Neural activity mapping with inducible transcription factors

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Abstract

Neurons respond to extracellular stimulation by modulating the expression of certain immediate-early genes. Inducible transcription factors (ITFs), such as c-Fos and Zif268, are coded by this class of gene and are among the first proteins to appear. The rapid accumulation of these products 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. However, neuroscientists have long recognized two major drawbacks of ITFs that limit their use in the CNS: cell-type expression specificity and stimulus–transcription coupling uncertainty. In this review, I discuss recent advances in the field that broaden our understanding of the molecular constraints on ITF expression as well as in techniques that may help to extend their utility in functional mapping.

Introduction

It is 10 years since the discovery that sensory stimulation can initiate rapid and transient induction of the c-fos gene in neurons. Since then, much effort has been directed at understanding the molecular mechanisms that mediate the expression of c-fos as well as other so-called immediate early genes (IEGs). Indeed, we now know much about the receptor systems and signal transduction pathways that are involved in coupling synaptic stimulation to various IEG transcriptional responses. As a result of these efforts, the early notion that c-Fos, the protein product of the c-fos gene, may be used as an endogenous marker of neuronal activity has now become widely accepted (for recent reviews1–3).

Although the products of both c-fos and another IEG, zif268, have become popular tools for mapping functional activity in the brain, neuroscientists have had to deal with two major drawbacks to their use: cell-type expression specificity and stimulus–coupling uncertainty. The first problem can produce regional and cellular limitations in expression that constrain the use of IEGs as general markers of activity throughout the brain. The second problem can affect the interpretation of IEG activity maps once they are obtained. The purpose of this mini review is to examine recent developments in IEG mapping techniques and how continuing advances in our understanding of the molecular mechanisms that govern IEG induction may help to extend their utility in functional mapping.

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 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. This can occur either through the NMDA receptor–Ca\(^{2+}\) ionophore complex after glutamate (G) binding or through voltage-sensitive calcium channels (VSCCs) following membrane depolarization. Thereafter several different enzyme systems are marshalled by Ca\(^{2+}\). These include various protein kinases that activate the transcription factor CREB and phospholipases that initiate the production of prostaglandins (PG) and leukotrienes (LK).6,7 The precise manner by which PGs and LKs regulate IEG expression remains unknown, though each has been shown to have different effects on c-fos and zif268 transcription.

NMDA receptor activation can also relay its effects through a second pathway involving extracellular signal-regulated kinases (ERKs).8 This pathway is generally linked to neurotrophins (NTs) which upon binding to Trk receptors ultimately activate the transcription factors SRF and TCF that together regulate IEG transcription.9,10 There is also
evidence that Ca\textsuperscript{2+} entry through NMDA receptors or VSCCs is sufficient to trigger SRF-mediated transcription of c-fos, possibly through the ERK signal transduction cascade. CREB phosphorylation can also be triggered by ERKs after NT stimulation. Thus, multiple signaling pathways appear to converge upon both SRF/TCF and CREB. The trans-activating effects of these complexes occur when they are bound to specific sequences (SRE and CaRE/CRE respectively) that are present in the promoter regions of both the c-fos and zif268 genes.

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. c-Fos must dimerize with a member of the Jun phosphoprotein family (c-Jun, JunB, or JunD) to produce a functional transcription factor, activating-protein 1 (AP-1). AP-1 may either stimulate or repress the candidate gene after binding to a specific promoter sequence (TRE). 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 the specific molecular roles of ITFs, 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.

**Neural activity and ITF expression specificity**

One of the early concerns with ITF mapping was that in some cases c-Fos expression did not correlate with 2-deoxyglucose (2-DG) accumulation, one of the standard procedures for imaging neural activity. We now know that only a subset of neurons express a particular ITF and that neurons in some brain structures may not express it at all. This is now recognized to be one of the major drawbacks to ITF use since they cannot be considered to be a general marker of neural activity that can be applied throughout the CNS.

A particularly striking example of the fact that neuronal activation alone may not lead to ITF expression was recently shown by Kimpo and Doupe. They examined c-Fos expression in the birdsong system of zebra finches and found that singing induced c-Fos expression in two sensorimotor nuclei, HVc and RA. However, Kimpo and Doupe showed that it was the motor act of singing itself that induced this expression rather than auditory stimulation of the bird’s own song. This was based on the finding that normal and deafened birds that sang showed similarly robust c-Fos expression in the two nuclei. Furthermore, exposure of normal birds to taped auditory stimuli, and in the absence of singing, failed to produce c-Fos expression.

Although the Kimpo and Doupe study gives an elegant demonstration of how ITF maps can provide a visual display of neural assemblies that are involved in a particular behaviour, it also illustrates the discordance that can exist between neural activity and c-Fos expression at several levels. First, they observed structural expression specificity in that only the HVc and RA nuclei showed c-Fos induction whereas other nuclei that are known to be essential in birdsong learning and production did not. Second, cellular expression specificity was apparent within a particular neural structure. HVc contains two separate but interconnected popula-
tion of neurons: those projecting to the downstream RA nucleus and those projecting to a nucleus, area X, in the anterior forebrain. Double labeling with retrograde tracers showed that the first set of neurons expressed c-Fos after singing but the second set did not. And third, gene expression specificity was apparent in that certain auditory areas that are related to the birdsong system express Zif268 but not c-Fos. This finding may be related to an important difference in the temporal induction pattern of these two ITFs.

**Sustained and transient patterns of ITF induction**

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.

It is now apparent that c-fos induction in stimulated neurons is also influenced by the temporal dynamics of CREB phosphorylation, as shown recently by Liu and Graybiel. They studied the developing rat striatum and found that the duration of CREB phosphorylation was critical to subsequent c-fos induction (i.e. transient phosphorylation did not yield c-fos expression whereas sustained phosphorylation favoured induction of downstream CRE-containing genes). The interplay between cell-type specific phosphatases and phosphatase inhibitors turns out to be an important determinant as to whether CREB phosphorylation can be maintained after stimulation. Liu and Graybiel showed that D-1 class dopamine receptors activated a potent phosphatase inhibitor (DARPP-32) that allows sustained CREB phosphorylation, and subsequent c-Fos induction, to occur in developing striosomes but not in the surrounding matrix neurons, which lack the inhibitor. Similarly, Ca\(^{2+}\) influx through VSCCs activates the phosphatase calcineurin and abbreviates the temporal span of CREB phosphorylation in striosomes but not in matrix neurons, which do not contain this phosphatase. Thus, the temporal characteristics of CREB phosphorylation in the developing striatum, and its accompanying effects on c-fos expression, are governed by cellular gating mechanisms that are spatially segregated in different cell types. The importance of the Liu and Graybiel study is that it provides insight into the molecular mechanisms that guide ITF expression in the striatum, and perhaps elsewhere in the brain, and why certain types of neurons readily show c-fos induction in response to stimulation whereas others do not.

**Neuronal stimulation and gene expression: the coupling uncertainty**

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 behavioural task or sensory stimulus that was applied, by some non-specific feature of the experimental condition, or by an unrelated mental (or endocrin) event. Of course suitable controls can be applied to verify the stimulus-response association, either at the stimulation stage or during evaluation of expression 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: they do not permit a positive association to be made at the cellular level for any of the individual ITF-stained neurons. However, one of the most appealing features of this technique is the cellular resolution that ITF activity maps offer. This is 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. The principle behind dual-activity map-
ping relies on the different spatial localization of ITF mRNA and protein (cytosol and nucleus respectively) and the different temporal patterns of their expression. The mRNA levels are effectively regulated in either direction within 20 min of stimulus onset or offset whereas the protein requires a longer period that can be up to 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 \textit{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, and those with overlapping sensitivity will be double-labeled.

To test this idea, adult monkeys were given selective light exposure in order to activate ocular dominance columns in cortical area V1. The two sets of columns that represent each of the eyes remain spatially segregated in this area and therefore provide an ideal system for examining the effects of separate stimulus applications. A reverse occlusion procedure with appropriate exposure times showed differential \textit{zif268} mRNA and protein accumulated in ocular dominance columns. The two sets of columns could be independently stained by ISH and ICC procedures to produce complementary, non-overlapping spatial profiles (Fig. 2). Simultaneous application of ICC and non-

![Diagram](image)

**FIG. 2.** Application of ITF dual activity mapping in primate visual system. Adult monkeys subjected to selective visual exposure showed elevated levels of \textit{zif268} mRNA and \textit{Zif268} protein in ocular dominance columns of area V1 (A). The two sets of stained columns were spatially coincident in a monocular deprivation condition whereas they were spatially complementary when reverse occlusion was applied. This is especially apparent in topographical view obtained after tangential sectioning (B). The delineated centres of mRNA and protein columns (1 and 2 respectively) show a complementary relationship when superimposed (3). This is corroborated by optical density measurements (4) taken from the rectangular windows in stained sections. Application of ICC and non-radioactive ISH can produce a display of activated neurons at the cellular level with either one-colour (1) or two-colour (2) processing (C). The border region of two ocular dominance columns from a reverse occlusion animal is shown here. Protein- and mRNA-positive neurons are indicated by blue and red arrowheads respectively whereas double-labeled cells are indicated with both. The first two are presumably monocular neurons separately driven by the first and second eyes whereas double-labeled cells were likely responding to stimulation through both eyes. Adapted from Chaudhuri et al.22
radioactive ISH procedures on the same tissue section produced a visual display of activated neurons at the cellular level. Neurons at the border of two ocular dominance columns showed a mixture of labeling: single labeling of protein (nuclear staining), of mRNA (cytoplasmic staining), and double-labeling. Thus, dual activity maps at the cellular level 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 provides 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.

Synthesis

The number and diversity of biological questions to which c-fos and zif268 expression have already been applied is truly impressive. IEG expression maps can serve different purposes depending upon the brain structure being examined and the behavioural or sensory condition being employed. Whether IEG expression is being used to map the neural correlates of learning and memory, developmental plasticity, or functional activity, the information contained in these profiles must be interpreted within the molecular context by which these products are induced. Thus, continuing developments in our understanding of the molecular mechanisms that guide ITF expression will likely have considerable impact on future mapping applications. For example, if we know why certain types of neurons express various ITF products whereas others do not then this would assist in interpreting activity maps because the molecular ground rules that apply can be taken into account in experimental design.

On the technical side, continuing refinements in non-radioactive ISH techniques should allow multiple maps to be obtained from the same brain region with greater ease than currently possible. The recent introduction of antibodies that specifically recognize phosphorylated epitopes of transcription factors (e.g. c-Jun, CREB) may also find applications in activity mapping. As discussed above, the persistence of phosphorylation is very short because of the fine control that is exerted by phosphatases. If, however, the phosphorylation state of a signaling enzyme or ITF can be veridically captured, then activity mapping with these probes can be initiated after much shorter stimulation periods than currently possible. This would yield an additional time point that can be used in multiple mapping strategies. Indeed, both technical developments in detection along with further understanding of molecular induction mechanisms will largely influence the next decade of activity mapping with ITFs.

References
