

Short communication

## CREB phosphorylation in the nucleus of the solitary tract and parabrachial nucleus is not altered by peripheral cholecystokinin that induces c-Fos

Thomas A. Houpt \*

*E.W. Bourne Behavioral Research Laboratory, Department of Psychiatry, Cornell University Medical College, 21 Bloomingdale Road, White Plains, NY 10605, USA*

Accepted 3 December 1996

### Abstract

Ca<sup>++</sup>/cAMP response element binding protein (CREB), phosphoCREB, and c-Fos-like (c-FL) immunoreactivity (IR) were examined in the nucleus of the solitary tract (NTS) and parabrachial nucleus (PBN) after peripheral cholecystokinin (CCK). c-FLIR was observed only after CCK, but CCK did not alter high basal levels of CREB-IR and phosphoCREB-IR. PhosphoCREB may be necessary but is not sufficient to induce c-Fos after CCK injection.

*Keywords:* Immediate-early gene; Neuropeptide; Transcription factor; Food intake; Immunohistochemistry

Several studies have demonstrated activation of the mNTS and latPBN as measured by *c-fos* expression following peripheral injection of CCK [2,3,5,13,14,16]. The mNTS and latPBN are the first and second central relays, respectively, of visceral sensory information conveyed by the vagus nerve in the rat, which mediates the behavioral effects of low doses of exogenous CCK (e.g., satiety of feeding) [20]. The role of CREB and other intracellular pathways in the induction of c-Fos in the mNTS and latPBN are unknown, however. Transcription of the *c-fos* gene is regulated by multiple signaling pathways and transcription factors. While *c-fos* mRNA or protein expression can be visualized in single cells by in situ hybridization or immunohistochemistry, the activation of transcription factors is usually approximately correlated with c-Fos induction using homogenized brain regions in ex vivo assays (e.g., gel mobility shifts which only demonstrate DNA binding, not transactivation).

Recently, however, antisera have been produced that specifically recognize the phosphorylated and activated forms of transcription factors (e.g., CREB [8]). In this study the specific antiserum against CREB phosphorylated at Ser 133 (phosphoCREB; the transactivatory form) was

employed to determine if CREB activation is correlated with c-Fos induction in the mNTS and latPBN after CCK.

After 17-h food deprivation, adult male rats ( $n = 8$ ) received intraperitoneal injections of 8  $\mu\text{g}/\text{ml}/\text{kg}$  sulfated cholecystokinin octapeptide (a kind gift of the Bristol-Myers-Squibb Pharmaceutical Research Institute) or 1 ml/kg isotonic saline vehicle injections ( $n = 4$ ). One hour after injection, the rats were transcardially perfused, and brains were post-fixed and cryoprotected.

To determine if CREB phosphorylation was time or dose dependent, additional rats were killed 15 min after 8  $\mu\text{g}/\text{kg}$  CCK injection ( $n = 2$ ), or 1 h after 32  $\mu\text{g}/\text{kg}$  CCK injection ( $n = 2$ ).

Forty micron coronal sections were cut on a microtome through the rostral-caudal extent of the NTS. Eighteen sections were cut through the pons at the level of the parabrachial nucleus. After permeabilization, alternating free-floating sections were incubated overnight with rabbit anti-CREB (1:5,000), rabbit anti-phosphoCREB (0.73  $\mu\text{g}/\text{ml}$ ; both from Upstate Biotechnology, Lake Placid, NY) or sheep anti-c-Fos peptide antibody (Genosys, The Woodlands, TX) at a dilution of 1:3,000. Antibody complexes were visualized by ABC and diaminobenzadine reactions (Vector Lab.).

Cells expressing positive, nuclear IR were quantified in the medial subpostremal NTS and the latPBN. Three adjacent sections, each stained for one of the three probes,

\* Corresponding author. Fax: +1 (914) 682-3795; e-mail: tah2@cornell.edu

were digitized at the level of densest c-FLIR as 8-bit monochrome images and immunoreactive nuclei were counted by hand using the cell scoring macros of the NIH Image software (W. Rasband, NIH). One section stained for each probe was counted for each brain region from each rat. NTS staining was quantified bilaterally medial to the solitary tract at the level of the area postrema; PBN staining lateral to the brachium conjunctivum and medial to the ventral spinocerebellar tract was quantified unilaterally. Cell counts for each section were normalized by area (mNTS =  $0.72 \pm 0.02 \text{ mm}^2$ ; latPBN =  $0.33 \pm 0.01 \text{ mm}^2$ ) to give IR density (nuclei per  $\text{mm}^2$ ). Counting was done blind to treatment and analyzed by ANOVA and Fisher's post-hoc test.

CREB-IR appeared to label every nuclei in the brainstem (Fig. 1A,B). No difference CREB-IR was found between saline ( $1730 \pm 137$ ) and CCK treated groups ( $1630 \pm 66$ ). PhosphoCREB-IR was much sparser throughout the brainstem (Fig. 1C,D). Dense labeling was seen, however, in the mNTS. PhosphoCREB-IR was approximately 50% of CREB-IR counted on adjacent sections. CCK did not change phosphoCREB-IR compared to saline treatment ( $959 \pm 164$  vs.  $886 \pm 104$ ).

CCK significantly increased the c-FLIR-positive cells in the mNTS compared to vehicle injection ( $17 \pm 5$  vs.  $221 \pm 28$ ,  $P < 0.0005$ ), and also increased c-FLIR in the adjacent area postrema (Fig. 1F). The density of c-FLIR after CCK was only 25% that of phosphoCREBIR and

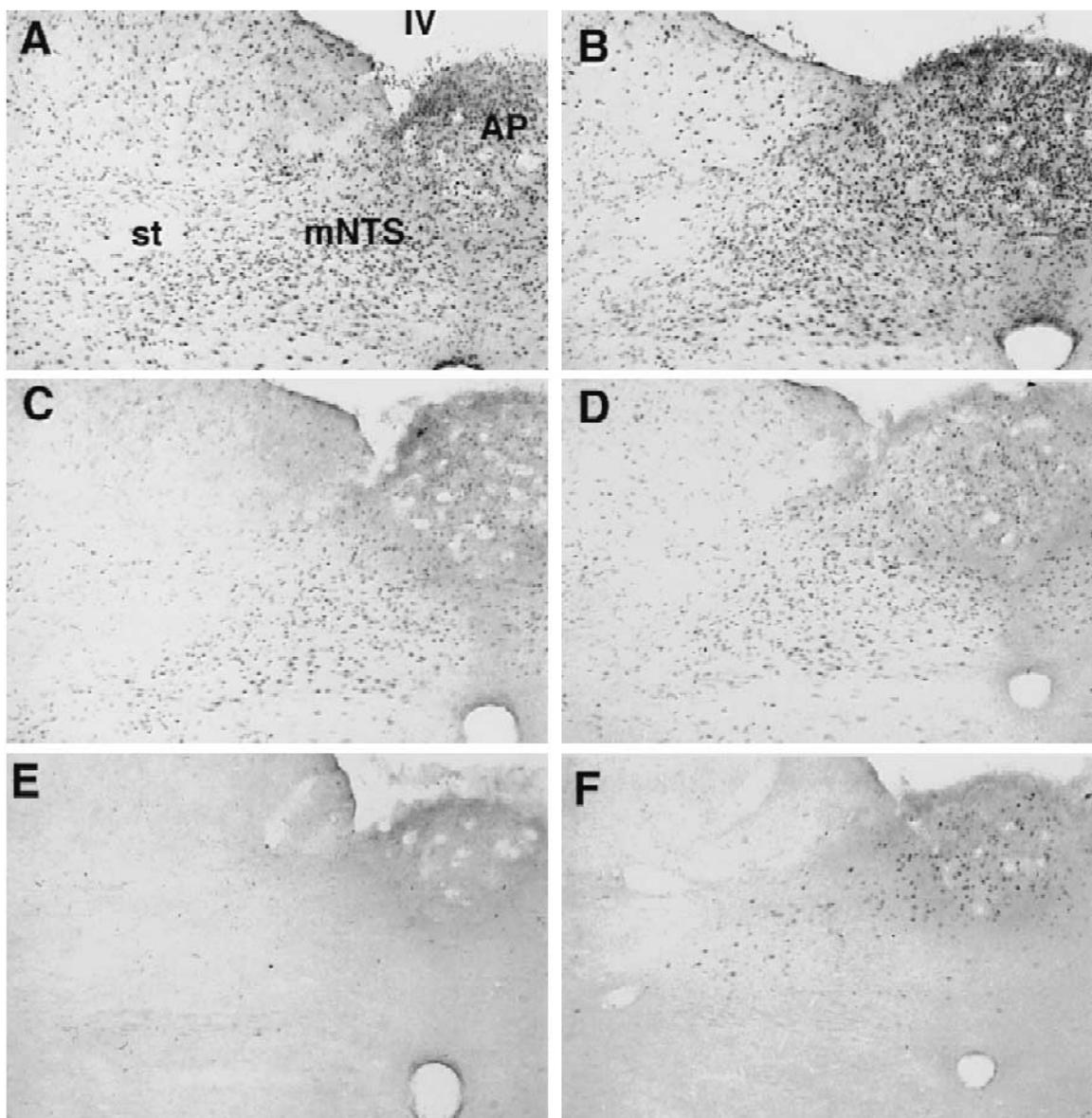


Fig. 1. Bright field photomicrograph of CREB (A,B), phosphoCREB (C,D) and c-Fos-like (E,F) immunoreactivity in the medial NTS after i.p. injection of saline (A,C,E) or CCK (B,D,F). There was no difference in the density of CREB-IR or phosphoCREB-IR between saline- and CCK-injected rats. Large numbers of c-FLIR-positive cells were only present in the mNTS and AP of the CCK-injected rat. st, solitary tract, mNTS, medial nucleus of the solitary tract, AP, area postrema, IV, fourth ventricle.

only 14% of CREB-IR. (Note that c-Fos may be induced primarily in neurons, while the cell counts for CREB and phosphoCREB may include glial as well as neuronal staining.)

PhosphoCREB-IR in the mNTS of rats killed 15 min after CCK (8  $\mu\text{g}/\text{kg}$ ) was not increased compared to saline or 1 h after CCK ( $740 \pm 96$  nuclei/ $\text{mm}^2$ ). c-FLIR at 15 min ( $21 \pm 8$  nuclei/ $\text{mm}^2$ ) was not different from saline-induced c-FLIR. A larger dose of CCK (32  $\mu\text{g}/\text{kg}$ ) also did not increase phosphoCREB-IR at 1 h ( $636 \pm 106$  nuclei/ $\text{mm}^2$ ), but did increase c-FLIR ( $381 \pm 143$  nuclei/ $\text{mm}^2$ ).

The pattern of CREB, phosphoCREB, and c-Fos-like IR in the latPBN paralleled the pattern observed in the mNTS. CREB-IR was dense throughout the latPBN (Fig. 2A,B). PhosphoCREB-IR was less than 50% as dense as CREB-IR, with notable densities of labeling in the external and ventral subnuclei of the latPBN (Fig. 2C,D). Neither CREB nor phosphoCREB density was altered by CCK injection ( $2360 \pm 207$ ,  $1174 \pm 155$ ) compared to vehicle levels ( $2187 \pm 279$ ,  $1141 \pm 167$ ). Although apparent increases in c-FLIR were seen in the external and ventral subnuclei of the lateral PBN after CCK (Fig. 2F), CCK did not significantly increase c-FLIR over vehicle as quantified across the entire extent

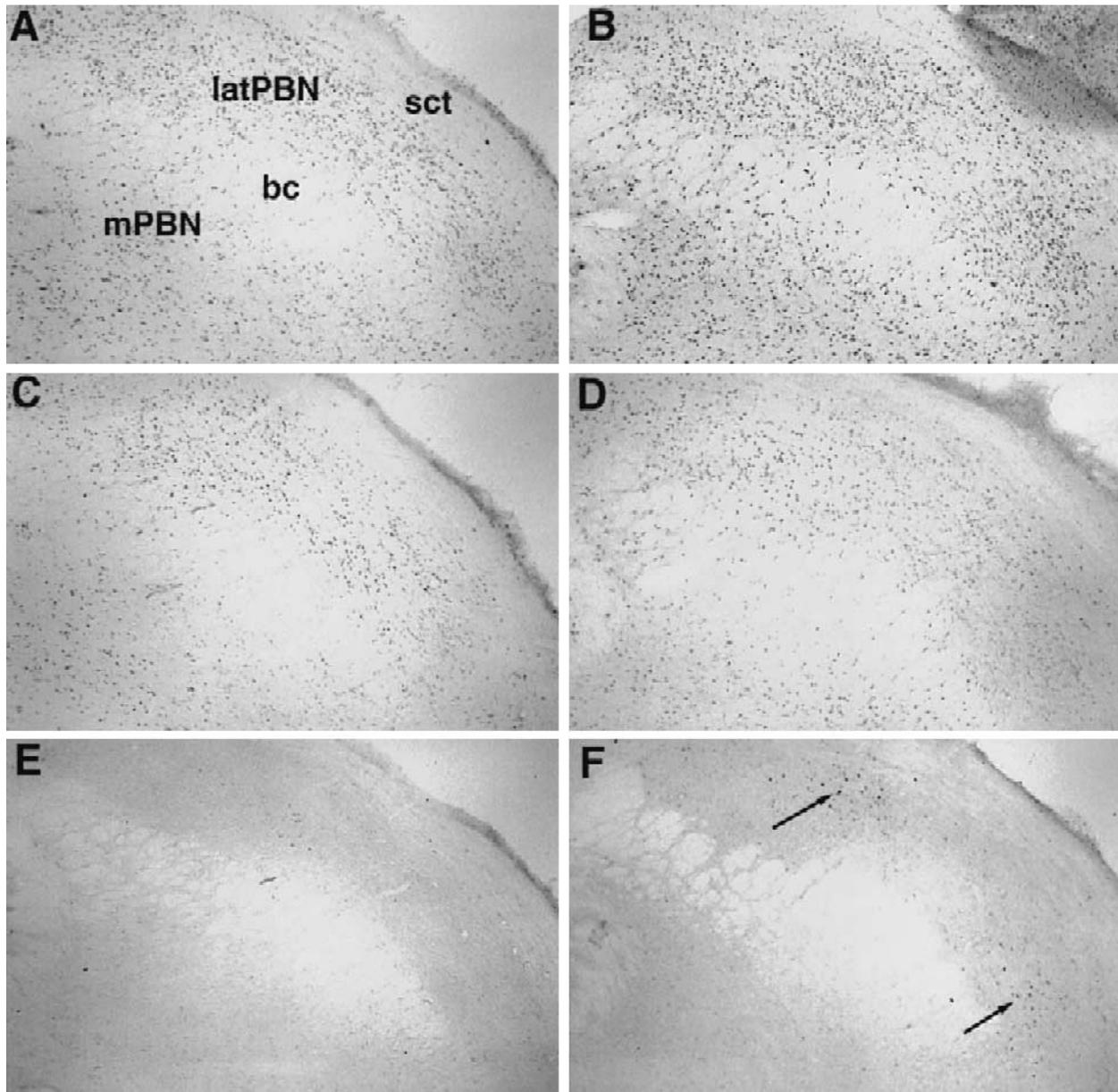


Fig. 2. Bright field photomicrograph of CREB (A,B), phosphoCREB (C,D) and c-Fos-like (E,F) immunoreactivity in the lateral PBN after i.p. injection of saline (A,C,E) or CCK (B,D,F). There was no difference in the density of CREB-IR or phosphoCREB-IR between saline- and CCK-injected rats. Cells containing c-FLIR-positive cells were densest in the external and ventral subnuclei of the lateral PBN in CCK-injected rats (arrows). sct, ventral spinocerebral tract, bc, brachium conjunctivum, latPBN, lateral parabrachial nucleus, mPBN, medial parabrachial nucleus.

of the latPBN ( $120 \pm 29$  vs.  $217 \pm 42$ ,  $P < 0.10$ ). c-FLIR after CCK injection was less than 20% and 10% the density of phosphoCREB-IR and CREB-IR, respectively.

In other brain regions, CREB-IR nuclei were ubiquitous, while phosphoCREB-IR was sparser than CREB labeling. Dense phosphoCREB-IR was seen in the mNTS (but not lateral NTS), the area postrema, dorsal motor nucleus, and C1/A1 cell groups, and the inferior olives contained dense phosphoCREB-IR in both saline- and CCK-treated rats. In the pons, the latPBN, medial PBN, locus ceruleus, posterodorsal tegmental gray, and dorsal central gray had dense phosphoCREB-IR.

CREB expression in the NTS and PBN was not acutely altered by CCK. CREB expression is not rapidly altered by other acute treatments [1,8,11,18,19], although there have been some reports of upregulated CREB expression (e.g., in glial cells following axotomy of neighboring neurons [9]).

While phosphorylation of CREB has been shown to induce *c-fos* expression in vitro and the presence of phosphoCREB-IR in other brain regions is correlated with *c-Fos* induction [1,8,11,18,19], this study found a high density of phosphoCREB-IR in the mNTS and latPBN that was not correlated with CCK treatment or *c-Fos* expression.

These results are consistent with other reports that in some brain regions stimulation increases c-FLIR without increasing phosphoCREB-IR above high control levels [10,18]. In other brain regions, however, increases in phosphoCREB-IR have been correlated with the induction of *c-Fos* [1,8,11,19]. Thus the utility of phosphoCREB-IR as a marker of stimulated neuronal activity may depend on the basal level of CREB phosphorylation in a particular brain region (or on the specific immunohistochemical procedures employed and the reproducibility of phospho-antibody results). In the mNTS and latPBN, phosphoCREB-IR is not a useful marker of activation compared to c-FLIR.

There are a number of caveats in interpreting the immunohistochemical visualization of phosphoCREB. While the presence of phosphoCREB supports the hypothesis that phosphoCREB is necessary for *c-fos* transcription, *c-Fos* expression may also depend on other factors within the stimulated cell. CREB phosphorylation at other sites can enhance (Ser 129 [4]) or inhibit (Ser 142 [6]) Ser133 phosphoCREB activity. CREB may also require association with other CREB-binding proteins (CBP [12] and p300 [15]) to initiate transcription.

Transgenic mice [17] and in vitro transfection studies [7] have shown that multiple *c-fos* promoter elements (e.g., the sis-inducible element, serum response element, and AP-1 element) are required for activation by any one transcription factor. Thus, the activation of other transcription factors (such as the serum response factor activated by the MAP kinase pathway), or the removal of competing CRE-binding factors (such as other members of the bZIP family including CREM, ATF-2, and c-Jun) may also be

required to induce *c-Fos*. Quantitation methods may also have obscured small changes in phosphoCREB quantity or concentration.

Despite these cautions, this study demonstrates that phosphorylation of CREB at Ser 133 is not a sufficient condition for *c-Fos* expression in the mNTS and latPBN, since 75–80% of all phosphoCREB-positive cells did not express *c-Fos*. The presence of pCREB may be a necessary condition for *c-Fos* expression, however, because it seems probable that all c-FLIR-positive cells also contained pCREB-IR. To establish a necessary role for CREB phosphorylation in vivo would require: (1) double-labeling studies to co-localize *c-Fos* and pCREB; and (2) in vivo blockade of CREB synthesis, phosphorylation or DNA binding.

### Acknowledgements

I would like to thank Sandra P. Frankmann for statistical advice, and Gerard P. Smith for a critical reading of the manuscript. This work was supported by the New York Obesity Center and the Whitehall Foundation.

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