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Activin mRNA Induced during Amygdala Kindling Shows a Spatiotemporal Progression That Tracks the Spread of Seizures

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ABSTRACT

The progressive development of seizures in rats by amygdala kindling, which models temporal lobe epilepsy, allows the study of molecular regulators of enduring synaptic changes. Neurotrophins play important roles in synaptic plasticity and neuroprotection. Activin, a member of the transforming growth factor-β superfamily of growth and differentiation factors, has recently been added to the list of candidate synaptic regulators. We mapped the induction of activin βA mRNA in amygdala and cortex at several stages of seizure development. Strong induction, measured 2 hours after the first stage 2 (partial) seizure, appeared in neurons of the ipsilateral amygdala (confined to the lateral, basal, and posterior cortical nuclei) and insular, piriform, orbital, and infralimbic cortices. Activin βA mRNA induction, after the first stage 5 (generalized) seizure, had spread to the contralateral amygdala (same nuclear distribution) and cortex, and the induced labeling covered much of the convexity of neocortex as well as piriform, perirhinal, and entorhinal cortices in a nearly bilaterally symmetrical pattern. This pattern had filled in by the sixth stage 5 seizure. Induced labeling in cortical neurons was confined mainly to layer II. A similar temporal and spatial pattern of increased mRNA expression of brain-derived neurotrophic factor (BDNF) was found in the amygdala and cortex. Activin βA and BDNF expression patterns were similar at 1, 2, and 6 hours after the last seizure, subsiding at 24 hours; in contrast, c-fos mRNA induction appeared only at 1 hour throughout cortex and then subsided. In double-label studies, activin βA mRNA-positive neurons were also BDNF mRNA positive, and they did not colocalize with GAD67 mRNA (a marker of γ-aminobutyric acidergic neurons). The data suggest that activin and BDNF transcriptional activities accurately mark excitatory neurons participating in seizure-induced synaptic alterations and may contribute to the enduring changes that underlie the kindled state. J. Comp. Neurol. 476:91–102, 2004.

Indexing terms: amygdala kindling; seizures; activin; in situ hybridization; epilepsy; neurotrophins; brain-derived neurotrophic factor

Amygdala kindling is an important model of temporal lobe epilepsy of the complex partial type with secondary generalization (McNamara, 1988; Mody, 1999). The model is useful for examination of molecular events that accompany the spread of seizure activity from the original focus, especially those responsible for the enduring synaptic changes that occur in the affected areas. Neurotrophins are known to play a role in synapse regulation-brain-derived neurotrophic factor (BDNF); neurotrophins-3, -4/5, and -6; nerve growth factor (NGF); and glial cell-derived neurotrophic factor (GDNF) have received the most attention in the context of seizure-induced changes (Lindvall et al., 1994; Nanobashvili et al., 2000; Racine et al., 2002). Another candidate in the neurotrophic family is activin A. Here we map induced mRNA expression of activin βA in amygdala-kindled rats and relate it to seizure progression.

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Activin A is a member of the transforming growth factor (TGF)-β superfamily of growth and differentiation factors. Activin A has been called a neuropoietic cytokine because it can regulate gene expression in both neurons and hematopoietic cells (Fann and Patterson, 1994). Activins have been studied mainly for their actions in endocrine systems on hormone secretion, but they have more recently been shown to promote neuronal survival (Iwahori et al., 1997; Schubert et al., 1990).

Activins are glycoprotein dimers composed of two β-subunits (βA and βB; for review see Vale, 1990). Three forms of activin have been characterized and shown to be functional in vivo. Activin A and activin B are homodimers (βA/βA and βB/βB, respectively), and activin AB is a heterodimer of subunits βAβB. The activins bind to a cell-surface receptor complex comprising type I and type II activin receptors (Massague, 1998; Mathews and Vale, 1993). Binding is thought to occur first to the type II receptor, which then complexes with the type I receptor, and the complex then activates the Smads (Massague, 1998; Miyazawa et al., 2002). Activin and TGFβ specifically use Smad2/3 plus Smad4 (the obligatory co-Smad), which combine to interact in the nucleus with a large number of DNA-binding proteins, including the Jun family of AP1 transcription factors (Liberati et al., 1999).

The type I receptors are members of the activin receptor-like kinase (ALK) family, and it is now thought that ALK-4 (ActR-IB) is the specific type I activin receptor (Miyazawa et al., 2002). Two type II receptors, ActR-II and ActR-IIB, have been identified (Mathews and Vale, 1993). Expression of mRNA and protein for activin subunits (Roberts et al., 1996) and activin receptor types (Bengtsson et al., 1995; Cameron et al., 1994; Funaba et al., 1997; Morita et al., 1996; Soderstrom et al., 1996) has been demonstrated and partially mapped in the CNS. Both activin subunit mRNAs, βA and βB, are constitutively expressed in the CNS (Andreasson and Worley, 1995). However, activin βA mRNA and its related protein, activin A, have been suggested to play an important role in CNS function. Activin A has been shown to be neurotrophic (Iwahori et al., 1997; Schubert et al., 1990) and to determine neuronal phenotype (Fann and Patterson, 1994) in vitro. In addition, several in vivo studies have suggested a specific role for activin A in neuronal function. The level of activin βA mRNA expression has been shown to be regulated by neuronal activity in the developing and adult brain (Andreasson and Worley, 1995). Activin βA mRNA is strongly induced following hypoxic-ischemic injury (Lai et al., 1996) and excitotoxic injury (Tretter et al., 1996), whereas the βB subunit mRNA does not show increased expression in these models. Additional experiments have demonstrated that activin A plays a neuroprotective role in these CNS injury models (Tretter et al., 2000; Wu et al., 1999).

There have been no studies examining activin A or its binding partners, the ActR-II and ActR-IIB receptors, in brain in the kindling model. The data here show strong time- and region-specific up-regulation of activin βA mRNA but not receptor mRNAs in amygdala and cortex, supporting an possible role for activin A in regulating the plastic changes associated with development of seizure susceptibility in these areas and perhaps kindling-based neuroprotective mechanisms (Kelly and McIntyre, 1994).

MATERIALS AND METHODS

Animals and amygdala kindling procedure

Fifty-nine Long-Evans hooded rats (Charles River Laboratories) were housed individually in hanging cages and were maintained on a 12-hour light-dark cycle (light phase 0700–1900 hours). At the time of surgery, 32 randomly selected rats were anaesthetized with sodium pentobarbital (Somnotol; 60 mg/kg, i.p.) and were implanted bilaterally in the amygdalae, with the stereotaxic coordinates: 0.2 mm posterior to bregma, 4.5 mm lateral to the midline, and 8.5 mm below the skull surface (Pellegrino et al., 1979). The bipolar stimulation/recording electrodes were constructed from twisted 0.127-mm Diamel-insulated, nichrome wire. A ground wire was inserted over the frontal pole for electroencephalogram (EEG) recordings. Dental cement and six jeweler screws were used to secure the electrode and ground wire to the skull. The electrode and ground pins were then inserted into a plastic head plug, which was held to the skull with dental acrylic (Molino and McIntyre, 1972).

Kindling was accomplished in the appropriate rats by exposing them to a 2-second, 60-Hz sine wave stimulus delivered to the left or right amygdala at its local afterdischarge threshold (ADT) intensity. The ADT was determined on the first kindling day by activating the amygdala with a low-intensity stimulus that was progressively increased (15, 25, 35, 50, 75, 100, 150, 200 μA), with a 1-minute interstimulation interval, until an electrographic afterdischarge (AD) was elicited. An AD had to outlast the stimulus by 2 seconds or more. Kindling was then commenced the next day in those rats by stimulating the amygdala (same side) once daily at its ADT until it reached the kindling criterion that defined its group. At the time of the final stimulation for the rats in each kindled group, the ADTs were redetermined.

Two basic experiments were conducted. The first addressed the spatial and temporal patterns of gene expression in the cortex and amygdala over the course of kindling (from the second kindling stimulation to the fully kindled state), and the second tracked the change in cortical expression levels in fully kindled rats over time since the last seizure. The seven groups involved in the first experiment were 1) normal control group: rats that received no surgical treatment but daily handling as in the other groups; 2) sham-operated control group: rats that were surgically implanted and handled daily as in the kindled rats but were never intracranially stimulated; 3) two afterdischarges (2AD) group: rats that received their second AD, which occurred on the day following their initial ADT test; 4) first stage 2 (1s2) group: rats that were stimulated daily until they exhibited their first stage 2 partial seizure (Racine, 1972); and 5) three groups that received either 1, 2, or 6 (1s5, 2s5, 6s5) stage 5 generalized convulsive seizures (Racine, 1972) before termination. Two hours after their final seizure, the rats in each group were killed by decapitation. This survival time was selected on the basis of earlier reports showing events related to neuroprotection ongoing at 2 hours (Kelly and McIntyre, 1994; Plata-Salaman et al., 2000). The second experiment involved four groups, which were 1) the normal controls from experiment 1 and 2) three groups that received six stage 5 generalized seizures (6s5) and were killed 1, 6, or 24 hours after their final stage 5 seizure. All surgical and behavioral procedures were approved by the

Carleton University Animal Care Committee, which adheres to the guidelines of the Canadian Council on Animal Care.

Brain section collection

Brains were removed and rapidly frozen by immersion in 2-methylbutane at –50°C and were stored at –70°C. Cryostat-cut 12-μm-thick coronal sections were thaw mounted onto gelatin-coated slides, dried, and stored at –35°C. Sections were collected from all cortical levels.

In situ hybridization

In situ hybridization procedures were performed as previously described for ribonucleotide (cRNA) probes (Whitfield et al., 1990). Tissue sections were fixed with 4% formaldehyde; acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine-HCl, pH 8.0; dehydrated; and delipidated with chloroform. Antisense ribonucleotide probes were transcribed from linearized plasmids containing cDNAs encoding rat activin βA (gift of Dr. Joanna Jankowsky, California Institute of Technology, Pasadena, CA), rat ActR-II and ActR-IIB (gifts of Dr. Wylie Vale, Salk Institute, CA), rat full-length GAD67 (gift of Drs. A. Tobin and N. Tillakaratne, UCLA, Los Angeles, CA), rat full-length BDNF (gift of Drs. J. Lauterborn and C. Gall, University of California Irvine, Irvine, CA), and rat c-fos (bases 1,256–2,116; gift of Dr. T. Curran, Roche Institute of Molecular Biology, Nutley, NJ) using the Riboprobe System (Promega Biotech, Madison, WI) with α-^{35}S-UTP (specific activity >1,000 Ci/mmol; Perkin Elmer, Boston, MA) and T3 or T7 polymerases. Radiolabeled probes were diluted in a hybridization buffer, pH 7.5, and applied to brain sections (approximately 500,000 CPM/section). Slides were incubated overnight at 55°C in a humidified chamber. To reduce nonspecific binding of the probe, slides were washed in 20 μg/ml RNase solution for 30 minutes at room temperature, followed by 1 hour each in 2× SSC at 50°C and 0.2× SSC at 55°C and 60°C. Slides were dehydrated and air dried for autoradiography.

Methods for double-label in situ hybridization were described previously (Hohmann and Herkenham, 2000). Slides bearing adjacent sections were hybridized with a cocktail of radiolabeled and digoxigenin (DIG)-labeled probes for BDNF or GAD67 combined with the ^{35}S-labeled probe for activin βA. After routine hybridization and washing steps, double-labeled sections were subsequently washed at 60°C in 0.1× SSC (1 hour) and subjected to DIG-labeling procedures. Slides were washed in 100 mM Tris, pH 7.4, with 150 mM NaCl added. Blocking was performed in 3% normal goat serum (Sigma, St. Louis, MO) in the same buffer with 0.3% Triton X-100 (Fisher, Fair Lawn, NJ) added (1–2 hours). Slides were incubated overnight in alkaline phosphate-conjugated anti-DIG serum (Boehringer Mannheim, Indianapolis, IN; diluted 1:1,000 in blocking solution) and washed in the Tris-NaCl buffer (3 × 5 minutes) and then in alkaline phosphatase reaction buffer (100 mM Trizma, pH 9.1, 100 mM NaCl, and 50 mM $MgCl_2$) for the same times. Slides were incubated (4°C) in chromogen solution containing 5-bromo-4-chloro-3-indoyl phosphate toluidium salt (180 μg/ml; Boehringer Mannheim), nitroblue tetrazolium salt (340 μg/ml; Boehringer Mannheim), and levamisole (240 μg/ml; Sigma) in the alkaline phosphatase reaction buffer overnight. The chromogen reaction was terminated by washing (3 × 5 minutes) in the original buffer with 10 mM EDTA added. Sections were rinsed in deionized water (3 × 5 minutes) and air dried.

Combined immunohistochemistry and hybridization histochemistry

Sections were processed for in situ hybridization with the activin βA cRNA probe, as described above, followed by immunohistochemistry with antineuronal nuclei (NeuN) monoclonal antibody (Chemicon International, Temecula, CA). For the immunohistochemistry, sections were fixed in 4% formaldehyde; rinsed three times in phosphate-buffered saline (PBS), pH 7.4; blocked for 1 hour in PBS, 0.1% Triton-X, 0.5% blocking reagent (Perkin Elmer); and incubated overnight with anti-NeuN (1:1,000). Sections were then rinsed three times in PBS, 0.1% Triton-X; incubated with a biotinylated secondary antibody (goat anti-mouse IgG, 1:500) for 1 hour; and reacted for visualization by the conventional avidin-biotin immunoperoxidase protocol (Vector, Burlingame, CA).

Autoradiography

Slides and ^{14}C plastic standards were placed in X-ray cassettes, apposed to film (BioMax MR; Eastman Kodak, Rochester, NY) for 3–6 days, and developed (X-Omat; Kodak). To determine the cellular localization of hybridized probes, sections were coated with nuclear track emulsion (NTB-2; Kodak), exposed for 3–6 weeks, developed (D19; Kodak), and counterstained with cresyl violet. Double-labeled sections were coated with LM-1 emulsion (Amersham, Arlington Heights, IL), exposed for 3 weeks, and developed.

Data analysis and presentation

Autoradiographic film images of brain sections and standards were digitized with a solid-state camera (CCD-72; Dage-MTI) and a Macintosh computer-based image-analysis system with NIH Image software (http://rsb.info.nih.gov/nih-image). For each brain, 11 predetermined levels, based on the Atlas of Paxinos and Watson (1998), were captured. The film images were visually scored for cortical labeling density (–, +, ++, +++) along the cortical contour, and the values were recorded in a spreadsheet. A representative surface view of labeling density in neocortical layer II, averaged from individual patterns, was generated in Adobe Illustrator for each kindling stage. The flattened cortical map was modeled after the map of mouse neocortex by Caviness (1975). Photography of the film images and of emulsion-coated, slide-mounted sections viewed through a Leica DMR microscope was accomplished with a CoolSnap cf digital camera (Photometrics, Tucson, AZ) and IPLab Spectrum Software (Scanalytics, Fairfax, VA). All images were processed in Adobe Photoshop, with the levels, filter (unsharp mask), and contrast features used to optimize them. Double-labeled material was photographed as a double exposure; first, the silver grains were captured by using epiillumination through a polarized light (POL) filter cube, and then the same field was captured with normal brightfield illumination. The separate images were taken into Image J (http://rsb.info.nih.gov/ij/), and the POL image was inverted, made binary, colorized red, and pasted (transparent) into the image of the DIG labeling. In this manner, no squelching of silver grain appearance by the underlying DIG reaction occurred. Statistical analysis of electrophys-

TABLE 1. Average (±SEM) Number of Stimulations to Reach Stage 2 and Stage 5 Seizure Levels (Experiment 1) and Full Generalized Seizure (6s5) with Different Survival Times after the Last Seizure (Experiment 2) Plus Final Afterdischarge Threshold (ADT) and Its Duration (ADD)

Groups	N	Stage-2	Stage-5	ADT (μA)	ADD (sec)
Experiment 1					
2 Stim	6	N/A	N/A	55.8 ± 20.4	15.3 ± 2.9[1]
1 Stage 2	6	11.5 ± 2.7	N/A	61.6 ± 17.9	34.0 ± 8.5
1 Stage 5	6	11.2 ± 3.3	16.8 ± 2.9	53.3 ± 11.7	49.5 ± 9.6
2 Stage 5	3	10.0 ± 2.0	12.3 ± 2.1	70.0 ± 40.1	75.0 ± 16.9
6 Stage 5	5	9.4 ± 1.3	17.0 ± 2.9	55.0 ± 9.3	42.4 ± 13.2
Experiment 2					
1 Hour	6	13.5 ± 3.0	21.5 ± 3.2	34.2 ± 3.7	62.3 ± 9.8
6 Hours	6	9.5 ± 1.0	17.0 ± 2.1	43.3 ± 12.0	69.3 ± 6.5
24 Hours	6	9.7 ± 1.1	15.7 ± 1.8	58.3 ± 12.3	55.5 ± 9.0

[1]Significantly different from all other groups; $P < 0.001$ by Bonferroni correction following ANOVA.
N, number of rats in each group.

iological and densitometric data were performed by ANOVA, followed by appropriate post hoc tests. $P < 0.05$ was set as the level of significance.

RESULTS

Physiology and behavior of kindled rats, with reference to stages

Animals in the kindled groups of both experiments developed their seizures at rates typical of the Long-Evans hooded rat. In the development of those seizures, ANOVA indicated that there were no significant differences between the stimulated groups in the numbers of stimulations to reach the two different stages of seizure development or in their final afterdischarge threshold and its duration. As the duration of the electrographic seizures in the two amygdalae were similar during both partial seizure development and generalized seizure expression, a single value for each of those two periods is presented (Table 1).

Anatomy

Induction of activin βA mRNA in cortex. Activin βA mRNA in normal or sham-operated animals was expressed at low levels in widely scattered neurons of neocortical layers II and upper VI, dentate gyrus and Ammon's horn of hippocampus, striatum, piriform and entorhinal cortices, amygdala (cortical nuclei), parafascicular nucleus, and supramammillary area (not shown). Kindling powerfully elevated expression levels in neurons in a subset of these structures. In all locations, the induced expression was neuronal (Fig. 1a). Within neocortex, the induced expression was greatest in layer II. Slightly elevated activin βA mRNA expression was present also in subjacent layers III and the upper part of layer VI and lower layer V. Increases occurred both by increased expression level per cell and increased numbers of expressing cells.

Cells that were strongly activin βA mRNA expressing after the second afterdischarge (2AD group) were located ipsilaterally along the ventral portion of the rhinal sulcus and throughout the superficial piriform cortex, although only three of the six animals showed induction (data not shown). The cells showing strong mRNA expression following the first partial seizure, 1s2, again were located along the rhinal sulcus but also included the ventromedial parts of the frontal lobe ipsilaterally (Fig. 2a–d), except for

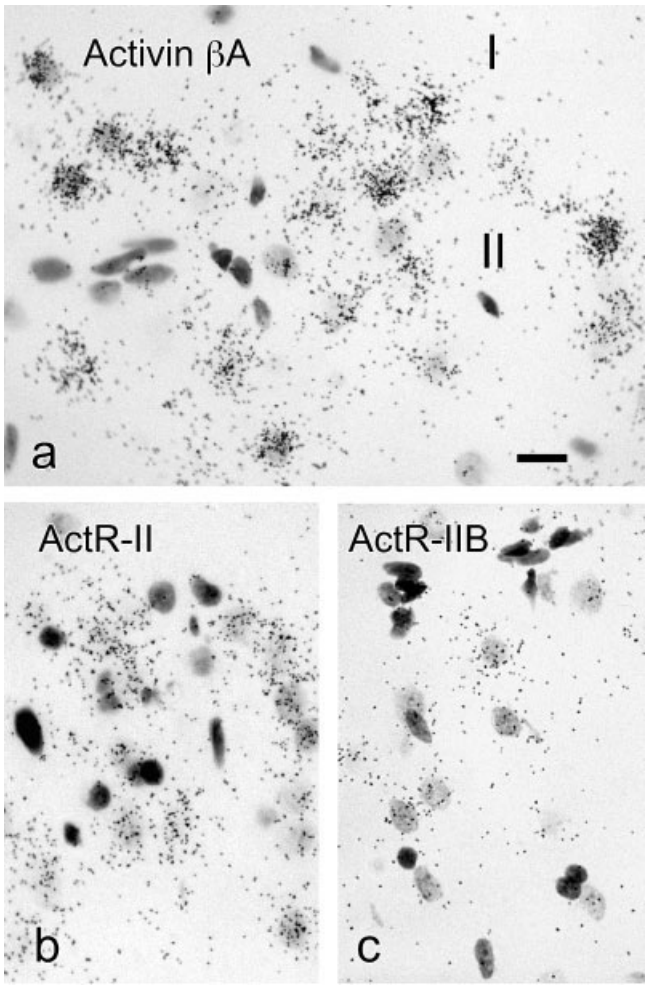

Fig. 1. Brightfield photomicrographs of Nissl-stained sections hybridized for mRNAs for activin βA (**a**), the ActR-II receptor (**b**), and the ActR-IIB receptor (**c**) in cerebral cortex. Silver grains mark hybridization signals over neurons, recognized as large with pale staining. The areas shown are in the outer part of cortical layer II (marked) in the insular (a), piriform (b), and somatosensory (c) cortices. Scale bar = 20 μm.

one case that showed bilateral label in the sulcal regions. In addition, another case showed almost no induction at all except in the ipsilateral posterior basomedial (BM) and posterior cortical (PCo) amygdaloid nuclei (data not shown). In this group, typical areas strongly labeled were the tenia tecta and prelimbic, lateral orbital, insular, piriform, perirhinal, and entorhinal cortices; endopiriform nucleus; and specific amygdaloid nuclei (see below). Activin βA mRNA was detected rostrally in the insular but not the piriform cortex (Fig. 2a), whereas caudally it was detected in piriform and not insular cortex (Fig. 2b–d). From midstriatal levels caudally, the elevated signal level in the rhinal fissure abruptly started in the middle of the fissure and extended ventrally into piriform and entorhinal areas. In the caudal half of convexity cortex (posterior somatosensory, auditory, and visual), there was weak bilateral activin βA mRNA induction (Figs. 2c,d, 3).

At the next progressive seizure stage examined, 1s5, the induced label became bilateral in most cases, with the

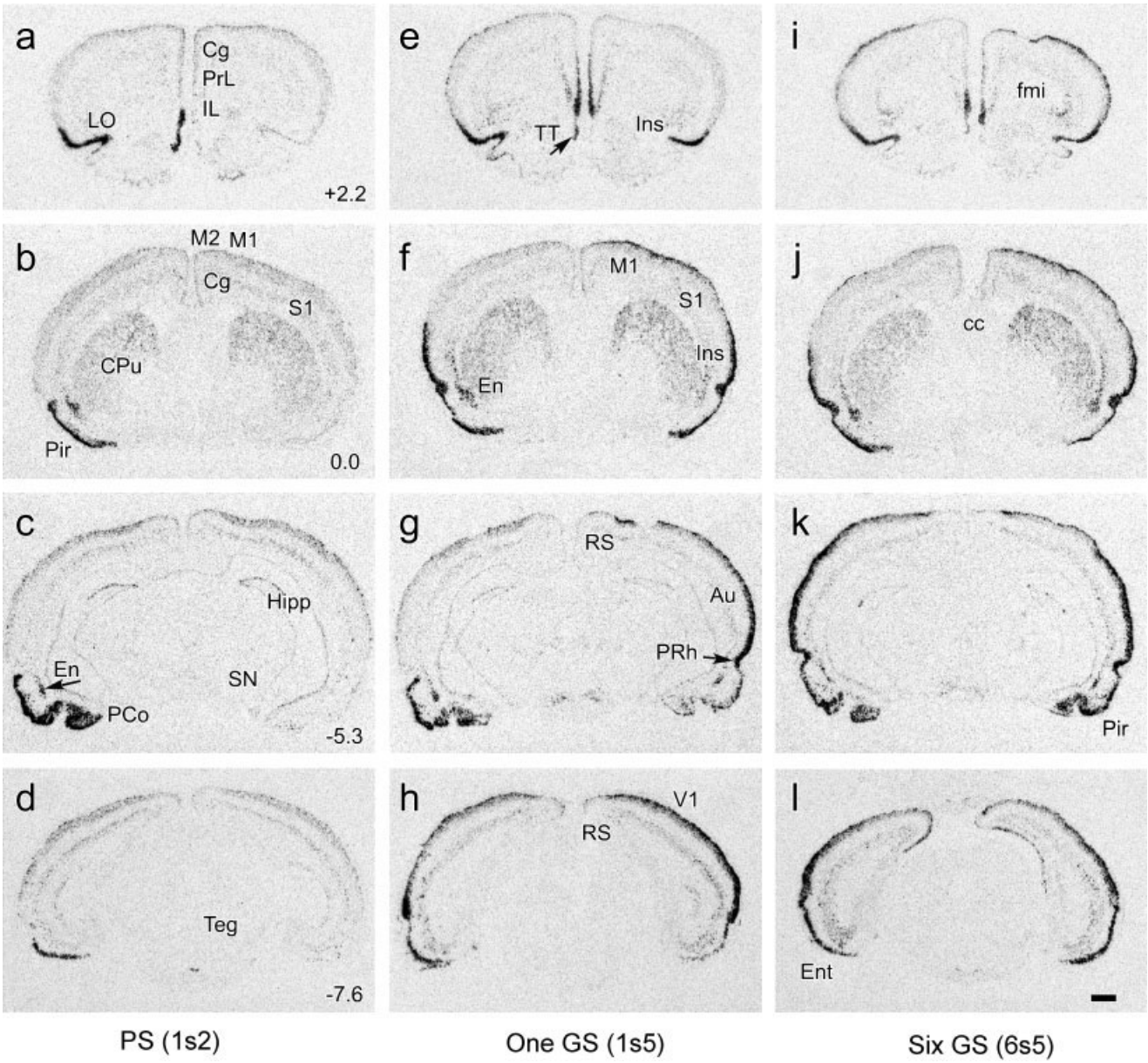

Fig. 2. Film autoradiographs show activin βA mRNA induction at four levels of brain and three seizure stages. The first stage 2 (partial) seizure (1s2) is shown in **a–d**; the coronal levels are numbered according to the levels of the atlas of Paxinos and Watson (1998). The first stage 5 (generalized) seizure (1s5) and sixth stage 5 seizure (6s5) are shown in **e–h** and **i–l** , respectively. Induced label appears in the superficial layer II of cortex and the amygdala. Au, auditory cortex; cc, corpus callosum; Cg, cingulate cortex; CPu, caudate-putamen; En, endopiriform nucleus; Ent, entorhinal cortex; fmi, forceps minor; Hipp, hippocampus; IL, infralimbic cortex; Ins, insular cortex; LO, lateral orbital cortex; M1, primary motor cortex; M2, secondary motor cortex; PCo, posterior cortical nucleus of amygdala; Pir, piriform cortex; PRh, perirhinal cortex, PrL, prelimbic cortex; RS, retrosplenial cortex; S1, primary somatosensory cortex; SN, substantia nigra; Teg, tegmentum; TT, tenia tecta; V1, primary visual cortex. Scale bar = 2 mm.

contralateral side typically showing higher mRNA expression levels especially on the convexity cortex (Figs. 2e–h, 3). In addition to the sulcal and frontal cortical areas labeled, additional areas labeled were the sensory and motor areas. Areas not labeled included cingulate cortex, motor cortex M2, parts of somatosensory cortex S1, and retrosplenial cortex. The same pattern, with small alterations, was seen at the final stage examined, 6s5 (Figs. 2i–l, 3).

The surface map shown in Figure 3 is a summary representation of the distribution of label at the different points of seizure development. Most animals at each seizure stage showed the majority of features depicted in the figure, notably, the strong progression of label along the rhinal sulcus and in the piriform and prelimbic cortices. However, each animal also showed unique variations from the general pattern depicted. Thus, the timing of spread was accelerated or delayed in some animals relative to

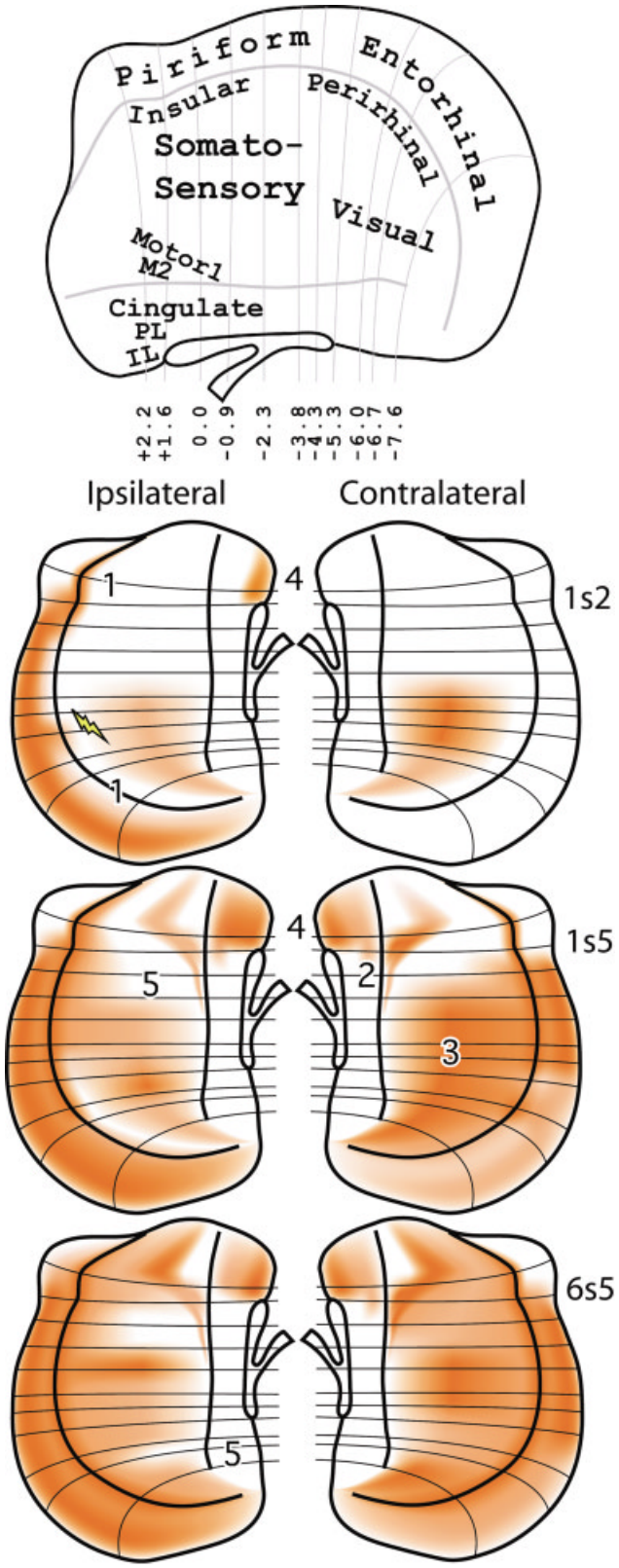

Fig. 3. Spread of activin βA mRNA induction in the cortex at four stages of seizure progression is transposed to a flattened map. The key at top shows the cortical areas mapped. The corpus callosum, dorsomedial convexity of cortex, and rhinal sulcus are drawn for reference, and the major cortical areas are marked. Vertically radiating thin lines on the topologic map mark atlas levels from which the data were taken. A lightning bolt marks the site of amygdala stimulation. Location and darkness of orange reflect the activin βA mRNA distribution and density. Numbers on the map illustrate the general points noted in the text. 1) Partial seizure (1s2): Activin βA mRNA is detected rostrally in the insular but not piriform cortex, whereas caudally it is detected in piriform and not insular cortex. 2) Generalized seizure (2s5): Activin βA mRNA signal is localized mainly to M1 cortex and absent in M2. 3) Contralateral signal was stronger than ipsilateral signal over most of the convexity cortex. 4) Progression of increased signal levels between adjacent related cortical regions was observed; e.g., for infralimbic, prelimbic, and cingulate cortices, signal in infralimbic precedes and persists. 5) General: There are regions where activin βA mRNA expression is not elevated, including parts of somatosensory cortex and retrosplenial cortex bilaterally.

others in the same seizure group. Also, some animals showed greater or lesser levels of expression in more remote areas, such as the somatosensory, entorhinal, and retrosplenial cortices. Additional general features of the kindling response are described in the Figure 3 legend.

Amygdala. Activin βA mRNA induction in the amygdala at 1s2 was restricted to specific ipsilateral nuclei (Fig. 4a,b,g,h). There was not much induction rostrally, where mRNA expression was confined to the lateral nucleus (La) in two cases. More caudally, the basolateral (BL), basomedial (BM), and posterior cortical (PCo) nuclei were labeled ipsilaterally. This ipsilateral labeling did not correspond to the site of the stimulating electrode, but this is not known for sure because reconstruction of the electrode track was not performed. By 1s5, half the brains showed bilateral, roughly symmetrical patterns of mRNA induction (Fig. 4c,d,i,j), and half showed still unilateral label. There were no changes in the pattern of expression. By 6s5, all patterns were bilateral and still unchanged.

Activin receptors. Two activin receptors were mapped, ActR-II and ActR-IIB. Both showed heterogeneous expression, similar to the distribution pattern that has been previously reported (Cameron et al., 1994). Both receptors were neuronally expressed (Fig. 1b,c), as reported from studies using immunohistochemical staining (Funaba et al., 1997). Examples of the mRNA distributions are shown in Figure 4e,f,k,i. No kindling-induced changes in expression level of either receptor were observed by examination of the film images (data not shown). The distribution of activin receptor mRNAs was much more extensive than the restricted area of activin βA mRNA induction. However, all areas that showed induced activin βA mRNA expression also showed expression of both receptor types, and the pattern of high activin βA mRNA induction in the amygdala (La, BL, BM, and PCo) corresponded to enriched expression of ActR-II mRNA (Fig. 4e,k).

BDNF mRNA. Two levels of brain (rostral caudate and midamygdala) were hybridized for BDNF mRNA expression. The global temporal and spatial pattern of regional activation at these levels closely matched that of activin βA mRNA seen in adjacent sections. As with activin βA mRNA, BDNF mRNA showed strong elevation first in ipsilateral amygdala and limbic cortical areas, then in a

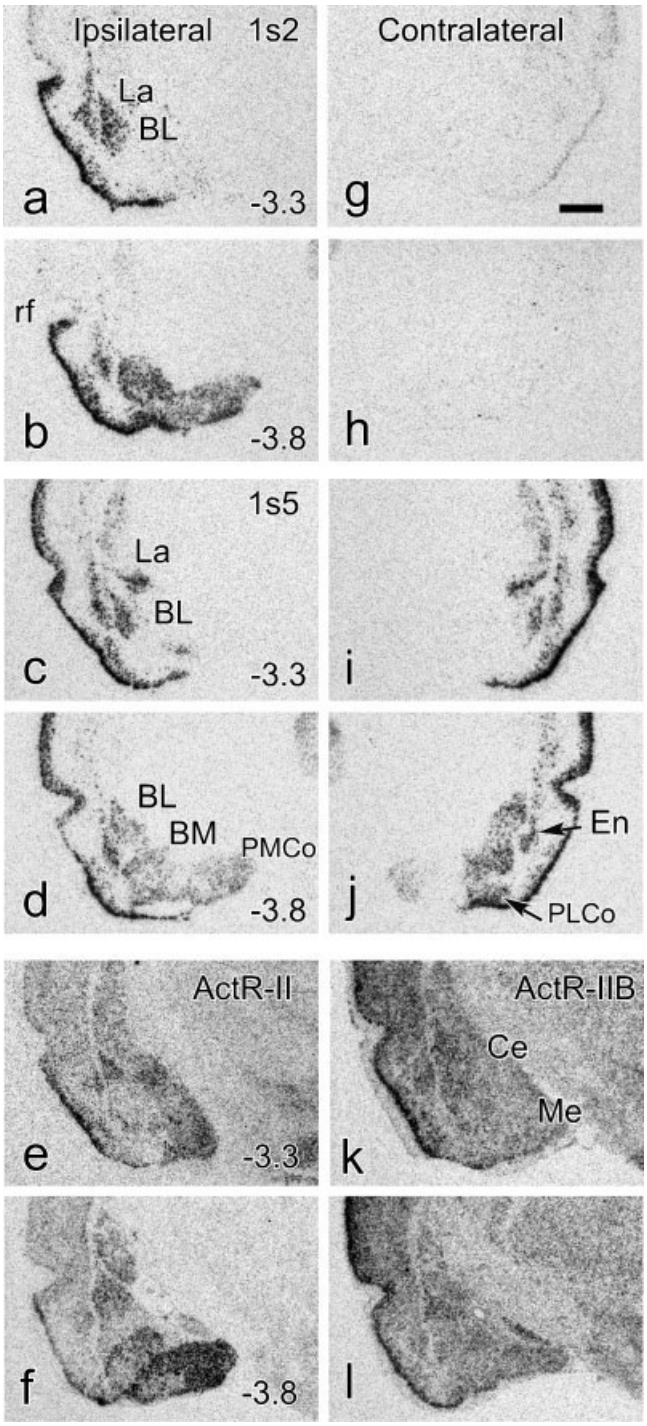

Fig. 4. Film autoradiographs show the amygdala kindling-induced activin βA mRNA expression and the constitutive expression of the two activin receptor mRNAs in the amygdala. For activin βA mRNA induction, the ipsilateral (**a–d**) and contralateral (**g–j**) sides are shown at two coronal levels of amygdala (–3.3 and –3.8) and two seizure stages (1s2 and 1s5), as indicated. The receptors [ActR-II (**e,f**) and ActR-IIB (**k,l**)] are shown at the same two levels. BL, basolateral nucleus, BM, basomedial nucleus; En, endopiriform nucleus; La, lateral nucleus; PLCo, posterior lateral cortical nucleus; PMCo, posterior medial cortical nucleus. Scale bar = 100 μm.

bilaterally symmetrical pattern of induction in amygdala and cortex. Within cortex, BDNF mRNA increases were greatest in layer II, but elevations were seen in all layers, rising above the overall higher level of constitutive expression seen throughout the cortical depth (Fig. 5). The patterns of elevated BDNF mRNA expression were not as striking as those for activin βA mRNA, therefore, because the constitutive expression level was greater, and the kindling-induced elevations were more widespread.

Double-label experiments

Combined radioactive and DIG in situ hybridization showed that, in the areas showing strong BDNF mRNA induction, including cortex and amygdala, the vast majority of DIG-positive cells (BDNF) were also ^{35}S labeled (activin βA; Fig. 6a). In contrast, none of the GAD67 mRNA-positive DIG-labeled cells showed induced activin βA mRNA expression in any subcortical or cortical location examined (Fig. 6b). Double labeling with the ^{35}S-labeled activin βA riboprobe and the NeuN antibody confirmed that activin expression was confined to neurons (Fig. 6c).

Time course after last seizure

In the time-course experiment, the rats were killed 1, 6, or 24 hours after their sixth stage 5 seizure, following normal development of kindled seizures, ADDs, and ADTs (Table 1). The mRNA expression level of activin βA was compared with that of the neurotrophin BDNF and the immediate-early gene c-fos to show the temporal and spatial relationship of the two kinds of transcripts. For all three mRNAs, robust induction was seen bilaterally in the cerebral cortex at 1 hour after the 6s5 seizure (Fig. 7). Activin βA and BDNF transcripts showed similar patterns of induction in the superficial layers (Fig. 7a,c), whereas c-fos was highly expressed throughout the depth of neocortex and piriform cortex (Fig. 7e). The activin βA and BDNF mRNA patterns at 1 hour were the same as those seen at 2 hours (Fig. 2j and not shown), whereas the c-fos mRNA at 2 hours was no longer elevated (not shown). The patterns at 6 hours were essentially the same as they had been at 2 hours, with activin βA and BDNF maintaining high expression levels in superficial cortex (Fig. 7b,d) and c-fos showing no induction (Fig. 7f). At 24 hours, all three probes were at baseline low levels of expression (not shown).

Analysis of densitometry performed on layers II/III of insular cortex (Fig. 8) showed significant main effects, an effect of survival time [time, $F(3, 23) = 244.9$, $P < 0.0001$], probe [probe, $F(2, 23) = 22.7$, $P < 0.0001$], and time × probe interaction [time · probe, $F(6, 23) = 107.2$, $P < 0.0001$]. Bonferroni/Dunn post hoc analysis showed that, for time · probe, activin βA and BDNF were not different from each other, but both activin βA and BDNF were different from c-fos ($P < 0.0001$). Closer inspection of the graphs showed that, although the mRNA distribution patterns for activin βA and BDNF mRNAs were very similar to each other, the activin βA mRNA levels were higher contralaterally than ipsilaterally, and they increased (on both sides) from 1 hour to 6 hours, whereas the opposite was the case for BDNF mRNA; its expression levels were stronger ipsilaterally than contralaterally, and they were stronger (on both sides) at 1 hour than at 6 hours. No individual comparison was significant by post hoc test, however.

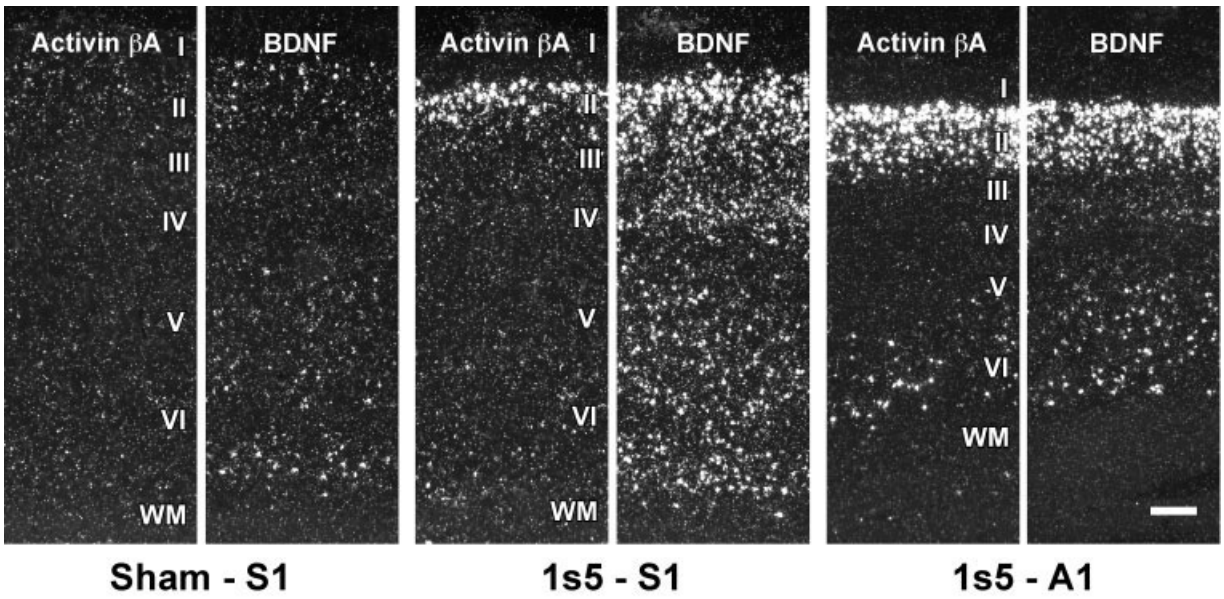

Fig. 5. Darkfield photomicrographs showing activin βA mRNA and BDNF mRNA expression in adjacent sections in two areas of neocortex in unstimulated (sham) and stimulated animals at the first stage 5 seizure (1s5). Areas shown are primary somatosensory (S1) and primary auditory (A1) cortex. Cortical layers are marked. WM, white matter. Scale bar = 200 μm.

DISCUSSION

Regional spread of activin βA mRNA induction

The relatively slow development of epileptogenesis in the amygdala-kindling model allows a detailed analysis of the progressive development of focal seizures with secondary generalization (Kalynchuk, 2000; Racine et al., 2002). Activin, as with BDNF, is a known neurotrophic factor, so cells showing elevated message levels may be implicated in roles underlying the enduring neuronal changes associated with epilepsy (Gall, 1993). The data show a spatiotemporal spread of gene induction across the amygdala and cortical areas affected by the seizures.

The 2 hours postseizure survival time in experiment 1 was selected because earlier reports showed that pharmacological intervention could be neuroprotective against status epilepticus (Kelly and McIntyre, 1994) at 2 hours and because kindling-induced up-regulation of mRNAs for cytokines such as TGFβ occurred at 2 hours (Plata-Salaman et al., 2000). At this time point, immediate-early genes (IEGs) such as c-fos mRNA are absent or no longer expressed, having peaked much earlier after the seizure (Burazin and Gundlach, 1996). Indeed, we performed parallel hybridizations with ribonucleotide probes for c-fos and zif268 (NGFI-A), and we saw only inconsistent, weak bilateral global cortical expression in the same animals (data not shown).

The functional markers c-fos and 2-deoxyglucose are responsive to most forms of excitatory neural activity and thereby show widespread elevations in cortical and subcortical labeling (Clark et al., 1991; Engel et al., 1978; Labiner et al., 1993), parts of which may not be relevant to the synaptic events responsible for enduring changes in kindled seizure susceptibility. Thus, it is not surprising that the activin βA mRNA expression pattern represents a discrete subportion of the early-appearing and transient IEG activation pattern. It is possible, of course, that the limited distribution of expressing cells is due to the inability of some neuronal groups to respond with elevated activin βA mRNA expression.

Results of this experiment can be compared with data based on functional markers used to map the spread of seizure effects following similar amygdala-kindling paradigms or status epilepticus. The previous studies were carried out with 2-deoxyglucose autoradiography (Engel et al., 1978; McIntyre et al., 1991), Fos immunohistochemistry (White and Price, 1993a), or IEG mRNA hybridization (Clark et al., 1991; Hosford et al., 1995; Sitcoske O'Shea et al., 2000). In these studies, brain areas responding early in the course of seizure development include the amygdala near the stimulation site and the adjacent piriform cortex. At later stages, widespread cortical and olfactory areas, hippocampus, and several subcortical structures—the ventral striatum (accumbens), basal forebrain and bed nucleus of the stria terminalis, thalamus (mediodorsal nucleus), and substantia nigra—show strong activation. Lesion studies have argued for the critical importance of the ipsilateral piriform cortex (Schwabe et al., 2000), insular cortex (Kodama et al., 2001), perirhinal cortex (Kelly and McIntyre, 1996; Kelly et al., 2002), and basal amygdala (White and Price, 1993b) in the seizure evolution process leading to convulsive behavior.

The induced activin βA mRNA expression pattern is strikingly discrete and anatomically maps several structures known to be important in seizure development and the epileptic state. However, some components of the temporal spread of activin labeling are not easily explained. Spread of activity-associated activin βA mRNA induction

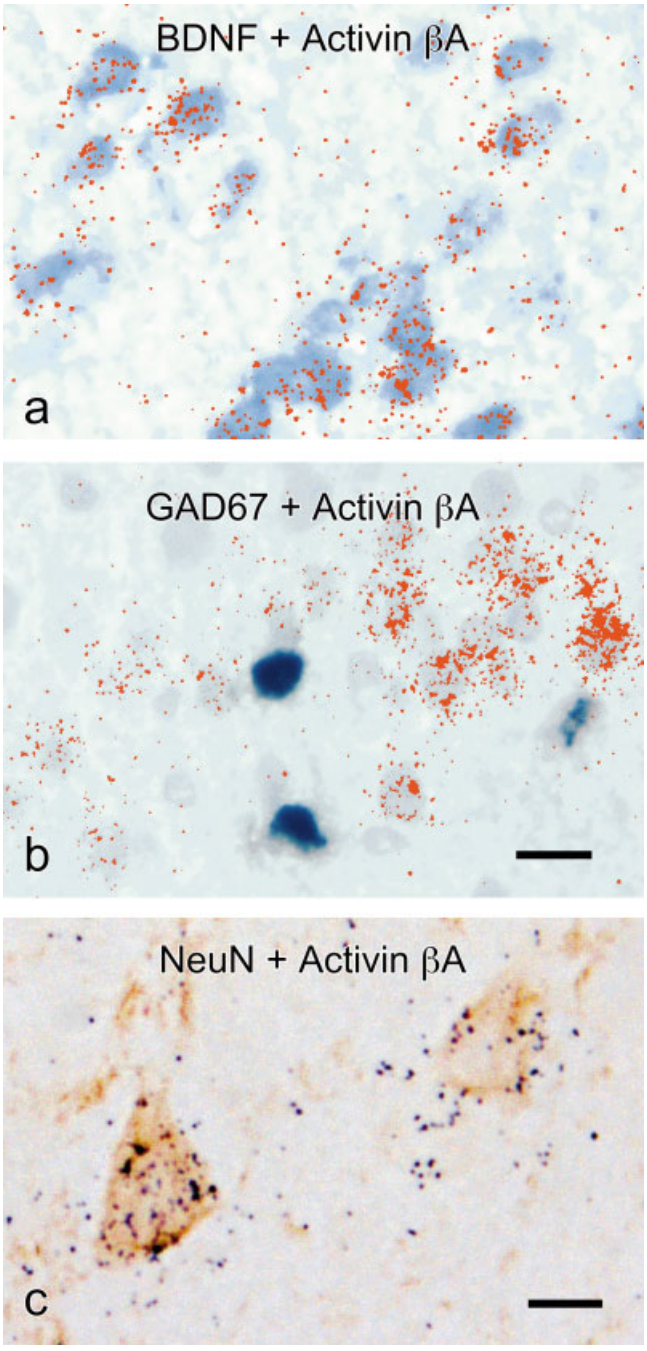

Fig. 6. Photomicrographs of neocortical layer II show that induced activin βA mRNA expression (^{35}S-labeled red grains) colocalizes with induced BDNF mRNA expression (DIG-labeled dark cells; **a**) but does not colocalize with GABAergic neurons marked by GAD67 mRNA (Dig-labeled; **b**). Activin βA mRNA (black grains) colocalizes with NeuN immunostained neocortical neurons (brown; **c**). Scale bar = 20 μm in b (applies to a,b); 10 μm in c.

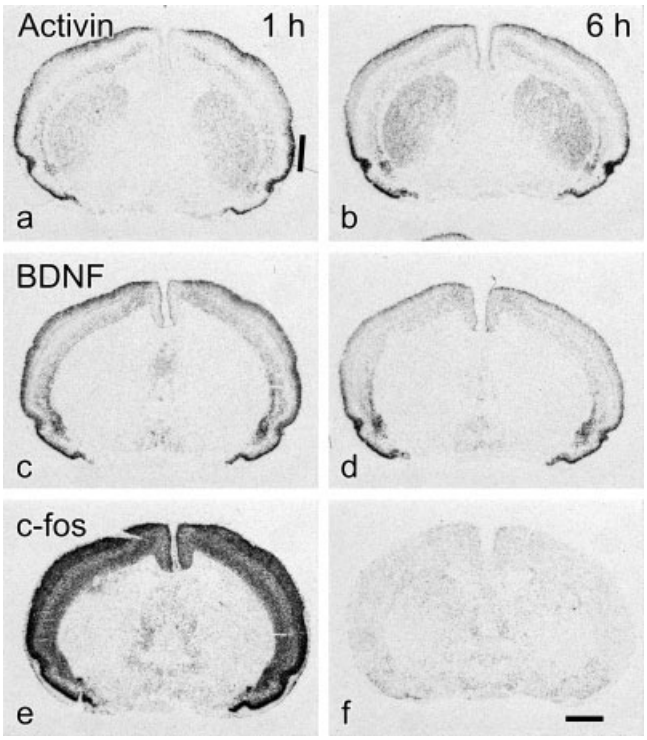

Fig. 7. Film autoradiographs show cortical induction patterns of mRNAs for activin βA (**a,b**), BDNF (**c,d**), and c-fos (**e,f**) at the level of the anterior commissure and striatum 1 hour (a,c,e) or 6 hours (b,d,f) after a full generalized seizure induced by amygdala stimulation on the left side. Line in a shows location of insular cortex, where densitometry was performed (see Fig. 8). Scale bar = 1 mm.

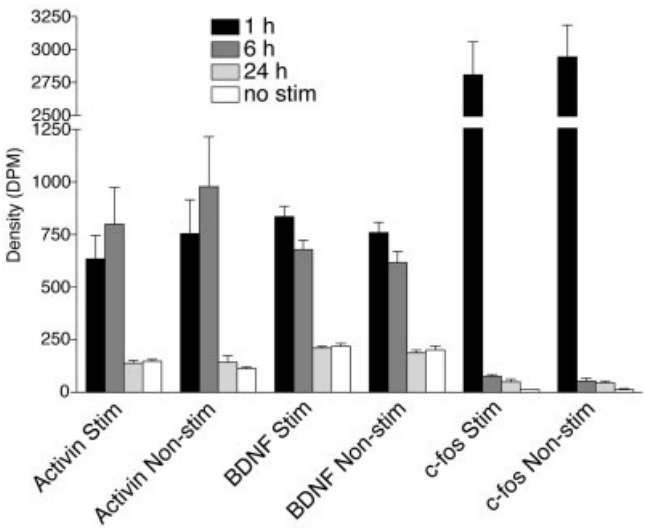

Fig. 8. Graph of mRNA expression levels (DPM/mg ± SEM) for three probes studied in experiment 2. Analysis of insular cortex on the stimulated (Stim) and nonstimulated (Non-stim) sides is shown for activin βA, BDNF, and c-fos at 1, 6, and 24 hours and in nonstimulated control animals.

from amygdala to cortex could occur via direct axonal projections from the BL to layers II and V of the perirhinal, insular, and prelimbic areas (Krettek and Price, 1977). These are the areas (and layers) that showed strong activin βA mRNA induction with the first stage 2 (partial)

seizures. Projections along this continuum are augmented by additional projections from the endopiriform nucleus (Behan and Haberly, 1999) and perirhinal cortex (Kelly and McIntyre, 1996; McIntyre et al., 1996) to insular and prelimbic cortices, all ipsilaterally. The first appearance of activin in the convexity cortex was bilateral (Figs. 1c,d, 2) and did not appear to require prior bilateral labeling along the sulcal cortex. Spread of activin βA message to the contralateral side occurred independently for amygdala and cortex. Thus, for example, in animals 4 (1s2) and 18 (1s5), neocortical labeling was stronger contralaterally than ipsilaterally, whereas amygdala labeling was predominantly ipsilateral. In another animal 19 (1s5), amygdala labeling was strictly and strongly ipsilateral, whereas cortical labeling was highly restricted bilaterally to the rostral insular and prelimbic areas and ipsilaterally to the caudal piriform cortex (data not shown).

The rapid switch to bilaterally symmetrical labeling in the amygdala does not closely resemble crossed amygdala projections, which spread beyond the homologous contralateral structure. For example, whereas the basolateral nuclei (notably the magnocellular basal nuclei) project to their contralateral homologous counterparts, they also project to nonhomologous structures, such as the central and anterior nuclei and the nucleus of the lateral olfactory tract (Savander et al., 1997), which did not show contralateral activin βA mRNA induction. It is noteworthy that the BL is necessary for spread of kindling, as determined by focal deactivation studies (White and Price, 1993b).

Corticocortical connections across the corpus callosum typically have a discontinuous, banded termination pattern (Cipolloni and Peters, 1979), which does not resemble the horizontally uniform pattern of mRNA for activin βA or other genes (see, e.g., Bengzon et al., 1993; Burazin and Gundlach, 1996) contralateral to the stimulation. Thus, the bilateral and rather symmetrical pattern of cortical activation is not easy to explain anatomically, which is analogous to the situation in the amygdala (see above). Horizontal spread of activity within the cortex might account for the uniformity of labeling strength.

Appearance of bilateral activin βA mRNA induction in the primary motor cortex (M1) at the first stage 5 seizure (1s5) shows early involvement of this area in the kindling phenomenon. The motor cortex is presumably the source of the behavioral responses (clonus) seen in the animals upon stimulation (Kelly et al., 1999). Synaptic changes here could underlie the lowered threshold for seizure recruitment and convulsive expression.

The distinctive pattern of activin βA mRNA expression overlaps with existing constitutive expression of activin receptors (ActR-II and ActR-IIB; Fig. 4; Cameron et al., 1994). These receptors are especially enriched in the endopiriform nucleus and the lateral, basal, and posterior cortical amygdaloid nuclei, which show elevated activin βA mRNA, but they are also strongly present in the central, medial, and anterior amygdaloid nuclei, which did not show activin βA mRNA elevations. This demonstrates that the discrete activin βA mRNA expression profile does coincide with some but not all areas of high densities of receptors.

Duration of mRNA induction after the seizure

Experiment 2 was designed to address the postseizure duration of activin βA mRNA expression in comparison with two other representative markers, c-fos, an immediate-early gene; and BDNF, a neurotrophic factor. After a full generalized (6s5) seizure, c-fos mRNA was strongly induced in the cerebral cortex bilaterally only at 1 hour, and its expression levels had returned to baseline by 2 hours. In contrast, both activin βA and BDNF showed coordinated bilateral induction in cortical layers II/III at 1, 2, and 6 hours, returning to baseline by 24 hours. The selectively extended period of elevated mRNA expression of activin βA and BDNF argues that these transcripts code for proteins that serve functions similar to those of neurotrophic factors regulating development and maintenance of the epileptic state and do not merely reflect transiently elevated neuronal activity. The subtle variations in laterality and time of peak expression between activin βA and BDNF mRNAs argue that they are under slightly different regulatory control.

Laminar and cellular analysis

Activin βA mRNA expression was largely confined to layer II neurons, which were typically pyramidal (e.g., Fig. 6c). These neurons presumably were not γ-aminobutyric acid (GABA)-ergic (being GAD67 mRNA negative); however, they did coexpress induced BDNF mRNA. Thus, both activin βA and BDNF mRNAs are selectively induced in presumably glutamatergic cortical projection neurons. Additional molecular markers of kindling-induced neocortical layer II neuronal mRNA expression include BDNF (present study; Ernfors et al., 1991; Isackson et al., 1991) and neuropsin (Okabe et al., 1996). BDNF is known to be neuroprotective and, in addition, may participate in synaptic changes that could support the epileptic state. Neuropsin is a serine protease that may affect synaptic architecture by proteolytic degradation of extracellular matrix proteins, such as fibronectin (Tomimatsu et al., 2002), and has been suggested to be neuroprotective to hyperexcitability (Davies et al., 2001). Activin is protective in some models of excitotoxic/ischemic brain injury (Hughes et al., 1999; Mattson, 2000; Tretter et al., 2000), and antiinflammatory actions, resembling those of TGFβ, have been shown (de Kretser et al., 1999). These molecules may play a role in the phenomenon of kindling-induced protection against kainate seizure-induced neuronal degeneration (Kelly and McIntyre, 1994).

Implications

Together the striking and distinctive induction pattern of activin βA mRNA, the selective localization to neocortex layer II pyramidal neurons, and the coincident expression of BDNF mRNA suggest that activin A may be an important molecule in the kindling process. We suggest that, as with BDNF and neuropsin, activin may play a neuroprotective role. Activin receptors are protein serine kinases (Mathews and Vale, 1993) that selectively activate Smad2/3 via activin receptor-like kinase-4 (ALK-4) signaling (Massague, 1998). The roles of these Smads in neuronal function are not well known, but Smads are involved in neuronal remodeling (Zheng et al., 2003), neuroprotection (Docagne et al., 2002), and inhibition of calcium influx (Williams et al., 2002). These Smads physically interact in the cell with many important molecules (Miyazawa et al., 2002), such as calmodulin (Zimmerman et al., 1998), nuclear factor-κB subunits (Lopez-Rovira et al., 2000), hormone receptors (Miyazawa et al., 2002), and the Jun family of AP-1 transcription factors (Liberati et al., 1999). In

contrast, BDNF acts through transmembrane receptor tyrosine kinases (Trk), notably TrkB, leading to important intracellular signaling cascades such as MAPK-mediated signaling and CREB phosphorylation. Evidence suggests that BDNF contributes to the epileptic state (Binder et al., 2001), largely because $BDNF^{+/-}$ mice show suppressed development of kindling (Kokaia et al., 1995). Future work with regard to activin will focus on its actions through the ActR-II receptors and Smad pathways. As with BDNF, work showing the results of activin application and blockade in physiological studies will further reveal its roles in adult neuronal plasticity.

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