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Short Communications

Activation of the zinc finger encoding gene *krox*-20 in adult rat brain: comparison with *zif*268

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Zif268 and *krox*-20 are transcription regulatory factors that contain highly homologous zinc finger DNA-binding domains. Recent studies have demonstrated that *zif268* expression is rapidly regulated in brain by neuronal stimulation. We now report that, like *zif268*, *krox*-20 is rapidly and transiently activated by electroconvulsive shock treatment (ECT), D_1 dopamine receptor activation, and opiate withdrawal. These studies indicate that, as found for the leucine zipper family of transcription factors, multiple members of the zinc finger family of transcription factors are induced by neuronal stimulation.

Zif268 (also termed *NGFI-A*[15], *krox*-24[7] or *egr-1*[21]), a putative transcription regulatory factor[14,15] that contains a zinc finger DNA-binding domain, is rapidly and transiently activated in brain neurons by a variety of pharmacological and physiological stimuli[8–10,19,23,24]. While *zif268* expression has been extensively studied in rat brain, little is known about the regulation of a related transcription factor, *krox*-20 (also termed *egr2*[13]), that is highly homologous to *zif268* only in its zinc finger DNA-binding domain and recognizes the same DNA binding consensus sequence[5,6,13,14]. Although, both *zif268* and *krox*-20 are rapidly activated in fibroblasts by growth factors[7,15], differential expression has been observed. In PC12 cells, *zif268*, but not *krox*-20, is activated by nerve growth factor[13]. On the other hand, in the developing mouse hindbrain, *krox*-20 but not *zif268*, is expressed in a discrete segmental fashion[5,22]. In previous studies, we have found that following seizure activity there is a marked increase in expression of Zif268 mRNA and protein in the hippocampus[17]. In addition, gel shift assays using the Zif268 DNA binding consensus sequence indicated that in addition to Zif268, other proteins that share high-affinity for the same DNA binding consensus sequence, such as *krox*-20, are induced as well[17]. Accordingly, in this study we ascertained whether *krox*-20 is induced in adult rat brain by seizure activity. In addition, we have examined the expression of *krox*-20 after dopamine receptor activation and opiate withdrawal, two pharmacological stimulation paradigms that induce activation of *zif268*[8,11].

Adult male Sprague–Dawley rats (175–200 g) were maintained in a light controlled (12 h light/12 h dark) room with access to food and water ad libitum. Electroconvulsive treatment (ECT) was used to induce seizures in rats[9]. Briefly, earclips were attached to unanesthetized rats and electroshock consisting of a 1 s, 100 Hz, 85 mA stimulus of 0.5 ms square wave pulses was delivered (UGO Basile ECT unit, Model 7801) which resulted in a generalized tonic-clonic convulsion with hindlimb extension.

To examine effects of dopamine receptor activation on *krox*-20, we utilized drug treatments demonstrated[8] to elicit marked activation of *zif268*. In previous studies, we have found that the dopamine receptor agonist apomorphine, when administered by itself, fails to induce *zif268* expression[8]. However, following pre-treatment with either 6-hydroxydopamine (6-OHDA) or reserpine, apomorphine elicits robust increases in *zif268* mRNA. Accordingly, rats were pretreated with reserpine (5 mg/kg, i.p.), 18 h and 3 h prior to receiving apomorphine 2 mg/kg, i.p. and then sacrificed 30 min after apomorphine injection. In some experiments, the D_1 receptor antagonist SCH 23390 (0.5 mg/kg) or the D_2 receptor antagonist haloperidol (1 mg/kg) was administered i.p. 15 min prior to apomorphine injections.

For experiments examining opiate withdrawal, rats un-

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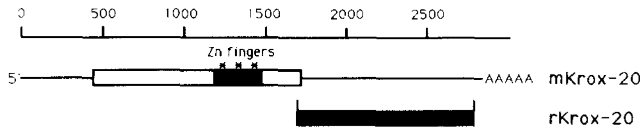

Fig. 1. Comparison of mouse *krox*-20 and rat *krox*-20 inserts used for in situ hybridization studies. The open box indicates the coding sequence of mouse *krox*-20 obtained from Chavrier et al.[7]. The three zinc fingers are represented by asterisks. The rat *krox*-20 insert depicted below the mouse *krox*-20 sequence does not overlap the zinc finger region.

derwent daily implantation of 75 mg morphine pellets for 5 days, a regimen previously found to induce tolerance to morphine in rats[1,11]. Withdrawal was initiated by a single subcutaneous injection of 100 mg/kg naltrexone as described in previous studies[11]. Rats were sacrificed at 1 h or 6 h after the naltrexone injection.

To analyze effects of drug treatment by in situ hybridization, the brains of control and treated rats were cut sagittally along the midline, aligned and embedded together in Tissue Tek (Miles Inc., Elkhart, IN) to minimize variability in processing sections from control and treated animals. Coronal sections (10 μm) were cut on a cryostat (Zeiss), thaw-mounted on gelatin/alum-subbed slides, fixed in 4% paraformaldehyde, and processed as described[8–10].

Single-stranded RNA probes were prepared from linearized plasmids containing rat *zif268* or rat *krox*-20 inserts and synthesized with $[^{35}S]\alpha$-UTP and T7 or T3 polymerase[8,9]. 0.5 μCi of ^{35}S probe in 50μl of hybridization buffer[8,9] was placed on each section. Incubation was performed overnight at 56°C in moist chambers. Slides were then washed at 31.5°C (for *krox*-20) or 35°C (for *zif268*) in 2 × SSC containing 10 μg/ml RNAse A (Worthington) for 20 min, rinsed and air-dried. Slides were apposed to autoradiographic film (Kodak XAR) for 3 days.

In previous studies, we have noted that use of species specific RNA probes markedly enhances the quality of in situ hybridization studies. Accordingly, a mouse *krox*-20 plasmid (provided by B. Christy) was used to obtain a partial rat cDNA sequence by screening a rat brain cDNA library. The insert was cloned into the Bluescript SK^+ (Stratagene, La Jolla, CA) plasmid. Partial sequencing from both ends of the insert confirmed a high degree of homology with mouse *krox*-20 and human *egr*-2. The insert corresponds to the non-coding region (nt 1707–2835) of mouse *krox*-20[7] and does not contain the zinc finger domain (nt 1198–1484) eliminating the possibility of cross-hybridization with rat *zif268* mRNA, or other mRNAs encoding homologous zinc finger regions (Fig. 1). In addition, Northern blot analysis of brain mRNA with cDNA probe prepared from this insert yields a single band[3] confirming its specificity.

In situ hybridization studies demonstrated a marked

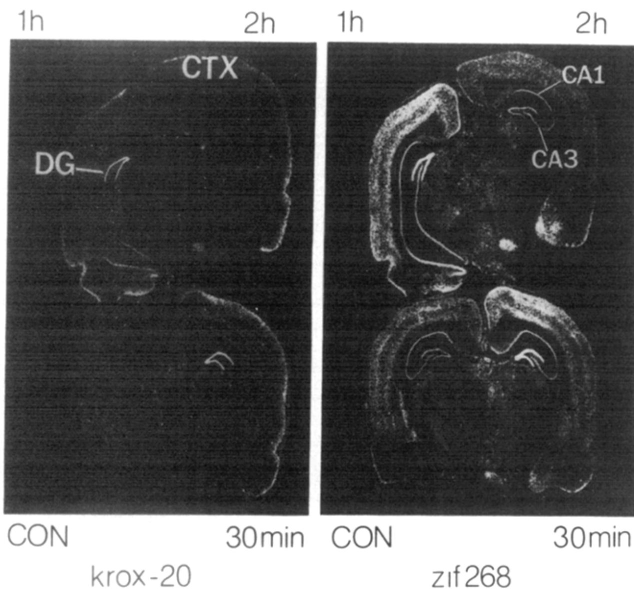

Fig. 2. Time course of rise in *krox*-20 and *zif268* mRNA levels following ECT. In situ autoradiograms of coronal sections of four hemibrains. Each brain hemisphere was taken from a control rat (CON), or from a rat 30 min, 1 h or 2 h following ECT (n = 3). DG, dentate gyrus; CTX, cortex; CA1 and CA3, hippocampal fields. In situ hybridization performed with *zif268* probes prepared from either full length insert or 3′ non-coding region give similar results ensuring that the *zif268* signal does not reflect cross-hybridization with *krox*-20 mRNA.

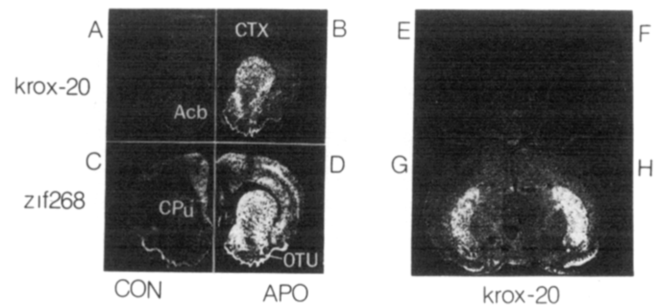

Fig. 3. Activation of *krox*-20 and *zif268* after administration of dopaminergic agents. Left panel displays in situ autoradiograms of coronal sections of hemibrains 30 min after apomorphine or vehicle injection. All rats were pretreated with reserpine 5 mg/kg i.p., 18 h and 3 h prior to sacrifice and then with either apomorphine or vehicle 30 min prior to sacrifice. Left brain hemispheres (A,C) from vehicle injected control (CON); right brain hemispheres (B,D) are from rats injected with apomorphine (APO) 2 mg/kg, i.p. CTX, cortex; Acb, nucleus accumbens; CPu, caudate putamen; OTU, olfactory tubercle. Right panel illustrates selective blockade of *krox-20* activation by SCH 23390, a D_1 dopamine receptor antagonist. In situ autoradiograms of *krox-20* mRNA in sections from rats treated as follows: E, control; F, apomorphine 2 mg/kg, given after SCH 23390 0.5 mg/kg, i.p. G, apomorphine 2 mg/kg; H, apomorphine 2 mg/kg, i.p. given after haloperidol 1 mg/kg, i.p. The antagonists SCH 23390 and haloperidol were injected 15 min prior to apomorphine. All rats were pretreated with 5 mg/kg reserpine 18 h and 3 h prior to sacrifice.

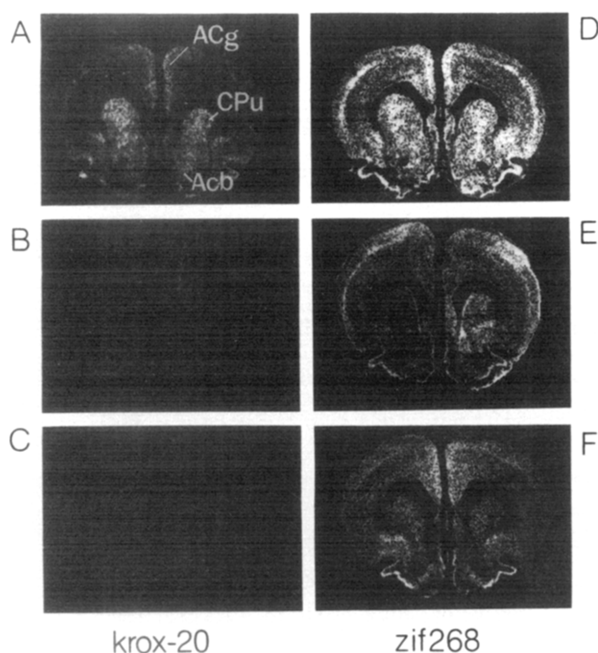

Fig. 4. Activation of *krox*-20 and *zif268* in response to opiate withdrawal. The top panels (A,D) are in situ autoradiograms of hemibrains of two rats 1 h after initiation of opiate withdrawal (n = 5) by naltrexone injection (100 mg/kg). The left brain hemispheres of the middle panels (B,E) are from a morphine tolerant rat 1 h after a saline injection and the right brain hemispheres are from a sham-operated rat. The left brain hemispheres of the bottom panels (C,F) are from a saline-injected naive rat and the right brain hemispheres from a naive rat receiving naltrexone (100 mg/kg) injection 1 h prior to sacrifice (n = 3). ACg, anterior cingulate cortex; CPu, caudate putamen; Acb, nucleus accumbens.

increase in *krox*-20 and *zif268* mRNA levels in dentate granule cells and pyriform cortex within 30 min after ECT that returned to basal levels within 2 h (n = 3) (Fig. 2). Although both *zif268* and *krox*-20 mRNAs display similar time course profiles, *krox*-20 expression appears to be more restricted with lower levels apparent in hippocampal pyramidal cells and in the neocortex.

Previous studies have demonstrated that dopaminergic stimulation elicits a robust immediate early gene response in the caudate putamen[8,12,18,25]. In order to determine whether pharmacological stimulation can activate *krox*-20 mRNA in adult rat brain, we examined the effect of the dopaminergic agonist apomorphine after reserpine pretreatment, a regimen previously found[8] to induce *zif268*. *Krox*-20 and *zif268* mRNAs are increased in the caudate-putamen and olfactory tubercle 30 min after an i.p. injection of apomorphine (2 mg/kg) in reserpine pre-treated rats (n = 3) (Fig. 3, left panel) and return to baseline within 2–4 h (not shown). In contrast to *zif268*, *krox*-20 induction is less apparent in the overlying cortex. As found for *zif268*[8], induction of *krox*-20 by apomorphine is selectively blocked by the D_1 receptor antagonist, SCH 23390 (0.5 mg/kg), but not by the D_2 receptor antagonist haloperidol (1 mg/kg) (Fig. 3, right panel, n = 3).

In addition, we examined whether *krox*-20 and *zif268* are activated during opiate withdrawal, a paradigm previously shown to elicit activation of c-*fos* in the striatum[11]. We found that basal levels of *krox*-20 in this region are extremely low as assayed by *in situ* hybridization and that chronic morphine treatment did not elicit an increase in *krox*-20 or *zif268* mRNA when compared to sham-operated controls (Fig. 4, middle panel). However, it appears that *zif268* mRNA levels are slightly decreased in the caudate putamen in chronic morphine-treated rats as previously noted for c-*fos*[11]. In assessing effects of opiate withdrawal on these mRNAs, we found that both *krox*-20 and *zif268* mRNAs, are increased in the caudate-putamen (CPu), nucleus accumbens (ACb) and the anterior cingulate cortex (ACg) 1 h after naltrexone injection in morphine tolerant rats (n = 5), but not by naltrexone in naive rats (Fig. 4). Both *zif268* and *krox*-20 mRNAs returned to control levels within 6 h (n = 2, data not shown).

These results demonstrate that, like *zif268*, *krox*-20 is activated by a variety of stimuli in adult rat brain. Recent studies have demonstrated that multiple members of the Fos and Jun families of transcription factors are induced in brain neurons[8,9,11,16,20,23]. Growing evidence suggests that individual members of these families differ in their time course of expression[15,17], their responsiveness to specific stimuli and in their function as transcriptional activators or inhibitors[2,4]. For example, *jun*-B, but not c-*jun*, is induced by membrane depolarization in PC12 cells[2] and AP-1 complexes containing *jun*-B appear to inhibit transcription[4]. In recent studies, we have obtained evidence for differential regulation of *zif268* and *krox*-20 in brain, as mRNA levels for *zif268*[10], but not *krox*-20 are markedly increased in dentate granule cells by brief tetanic stimulation of perforant path afferents (Worley et al., in preparation). Taken together, these findings focus attention on identifying how co-ordinate or differential expression of these zinc finger transcription factors affect regulation of target genes.

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