels has not been established rigorously. Saturation effects are expected. With increased transgene expression, the remaining unfilled area is diminished, and substantial signal overlap may occur, leading to an underestimate of true induction levels. In a previous study of transgene induction, this filled-area method gave

functionally equivalent results compared with quantitation of transgene expression by means of counting the number of induced cells, with an induced cell defined as any cell within the PVN with a staining profile larger than a defined basal profile (Borsook et al., 1994b). Thus, we are confident that the filled-area quantitation method used here is a reasonable measure of transgene expression, allowing for objective relative comparison between animals and across experimental groups.

Potential pathways

Systemic LPS and IL-1β. The mechanisms by which LPS or IL-1β administration lead to up-regulation of the proenkephalin-β-galactosidase transgene must be addressed first at the systems level. The effects of LPS administration on function of the neuroendocrine axis have been attributed largely to the release of cytokines, IL-1β in particular, from activated peripheral immune cells (Cavaillon and Haeffner-Cavaillon, 1990; Molloy et al., 1993). Recent studies of transgenic IL-1β knock-out mice have demonstrated that expression of IL-1β is not strictly necessary for generation of inflammatory responses to LPS, such as anorexia, activation of the hypothalamopituitary-adrenal axis, and increases in other inflammatory cytokines, such as IL-1α, tumor necrosis factor-α and IL-6 (Zheng et al., 1995; Fantuzzi and Dinarello, 1996; Alheim et al., 1997), although some aspects of the response are altered in the absence of IL-1β (Alheim et al., 1997; Fanggioni et al., 1998). These studies suggest a significant degree of functional redundancy in the cascade of proinflammatory cytokines following LPS administration, with IL-1β playing an important but not obligatory role. In addition to its role in up-regulating production of IL-1β and other cytokines, LPS also can act directly through the CD14 receptor without participation of any proinflammatory cytokines (Becher et al., 1996; Schumann et al., 1998).

Obligatory participation of systemic IL-1β in LPS-induced up-regulation of proenkephalin gene expression in the PVN remains to be established. However, systemic administration of IL-1β is known to elicit a set of responses that is similar to that observed following LPS (Dinarello, 1996). This study demonstrates that this includes up-regulation of the proenkephalin gene in the PVN. Induction of the transgene in response to IL-1β was transient (4–6 hours) compared with that following LPS (12 hours). This shorter time course of response may reflect a more limited cascade of signals initiated by IL-1β compared with LPS, or it may reflect a capacity to rapidly clear this proinflammatory cytokine from circulation (Klapproth et al., 1989; Oliver et al., 1993). In addition to multiple systemic signals that are initiated following LPS and IL-1β, activation of vagal fibers comprises a further potential signaling pathway (Gaykema et al., 1995; Goehler et al., 1997).

Central IL-1β. In addition to stimulating the production and secretion of IL-1β and other cytokines in the periphery, LPS administration at relatively high doses is known to induce production of IL-1β in the central nervous system, primarily in choroid plexus, meninges, perivascular cells, and microglia (Van Dam et al., 1992, 1995; Hillhouse and Mosley, 1993; Laye et al., 1994; Buttini and Boddeke, 1995; Gabellec et al., 1995). Systemic IL-1β administration also can lead to an induction of IL-1β mRNA in the central nervous system (Hansen et al., 1998). One recent study indicates that similar induction of brain IL-1β may be observed following a nonimmunologic stressor (Nguyen et al., 1998). Although specific signaling targets for this brain IL-1β have yet to be defined, administration of IL-1β directly into the cerebroventricular system can replicate the effects of peripherally applied IL-1β, such as fever, slow-wave sleep, hyperalgesia, reduced food and water intake, and reduced social interaction, with a lower dose requirement (Rothwell and Hopkins, 1995). Consistent with these findings, IL-1β administered directly into the cerebroventricular system is a more potent stimulus for up-regulation of the proenkephalin-β-galactosidase transgene than IL-1β delivered i.p. It remains an open question whether the signals invoked by i.c.v.-administered IL-1β truly mimic those used by the endogenously produced cytokine. For example, one potential explanation for the efficiency with which i.c.v.-administered IL-1β induces activation of the transgene may lie in the high levels of IL-1 receptor expressed in the choroid plexus, meninges, and cerebral blood vessels to which IL-1β delivered into the ventricle through an indwelling chronic cannula would have immediate and direct access (Cunningham et al., 1992; Cunningham and De Souza, 1993; Ericsson et al., 1995).

Direct action of i.c.v.-delivered IL-1β on PVN neurons or immediately surrounding cells is another potential explanation for its efficiency of action, but our results do not support such a hypothesis. Isolated coronal slices of the hypothalamus derived from P6–P8 transgenic mice are responsive to forskolin treatment (Borsook et al., 1998). However, such slice cultures demonstrate no change in proenkephalin-β-galactosidase transgene expression when treated directly with IL-1β. Based on these data, we conclude that, despite the potential availability of IL-1β in the vicinity of the PVN (either induced following LPS or IL-1β administration or applied directly), transgene induction is not the result of direct action of IL-1β on neurons of the PVN. This conclusion is not surprising in light of the fact that in situ hybridization studies of IL-1β receptor distribution have failed to detect appreciable receptor expression in the PVN (Cunningham et al., 1992; Cunningham and De Souza, 1993; Ericsson et al., 1995). However, it is important that caution be used in interpreting such in vitro studies. It remains possible that, under culture conditions, paraventricular neurons do not respond to IL-1β as they might in vivo. Activation of glial cell populations (which can produce IL-1β under certain conditions) could alter the local environment substantially, for example, inducing IL-1β receptor desensitization (Lee et al., 1995; Coltman and Ide, 1996). Local microvasculature, an important potential target of IL-1β, also is disrupted under culture condition. However unlikely, given the available receptor expression data, it also remains possible that direct IL-1β action at the level of the PVN is a necessary but insufficient stimulus for transgene induction in the absence of other significant efferent inputs that are not present under slice culture conditions.

We have demonstrated proenkephalin transgene up-regulation in the PVN in response to i.p. LPS, i.p. IL-1β, and i.c.v. IL-1β administration. It remains to be determined whether overlapping or distinct signaling pathways are involved in activating PVN neurons and inducing this up-regulation in each case. Determination of the relative importance of signaling pathways known to be active following immune challenge, such as prostaglandin-

dependent, catecholaminergic, and vagal pathways, to up-regulation of the proenkephalin gene following these stimuli may lend some insight (Ericsson et al., 1996, 1997; Scammell et al., 1996; Goehler et al., 1997). Our dose-response data provide evidence against an obligatory role for central IL-1β as an essential component in transgene induction following LPS administration. Although LPS administration has been shown to induce IL-1β mRNA expression throughout the brain, the doses of LPS administered in these studies tend to be relatively high (400–2,500 μg/kg; Van Dam et al., 1992, 1995; Hillhouse and Mosley, 1993; Laye et al., 1994; Buttini and Boddeke, 1995; Gabellec et al., 1995). We demonstrate significant induction of transgene expression following 16 μg/kg LPS (0.4 μg/25g mouse), a dose at which induction of central IL-1β expression, to our knowledge, has not been reported. Intracerebroventricular administration of IL-1β receptor antagonist or a blocking antibody in conjunction with systemic LPS or IL-1β administration will be necessary to address this issue directly.

Our observation that up-regulation of transgene expression is limited to a subset of transgene-expressing cells in the PVN deserves mention to emphasize the fact that, although there appears to be some overlap, the neuronal signaling pathways activated following immune challenge demonstrate clear differences from those activated by other stress paradigms that we have investigated (Borsook et al., 1994a,c). In particular, transgene expression clearly is induced in the SON under conditions of hypertonic saline stress and following naloxone-precipitated opiate withdrawal, whereas it clearly is absent in response to immune challenge. An explanation for these differences awaits further elucidation of the signaling mechanisms underlying proenkephalin-β-galactosidase transgene induction in vivo.

Intracellular signals

Knowledge of preproenkephalin gene regulation in other systems provides a valuable guide in searching out the mechanism of transcriptional control of proenkephalin gene expression in vivo. For example, CREB, a constitutively expressed transcription factor that is activated by phosphorylation, has been shown to be an important intracellular regulator of proenkephalin transcription (Hyman et al., 1988; Konradi et al., 1993, 1995). Phosphorylated CREB also is thought to be an important mediator of transgene expression in the PVN in response to hypertonic saline stress and is known to interact with a CRE element in the promoter of the proenkephalin gene that is contained within our transgene construct (Borsook et al., 1994c). In this study, we demonstrate that up-regulation of the proenkephalin transgene is correlated with induction of phospho-CREB immunoreactivity in the PVN following immune challenge. We demonstrate that phospho-CREB is induced at 15 minutes and 30 minutes following LPS administration. Thus, CREB phosphorylation precedes induction of the transgene. We recently demonstrated that phospho-CREB is induced for many hours following a single injection of LPS (Borsook, unpublished data). This may provide a partial explanation for the prolonged induction of the transgene. In addition, one reason for the decrease in proenkephalin gene expression observed at 24 hours and 48 hours following LPS administration may be due to the relative effects of inhibitory transcriptional regulators, such as cAMP-responsive element modulator/inducible cAMP early repressor (CREM/ICER), which is present in the PVN (Foulkes et al., 1991, 1996; Mellstrom et al., 1993). ICER is known to inhibit proenkephalin gene expression in cell line cultures (Foulkes et al., 1991). Furthermore, we have shown recently that CREM/ICER is induced in the PVN following LPS (Smirnova et al., 1998) and also following muscimol, a treatment that is known to inhibit proenkephalin gene expression in the PVN (Borsook et al., 1998). At present, we do not know whether phosphorylation of CREB is localized within neurons demonstrating induction of the transgene. We previously demonstrated that CREB is present in essentially all hypothalamic neurons, including those expressing the transgene; however, due to the time sequences of CREB phosphorylation followed by expression of the β-galactosidase protein, phospho-CREB localization within "induced" neurons has proven difficult (Borsook et al., 1994c). The observed induction of a transgene under the control of defined sequences and of CREB phosphorylation is only a first step in understanding the signal transduction in PVN neurons initiated by immunologic stressors, and it remains to be demonstrated that CREB is involved directly in the induction of transgene expression in vivo.

Although phosphorylated CREB certainly plays a key role in regulation of proenkephalin gene expression, other potential mechanisms also must be considered. In the context of proenkephalin gene regulation under conditions of stress, it is important to evaluate the potential role for glucocorticoid-mediated mechanisms. Glucocorticoids have been demonstrated to act in concert with cAMP mechanisms to positively regulate expression of the endogenous rat proenkephalin gene in C6 glioma cells, although the signaling mechanisms and regulatory elements by which this regulation occurs remain unclear (Yoshikawa and Sabol, 1986a,b). Glucocorticoids also have been show to influence striatal preproenkephalin expression in vivo, although, again, the mechanisms underlying this regulation are not understood (Chao and McEwen, 1990). A search by computer through the human proenkephalin 5′-flanking regions included in our transgene construct revealed no obvious elements resembling the positive or negative GRE consensus sequences, suggesting an indirect mechanism of glucocorticoid action.

Opioid inhibition of transgene induction

Opioids are known to have profound effects within the neuroendocrine hypothalamus. A potential endogenous substrate for these effects exists in the expression of opiate peptides, such as enkephalin and dynorphin, within effector neurons of the PVN (Merchenthaler, 1992). However, there is also evidence that PVN neurons themselves are under the influence of opioid systems (Pfeiffer and Herz, 1984; Tsagarakis et al., 1990; Koch et al., 1995; Kotz et al., 1995). In this study, we demonstrate that pretreatment with the opiate antagonist naltrexone can block proenkephalin-β-galactosidase transgene induction following LPS administration. The effects of naltrexone on transgene expression are not restricted to transgene up-regulation by LPS. A previous study determined that pretreatment with naltrexone can inhibit the transgene response to a subsequent hypertonic saline stress (Borsook et al., 1994b). Targets responsible for this opioid modulation may occur at multiple levels, both peripheral and central. For example, enkephalins can modulate the production and activation of immune cells in the periphery

(Sibinga and Goldstein, 1998). There is also the potential for direct action on PVN neurons. Opiate receptors are expressed within the PVN (Desjardins et al., 1990; Mansour et al., 1994, 1995), and there is substantial opioid innervation of the PVN arising from limbic, hypothalamic, and brainstem regions (Beaulieu et al., 1996). The direct physiological effects of opioids, in general, are inhibitory. The predicted result of removing direct opioid influence would be an increase in response output rather than the observed inhibition of transgene up-regulation following naltrexone pretreatment. We previously proposed γ-aminobutyric acid (GABA)ergic interneurons as a possible target of opioid modulation in the hypothalamus (Borsook et al., 1994b), and we recently demonstrated the existence of tonic GABAergic input to transgene-expressing PVN neurons (Borsook et al., 1998). Acute, anatomically selective inhibition of opioid function will be necessary to address the validity of these speculations. The ability of opioid systems to modulate the response of PVN neurons to immune challenge is a further indication of the complex systemic and local interactions that underlie the response of PVN neurons to stress.

CONCLUSIONS

The present study demonstrates that, in our transgenic mice, transcription of the proenkephalin gene is up-regulated in a subset of PVN neurons following administration of LPS (i.p.) or IL-1β (i.p. or i.c.v.). This up-regulation of transgene expression does not appear to result from direct action of IL-1β at the level of the PVN but, rather, through as yet unidentified intermediates. Furthermore, a role for endogenous opioid systems in modulating the transcriptional response of PVN neurons to immune challenge was demonstrated. Our transgenic model allows us to follow the transcriptional regulation of a well-defined gene in an anatomically complex brain region. In future studies, the model will allow us to evaluate the potential contribution of other neurotransmitter systems and neuromodulators to the control of transcriptional regulation within PVN neurons in response to immune challenge.

ACKNOWLEDGMENTS

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