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Molecular maps of neural activity and quiescence

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Abstract. The rapid accumulation of inducible transcription factors (ITFs), such as c-Fos and Zif268, in activated neurons combined with histological methods that offer detection at the cellular level are key features that have led to their wide use in visualizing activated neurons. There are two major drawbacks of ITFs that limit their use in the CNS - cell-type expression specificity and stimulus-transcription coupling uncertainty. Recent technical advances in the field of molecular activity mapping now permit dual-labeling approaches that help resolve some of these ambiguities and identify neurons that are activated by different sensory stimuli. Furthermore, the recent identification of the *robl*/*LC*7-like gene, which shows immediate-early repression after stimulation, may have utility in functional mapping where it can be used to delineate quiescent neurons and serve as a complement to molecular activity markers.

Key words: immediate-early genes, histochemistry, neocortex, plasticity, sensory stimulation, *c-fos*

INTRODUCTION

The immediate early genes (IEGs) *c-fos* and *zif268* have become popular neurobiological tools for mapping functional activity (Sharp et al. 1993, Hughes and Dragunow 1995, Chaudhuri 1997, Kaczmarek and Chaudhuri 1997). We now know that the expression of these genes is strongly, though not exclusively, linked to synaptic stimulation and that their products may be involved in key aspects of normal cellular function. Although much progress has been made toward understanding the intracellular processes that guide the expression of these genes, the precise physiological roles of the proteins encoded by them remain largely unknown. Many members of the IEG family are activated shortly after cell stimulation and without the requirement for *de novo* protein synthesis. The products of many of these genes have been observed in a wide range of tissues and under a variety of stimulation conditions (Herrera and Robertson 1996, Herdegen and Leah 1998).

In this paper, we describe two recent developments in neural activity mapping with the use of immediate-early gene products. First, the different time course of IEG mRNA and protein expression allows dual-labeling approaches that can be used to clarify expression specificity in a subpopulation of neurons. And secondly, the recent cloning of a cDNA encoding rat dynein light chain protein, *robl*/*LC*7-like (class 1), from visual cortex allows labeling of quiescent neurons. It has been shown that *robl*/*LC*7-like is rapidly down-regulated by neural activity. This feature allows its use in conjunction with inducible transcription factors to visualize both active and quiescent neural populations after stimulus application.

CHARACTERISTICS OF INDUCIBLE TRANSCRIPTION FACTORS

The various members of the IEG family are known to encode proteins with diverse cellular functions. In the brain, IEGs that are linked to neural activity and that have mapping applications generally encode proteins that serve as transcription factors - i.e., complexes that bind to the promoter regions of certain genes and then either activate or repress their expression. These so–called inducible transcription factors (ITFs) are distinguished from other proteins that normally reside in the nucleus and which also serve as regulators of ongoing transcriptional activity.

The ITFs c-Fos and Zif268 are both induced in neurons after extracellular stimulation by neurotransmitters and trophic substances (Fig. 1). The sequence of events that leads to ITF induction is largely coordinated by Ca^{2+} influx into the cell (Ghosh et al. 1994). This can occur either through the NMDA receptor-Ca^{2+} ionophore complex after glutamate binding or through voltage--sensitive calcium channels (VSCCs) following membrane depolarization. Thereafter, several different enzyme systems are marshaled by Ca^{2+} . These include various protein kinases that activate the transcription factor CREB (Sheng and Greenberg 1990) and phospholipases that initiate the production of prostaglandins (PG) and leukotrienes (LK) (Lerea et al. 1995). NMDA receptor activation can also relay its effects through a second pathway involving extracellular signal–regulated kinases (ERKs) (Xia et al. 1996, Kaminska et al. 1999).

After transcription is completed, the ITF mRNAs are translated into a protein product (c-Fos and Zif268) in the cytosol. These products rapidly migrate into the nucleus where they themselves influence the expression of another set of genes, the late–response genes. In the case of c-Fos, it must dimerize with a member of the Jun phosphoprotein family (c-Jun, JunB, or JunD) to produce a functional transcription factor that is called activating–protein 1 (AP-1). By regulating the expression of a host of late–response genes, both AP-1 and Zif268 are able to have a commanding influence on short- and long-term cellular homeostasis. Regardless of what the specific molecular roles of ITFs may be, their accumulation in the neuron generally signifies a prior state of activity and thus forms the logic for obtaining functional maps based on ITF staining.

TIME-COURSE OF IEG EXPRESSION

One of the striking advantages of IEG use in activity mapping is the short time course of induction. It appears that the intracellular mechanisms outlined in Fig. 1 operate in a very rapid manner such that mRNA levels are detectable in neurons within 20 min of stimulation (Worley et al. 1990, Chaudhuri et al. 1997). However, mRNA detection is technically difficult and generally relies on autoradiographic visualization of radiolabeled antisense probes. The more preferred technique is to apply immunocytochemical methods to detect IEG protein expression. The time course for IEG protein expression, however, is somewhat greater than for mRNA expres-

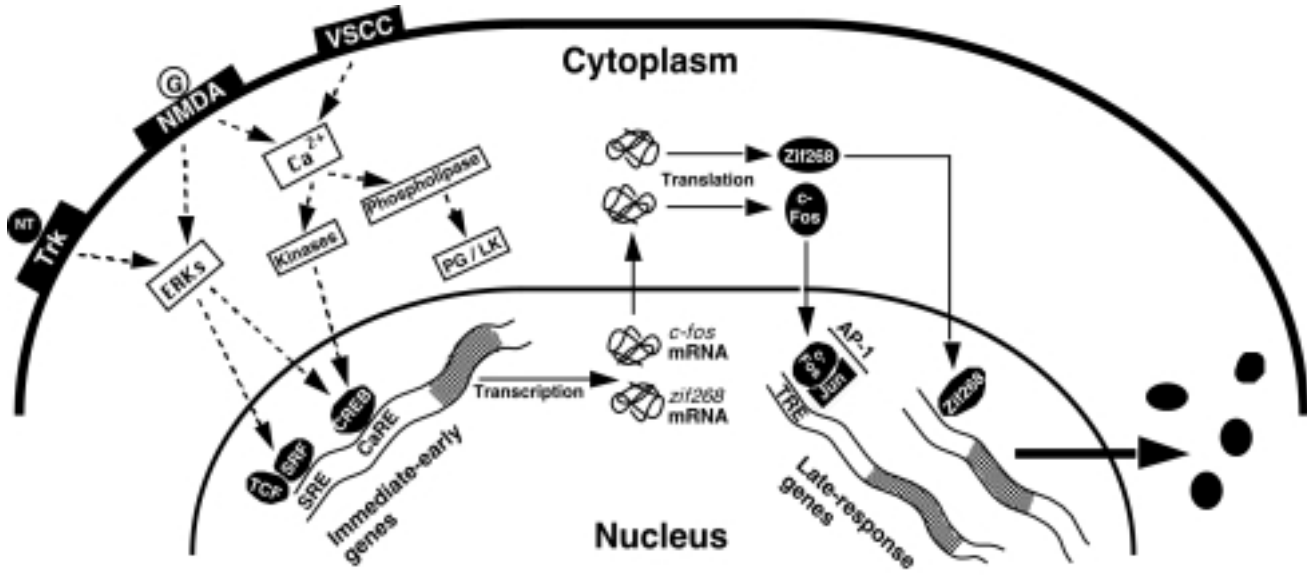

Fig. 1. Molecular processes involved in induction of two inducible transcription factors, c-Fos and Zif268, in neurons.

sion, most likely due to the added requirement for translation to be completed.

The temporal profile for c-Fos protein expression is shown in Fig. 2. These data were gathered from visual cortex of rats subjected to various periods of light exposure after 24 h of dark rearing. The dark control animal, which did not receive any light exposure, shows very few c-Fos immunopositive cells. As light exposure is increased, a greater degree of c-Fos expression is found. This expression peaks at approximately 1.5 h to 2 h after stimulus onset. Thereafter, c-Fos expression declines rapidly. This has now been shown from both immunocytochemical staining studies and by gel shift analysis of AP-1 DNA-binding activity (Kaminska et al. 1996). The light control animal, interestingly, shows very few immunopositive cells. In fact, the visual cortex of this animal is barely distinguishable from that of the dark control animal. This is in sharp contrast to Zif268 staining which remains elevated as long as sensory stimulation is applied (Chaudhuri et al. 1995).

While both *c-fos* and *zif268* can be induced by synaptic stimulation, the difference in their basal expression levels has certain implications for their use in activity mapping. The relative instability of *c-fos* mRNA and the presence of a negative feedback loop whereby the protein down–regulates its own transcription result in low constitutive c-Fos levels. As such, c-Fos staining is most informative when novel stimuli are applied or when the animal is stimulated after a period of sensory deprivation. Only in these situations will those neurons that are specifically responsive to the stimulus undergo *c-fos* induction in a rapid and transient manner. By the same argument, c-Fos staining after prolonged stimulation will yield negligible results. Because Zif268 does not appear to share the autoregulation features of c-Fos, it will display persistent expression and therefore high basal levels in many neural structures. This leads to the notion that *zif268* expression is linked to ongoing synaptic activity whereas *c-fos* expression is reliant upon activity being triggered after a period of neural quiescence or after exposure to a novel stimulus.

STRATEGIES FOR DETERMINING EXPRESSION SPECIFICITY

Given that IEG expression can be influenced by multiple receptor systems and coordinated by different signal transduction pathways, a causal link between ITF gene expression and a particular triggering event is often difficult to establish. Furthermore, functional mapping with *in vivo* preparations suffers from the usual uncertainty as to whether the pattern of ITF expression was produced by the specific behavioral task or sensory stimulus that was applied, by some non–specific feature of the experimental condition, or by an unrelated mental (or endocrine) event. Of course, suitable controls can be applied to verify the stimulus–response association, either at the stimulation stage or during evaluation of expres-

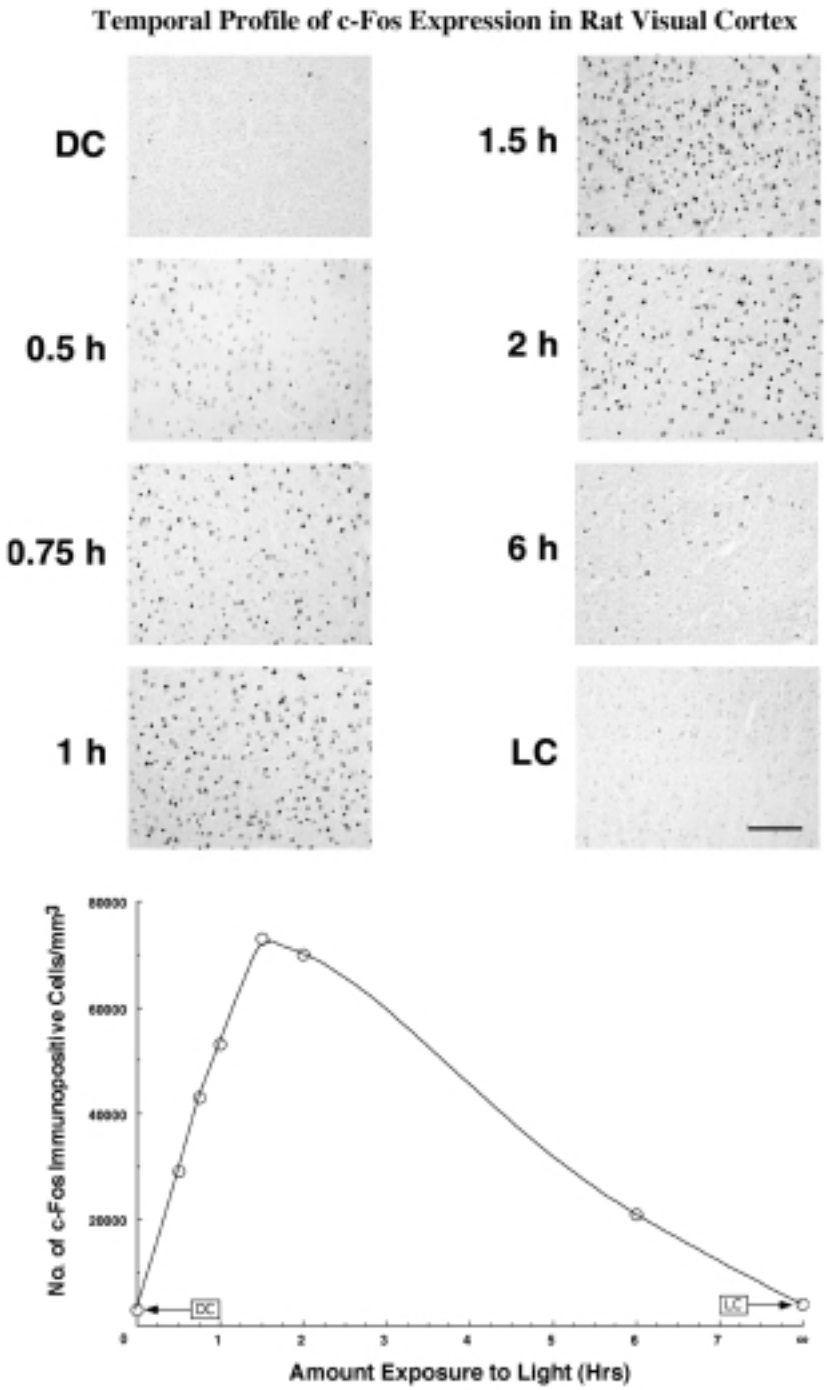

Fig. 2. Time course of c-Fos protein expression in rat visual cortex. The greatest level of expression is seen around 1.5 to 2 h after onset of visual stimulation. Both dark control (DC) and light control (LC) animals show very little c-Fos expression.

sion maps by comparison with other related neural compartments or activity markers (e.g., 2-DG, cytochrome oxidase, etc.). However, these controls can only resolve the uncertainty at a regional level, i.e., they do not permit a positive association to be made at the cellular level for any of the individual ITF–stained neurons. And yet, one of the most appealing features of this technique is the cellular resolution that ITF activity maps offer - a feature that often cannot be fully exploited due to the stimulus–coupling uncertainty.

The recent development of an ITF dual activity mapping technique may allow for an internal control to resolve such ambiguities at the cellular level (Chaudhuri et al. 1997). The principle behind dual–activity mapping relies on the different spatial localization of ITF mRNA and protein (cytosol and nucleus respectively) and the different temporal patterns of their expression. This is illustrated in Fig. 3. Both *c-fos* and *zif268* mRNA levels are effectively regulated in either direction within 20 min. of stimulus onset or offset whereas the protein product requires a longer period that can be upto 90 min. The discrepant time course of these two products may be exploited to provide a visual display of neurons that are stimulated under two different conditions. By first applying a stimulus for a prolonged period (say 90 min or more) followed by a different stimulus for another 20 min, it is theoretically possible to stain for the neurons that are selective to each stimulus by combined application of immunocytochemistry (ICC) and *in situ* hybridization histochemistry (ISH). Neurons that were triggered by the first stimulus (and unaffected by the second) will be immunopositive only, neurons stimulated by the second stimulus only will be mRNA–positive only, and those with overlapping sensitivity will be double–labeled. Therefore, dual activity maps at the cellular level should allow identification of neurons that were presumably triggered by each stimulus as well as those neurons that were likely to have been stimulated by some measure of both. This feature can provide an internal control for coding specificity of mRNA–positive neurons because it is unlikely that non–specific effects could arise only during the terminal period of stimulation and not before.

The basic idea behind dual-IEG labeling has now been verified in monkey visual cortex by differential staining of the two sets of ocular dominance columns (Chaudhuri et al. 1997). A reverse occlusion procedure can sequentially and separately stimulate each of the two ocular dominance column sets. By using appropriate durations of stimulation through each eye, it was shown

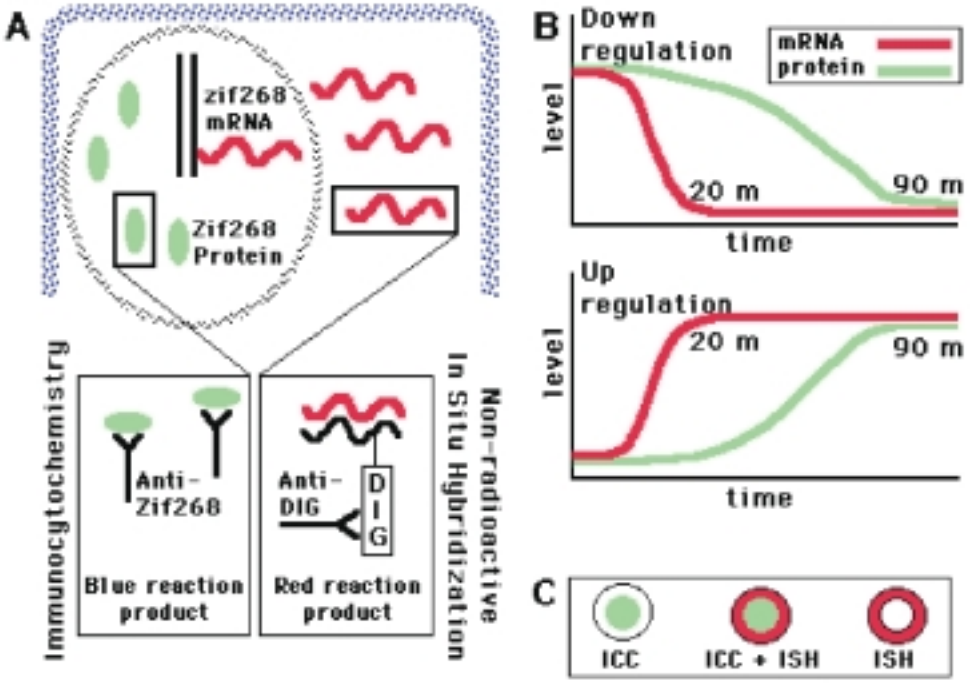

Fig. 3. The logic behind dual-labeling of IEG mRNA and protein in neurons. Shortly after onset of sensory stimulation, *zif268* mRNA levels accumulate in the cytosol and Zif268 protein in the nucleus (A). These products can be separately visualized by immunocytochemical (ICC) and *in situ* hybridization (ISH) techniques for protein and mRNA staining respectively. The time course of mRNA and protein expression, however, is quite different. In general, mRNA levels peak at around 20-30 min. after stimulus onset whereas protein accumulation peaks at about 90 min. (B). Sequential application of two different stimuli at these durations will lead to accumulation of protein in neurons selective to the first stimulus, mRNA in neurons selective to the second stimulus, and both products in neurons that have overlapping sensitivity (C).

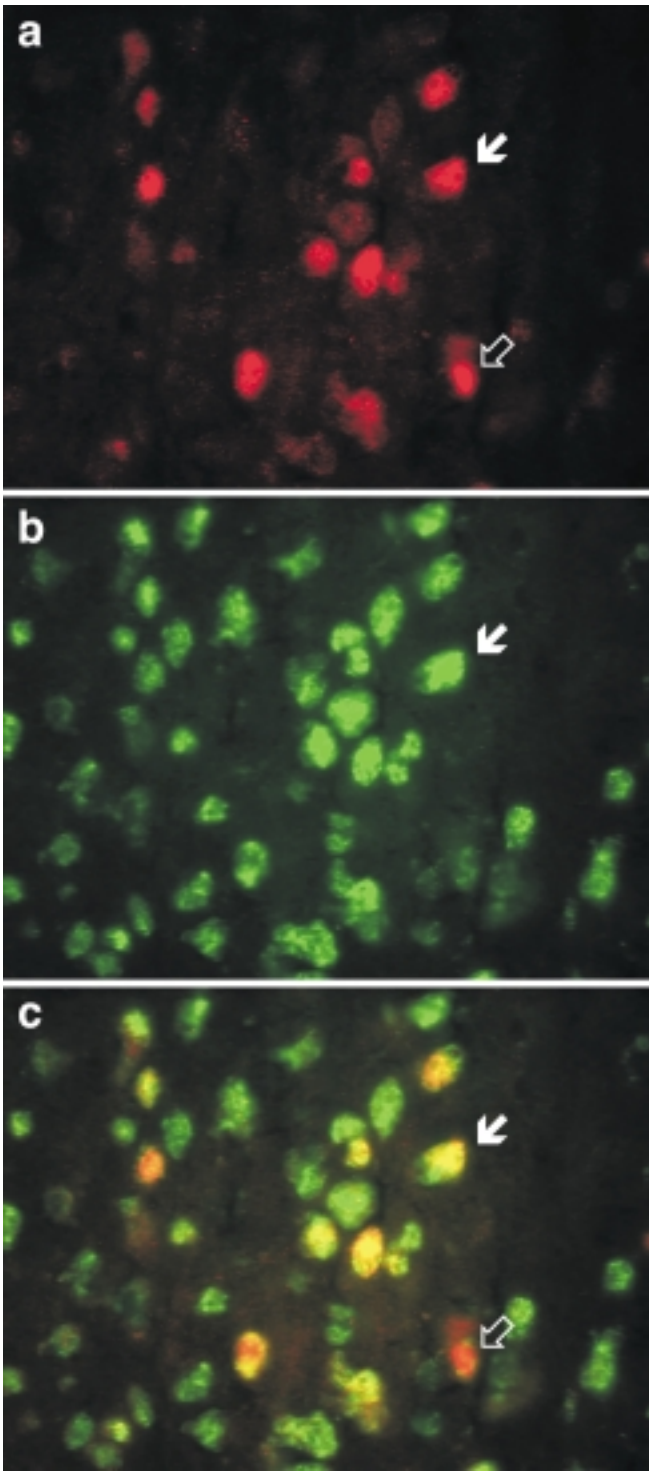

Fig. 4. Dual activity labeling in superior colliculus. Rats were deprived of accoustic and visual stimulation for 72 h followed by 2 h of sound and 30 min of light stimulation. Zif268 protein staining reveals neurons in the deep layers of the superior colliculus that were likely activated by accoustic stimulation (A). *In situ* hybridization histochemistry shows mRNA staining in neurons that were likely activated by visual stimulation (B). Simulataneous visualization of both products reveals that the majority of deep SC auditory neurons are polysensory and therefore also activated by visual stimulation (open arrowheads) whereas a few neurons were activated only by visual stimulation (closed arrowheads).

that one set of columns can be stained by Zif268 protein whereas the alternate set is stained by *zif268* mRNA. Cellular level staining showed that dual-labeled cells (i.e., binocular neurons) were predominantly found along the border regions of the ocular dominance columns. This dual activity labeling strategy would have particular appeal to neural systems that process multimodal sensory information. One such structure is the deep layers of the superior colliculus where both visual and auditory information is processed. The dual labeling technique can be used to dilineate those neurons that process only visual or auditory signals and those that are polysensory. As Fig. 4 shows, cellular level mapping can indeed provide activity maps generated by selective sensory stimulation. In this example, rats were acoustically stimulated for two hours in the dark to activate auditory neurons only followed by visual stimulation for 30 min in acoustic silence to activate visual neurons. Immunocyto- chemical mapping at the cellular level revealed Zif268 protein-labeled cells (Fig. 4A) that were presumably auditory-selective neurons. In situ hybridization mapping at the cellular level revealed Zif268 mRNA-labeled cells that were presumably visually driven (Fig. 4B). Simultaneous staining for both mRNA and protein products revealed that a large majority of the deep SC auditory neurons were multisensory and therefore also activated by visual stimuli (Fig. 4C). However, there were a few neurons that were stained only for the mRNA product, suggesting the presence of a subpopulation of unisensory visual neurons. In our sample, we failed to find unisensory auditory neurons.

VISUALIZING QUIESCENT NEURONS: NEW APPROACHES TO LABELING INACTIVE NEURONS

While a number of IEGs and late-response genes are induced by membrane depolarization, the opposite effect on gene expression is far less common. To date, only a handful of genes have been shown to be down-regulated after stimulation. The most notable in this class include the Kv1.2, Kv1.5, and Kv4.2 voltage-gated K + channels (Tsaur et al. 1992, Levitan et al. 1995). Depo-

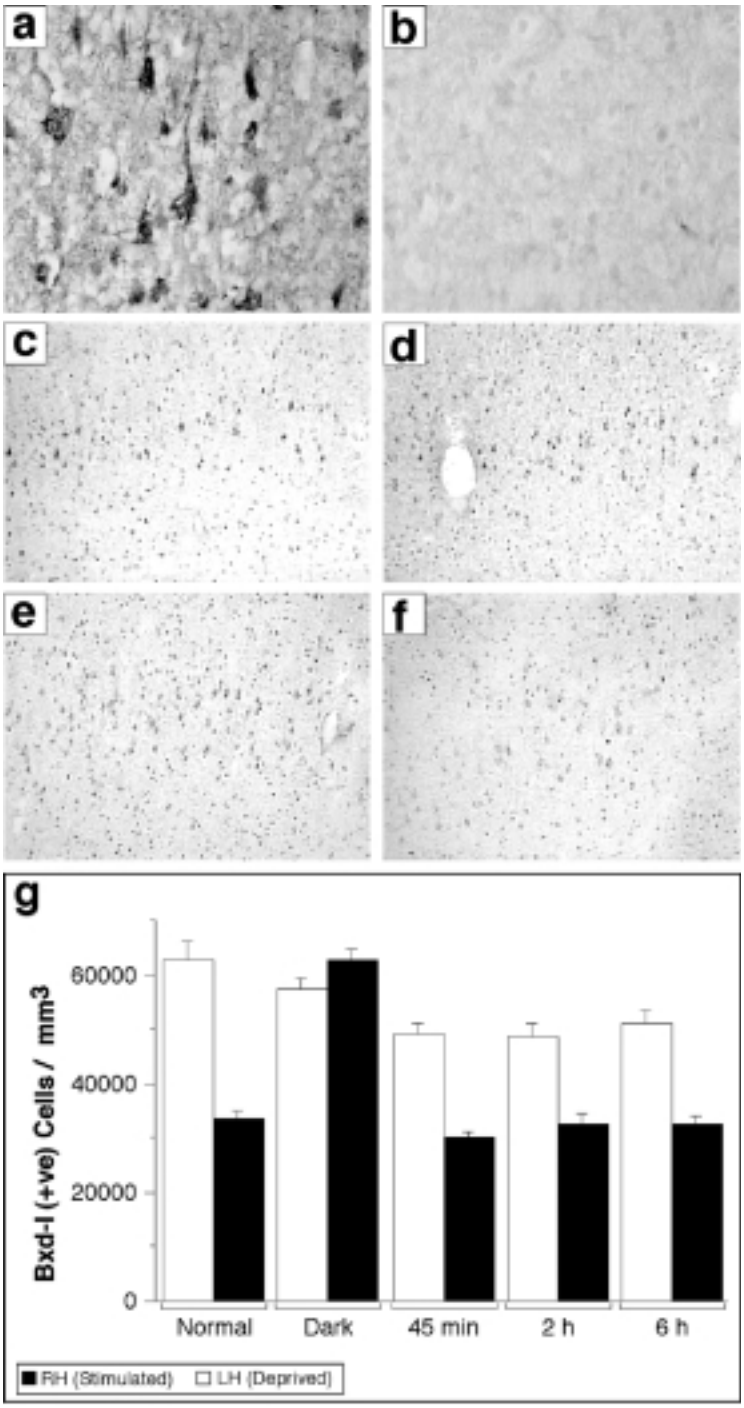

Fig. 5. Rat dynein light chain protein, *robl*/*LC*7-like (class 1), from visual cortex shows intense immunostaining in neocortex (a) that was highly specific, as shown by the positive control (b). Monocularly enucleated rats that were dark reared show no hemispheric difference in staining (c, d). However, if briefly exposed to light, then the light stimulated hemisphere (f) shows fewer labeled neurons compared to the inactive hemisphere (e). Light-driven repression of *robl*/*LC*7-like protein is seen in as little as 45 min. after stimulus onset, an effect that persists with prolonged stimulation (g).

larization-induced inhibition of the Kv1.5 subtype has been shown in clonal pituitory cells at levels of both message transcription and channel protein expression (Levitan et al. 1995) whereas suppression of Kv1.2 and Kv4.2 K + channel genes was shown at the mRNA level in dentate granule cells after seizure induction (Tsuar et al. 1992). The expression of CaMKII alpha subunit in mammalian neocortex has also been shown to be reduced after both electrical and sensory stimulation (Jones et al. 1996, Liang et al. 1996). However, in all of these cases, the down-regulation occurred several hours to days after onset of stimulation. As such, these genes by definition would not belong to the immediate-early response class.

The recent cloning of a full-length cDNA encoding rat dynein light chain protein, *robl*/*LC*7- like (class 1),

from visual cortex may now open the way for staining inactive neurons (Ye et al., unpublished). It was shown that rat *robl/LC7*-like gene is highly expressed in neocortex and displays the unusual feature of being rapidly down-regulated by sensory stimulation. This effect was seen at both mRNA and protein levels in visual cortex, being detectable in as little as 45 min. after the onset of visual stimulation (Fig. 5). Down-regulation by sensory stimulation was also found within ocular dominance columns of area V1 in monocularly deprived monkeys. These results suggest a high turnover rate of the *robl/LC7*-like protein and the presence of a repressor mechanism in neurons that is tightly coupled to synaptic stimulation. This unusual feature of may be of use in functional mapping studies to delineate neuronal populations that remain quiescent after exposure to a particular sensory stimulus and therefore provide a complement to immediate-early gene products that stain activated neurons. It is likely that other members of immediate-early repressors will emerge in the future, thereby expanding the number of available tools that can be applied in molecular labeling of neural activity.

REFERENCES

Chaudhuri A. (1997) Neural activity mapping with inducible transcription factors. NeuroReport 8: iii-vii.

Chaudhuri A., Matsubara J.A., Cynader M.S. (1995) Neuronal activity in primate visual cortex assessed by immunostaining for the transcription factor Zif268. Vis. Neurosci. 12: 35-50.

Chaudhuri A., Nissanov J., Larocque S., Rioux L. (1997) Dual activity maps in primate visual cortex produced by different temporal patterns of zif268 mRNA and protein expression. Proc. Natl Acad. Sci. USA 94: 2671-2675.

Ghosh A., Ginty D.D., Bading H., Greenberg M.E. (1994) Calcium regulation of gene expression in neuronal cells. J. Neurobiol. 25: 294-303.

Herdegen T., Leah J.D. (1998) Inducible and constitutive transcription factors in the mammalian nervous system: control of gene expressionby Jun, Fos and Krox, and CREB/ATF proteins. Brain Res. Rev. 28: 370-490.

Herrera D.G., Robertson H.A. (1996) Activation of c-fos in the brain. Prog. Neurobiol. 50: 83-107.

Hughes P., Dragunow M. (1995) Induction of immediate-early genes and the control of neurotransmitter-regulated gene expression within the nervous system. Pharmacol. Rev. 47: 133-178.

Jones E.G., Huntley G.W., Benson D.L. (1994) Alpha calcium/calmodulin-dependent protein kinase II selectively expressed in a subpopulation of excitatory neurons in monkey sensory- motor cortex: comparison with GAD-67 expression. J. Neurosci. 14: 611-629.

Kaczmarek L., Chaudhuri A. (1997) Sensory regulation of immediate-early gene expression in mammalian visual cortex: Implications for functional mapping and neural plasticity. Brain Res. Rev. 23: 237-356.

Kaminska B., Kaczmarek L., Chaudhuri A. (1996) Visual stimulation regulates the expression of transcription factors and modulates the composition of AP-1 in visual cortex. J. Neurosci. 16: 3968-3978.

Kaminska B., Kaczmarek L., Zangenehpour S., Cahudhuri A. (1999) Rapid phosphorylation of Elk-1 transcription factor and activationof MAP kinase signal transduction pathways in response to visual stimulation. Mol. Cell. Neurosci. 13: 405-414.

Lerea L.S., Carlson N.G., McNamara J.O. (1995) N-methyl-D-aspartate receptors activate transcription of c-fos and NGFI-A by distinct phospholipase A2-requiring intracellular signaling pathways. Mol. Pharmacol. 47: 1119-1125.

Levitan E.S., Gealy R., Trimmer J.S., Takimoto K. (1995) Membrane depolarization inhibits Kv1.5 voltage-gated K+ channel gene transcription and protein expression in pituitary cells. J. Biol. Chem. 270: 6036-6041.

Liang F., Isackson P.J., Jones E.G. (1996) Stimulus-dependent, reciprocal up- and down-regulation of glutamic acid decarboxylase and Ca^{2+}/calmodulin-dependent protein kinase II gene expression in rat cerebral cortex. Exp. Brain Res.110: 163-174.

Sharp F.R., Sagar S.M., Swanson R.A. (1993) Metabolic mapping with cellular resolution: c-fos vs. 2-deoxy-glucose. Crit. Rev. Neurobiol. 7: 205-228.

Sheng M., Greenberg M.E. (1990) The regulation and function of c-fos and other immediate early genes in the nervous system. Neuron 4: 477-485.

Tsaur M.L., Sheng M., Lowenstein D.H., Jan Y.N., Jan L.Y. (1992) Differential expression of K+ channel mRNAs in the rat brain and down- regulation in the hippocampus following seizures. Neuron 8: 1055-1067.

Worley P.F., Cole A.J., Murphy T.H., Christy B..A., Nakabeppu Y., Baraban J.M. (1990) Synaptic regulation of immediate-early genes in brain. Cold Spring Harb.Symp. Quant. Biol. 55: 213-223.

Xia Z., Dudek H., Miranti C.K., Greenberg M.E. (1996) Calcium influx via the NMDA receptor induces immediate early gene transcription by a MAP kinase/ERK-dependent mechanism. J. Neurosci. 16: 5425-5436.