

TRACING FUNCTIONAL CIRCUITS USING C-FOS REGULATED EXPRESSION OF MARKER GENES TARGETED TO NEURONAL PROJECTIONS

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1. ABSTRACT

We have developed novel techniques to trace functionally activated circuits and synaptic plasticity within the brain. We have generated transgenic mice, *FTL*, which contain a *tau-lacZ* fusion gene regulated by the promoter for *c-fos*. Following a particular nervous system stimulation in these mice, only neurons, which are functionally activated, will express LacZ, which is targeted to neuronal processes by the tau protein. In the *FTL* mice, we found highly inducible expression of lacZ by a range of different stimuli, and successful targeting of expression to neuronal cell bodies, axons and dendrites. To test if a functionally activated circuit could be visualized, the mice were deprived of water, which activates nuclei involved in body fluid homeostasis. LacZ was induced in these nuclei and their projections, allowing the mapping of a neuroendocrine circuit. Further studies have employed these mice in the analysis of neurons and circuits activated in vision, and learning and memory. We have also developed methods to measure markers of synaptic plasticity in the brain, and found significant experience dependent changes in the levels of these markers in different parts of the brain. We believe these techniques will aid in the identification of circuits for many different brain functions, and within those circuits, the locations of synaptic plasticity.

2. INTRODUCTION

A central aim in neurobiology is to determine the neural components and circuitry which are responsible for a given brain function. Moreover, storage of new information in learning and memory is believed to occur through

plasticity within these neural components and circuits. Traditionally, the connections a neuron makes have been identified using techniques such as electrophysiological recordings, histochemical techniques, anterograde or retrograde tracers, and functional imaging. While these approaches have particular and powerful benefits, they also all have drawbacks that limit their usefulness in the mapping of functional circuits. For example, electrophysiology and functional imaging do not visualize connectivity, and tracers are not targeted to functionally activated neurons.

An approach to determine which parts of the brain are involved in a particular function is to look for markers of neuronal activation. One group of markers, the immediate early genes, code for inducible transcription factors and are rapidly transcribed following neuronal stimulation. These genes are thought to couple short-term neuronal activity with changes at the level of transcription, and there is significant evidence for such a role in memory formation (1). Of these genes, *c-fos* is the most studied and is induced by a wide range of different stimuli, including many different models of learning and memory (2-5). Expression of *c-fos* correlates with functional activation in many different systems in the brain. In many systems, *c-fos* expression correlates with deoxy-glucose uptake studies, which implicate a given brain region being activated in response to a given stimulus (4). However, *c-fos* is expressed exclusively in the cell nucleus and what are identified in these studies are only the nuclei of activated neurons. For this reason, such markers give no indication of

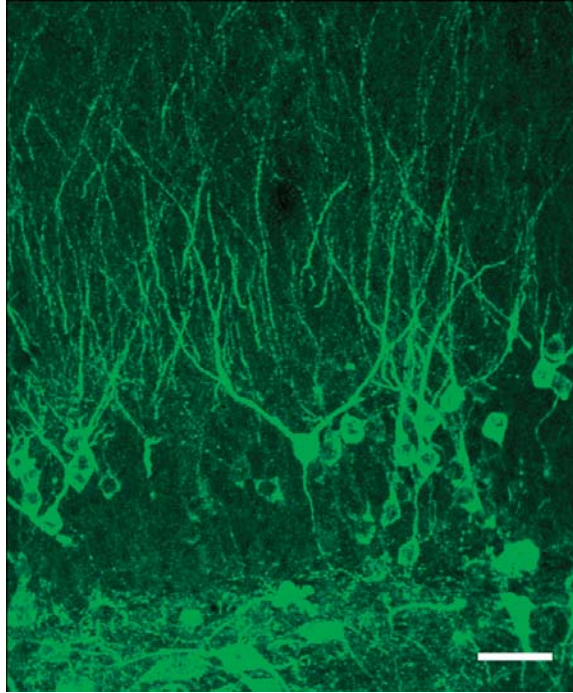


Figure 1. beta-gal⁺ neurons, axons and dendrites in *FTL* mice. Scale bar = 50 μ m

connectivity or the morphology of activated neurons within the nervous system. Even when combined with tracers (FOS⁺ nuclei and tracer labeled cell body with retrograde tracing), the mapping of neuronal projections and connectivity is a correlative and indirect process or the projection patterns of the FOS⁺ cells are inferred from anterograde tracing studies.

3. FTL MOUSE

Callahan and Thomas (6) first described a method of specifically labeling neuronal processes, which utilizes the enzyme reporter molecule, *E. coli* beta-galactosidase (beta-gal) and the microtubule-binding protein, Tau. They constructed an axon-targeted beta-gal reporter by fusing the cDNA encoding Tau, to *lacZ*, the *E. coli* gene encoding beta-gal. This reporter labels cell bodies and axons when expressed by developing and adult *Drosophila* neurons. In our current research, we have used a related genetic approach as a first step to directly visualize functionally activated circuitry in the brain. We have generated transgenic mice in which the *tau-lacZ* fusion gene is under the regulation of the promoter for the *c-fos* gene, the *FTL* mouse (7). In these mice, expression of beta-gal in positive neurons has clearly been targeted to cell bodies, axons, and dendrites (Figure 1). Further, constitutive expression of beta-gal was in accord with previous reports of *c-fos* expression. Treatment of the mice with kainic acid, a strong inducer of *c-fos*, resulted in high induction of beta-gal. beta-gal was induced in the same defined populations of neurons in the brain as those that express *c-fos* after kainic acid induction (8). Furthermore, the pattern of beta-gal expression within the neurons changed over time after

kainic acid treatment. Early after kainate treatment, beta-gal was found mainly in cell bodies; at later times, expression extended further along the neuronal processes (7). This expression pattern is consistent with induction and anterograde transport of the FTL protein in the neurons.

4. CIRCUITS INVOLVED IN BODY FLUID HOMEOSTASIS

In order to test if the circuitry of a physiologically regulated system could be visualized in the *FTL* mice, the mice were deprived of water to activate a specific and well-defined osmoregulatory pathway, which is involved in body fluid homeostasis. The key brain nuclei, which respond to changes in body fluids, surround the third ventricle. The regulatory path begins with the stimulation of osmotically sensitive neurons in the organum vasculosum of the lamina terminalis (OVLT), subfornical organ (SFO) and median preoptic nucleus (MnPO). These nuclei have efferent neural connections to the magnocellular neurons of the supraoptic (SON) and paraventricular (PVN) nuclei in the hypothalamus. In dehydrated animals, both the hypertonicity and hypovolemia of body fluids provide stimuli that activate neurons in the SON and PVN, as well as sensor sites in the lamina terminalis projecting to these two nuclei (18). These magnocellular neurons, in the SON and PVN, project their axons to and along the base of the third ventricle, in the internal zone of the median eminence, to the posterior pituitary at which site the neurosecretion of vasopressin occurs (16). Vasopressin plays a crucial role in regulating the volume and concentration of urine excreted by the kidneys (30, 31). Neurons at all levels of this pathway express *c-fos* in response to water deprivation (17-19).

In our experiments, water deprivation resulted in expression of beta-gal in neuronal cell bodies and processes throughout the lamina terminalis, and magnocellular neurons in both SON and PVN (7). Distinct labeling was also present in the hypothalamo-neurohypophyseal tract extending from the SON and PVN through the internal zone of the median eminence to the posterior pituitary. As shown in Figure 2, there is clear expression of beta-gal (yellow in dark field view) in the median eminence in water-deprived animals, and very little expression in water-replete animals (in dark field, negative axons are white). The median eminence is comprised only of axon tracts, and high power views show that beta-gal expression in this region appears to be localized to discrete puncta, which may represent individual axons or small bundles of axons in cross section (Figure 2). Thus, the detection of beta-gal throughout this tract demonstrates that we can trace functionally activated axons to their termination zones. In the posterior pituitary, beta-gal expression formed a loose semicircle at the perimeter of the posterior lobe of the pituitary, corresponding to the tract projecting from the median eminence (7). We have thus tracked this functionally activated pathway from sensors (osmoreceptors) in the lamina terminalis, to hypothalamic magnocellular neurons in the SON and PVN, and their axons in the median eminence, and finally to their neurosecretory terminals in the posterior pituitary.

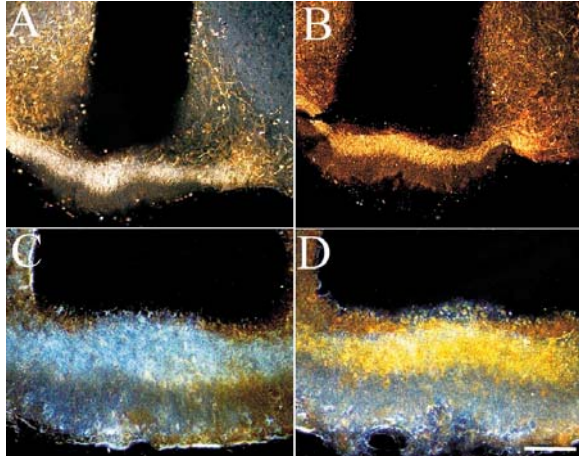


Figure 2. Expression of beta-gal in *FTL* mice following water deprivation. Mice were A. water-replete (A and C) water deprived (B and D). Shown are sections through the median eminence (C and D at high power), stained for beta-galactosidase using immunoperoxidase and viewed under dark field. Scale bar = 250 μ m

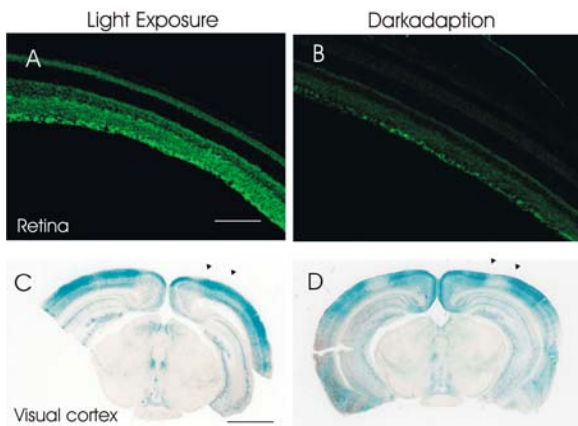


Figure 3. *FTL* transgene expression in retina and primary visual cortex. A, B; Immunofluorescence staining of retinal sections of illuminated (A) and dark-adapted (B) animal. C, D; beta-gal histochemistry of coronal brain sections of illuminated (C) and dark-adapted (D) animal. Dark adaptation suppressed *FTL* transgene expression in the retina (B), and area 17 (D). Arrows in C and D indicate the monocular region of area 17. Scale bar: A,B = 200 μ m; C, D = 1.5 mm.

This result indicates that a physiological stimulus, known to cause *c-fos* expression in the lamina terminalis, SON and PVN, also results in the production of FTL fusion protein under the control of the *c-fos* promoter in these transgenic mice. As a result, the fusion protein is transported throughout the neural processes of the magnocellular neurons enabling visualization of the entire neurosecretory pathway from hypothalamus to neurohypophysis. The visualization of osmoregulatory

neurons and their processes in the lamina terminalis was also made possible (7).

5. ANALYSIS OF CIRCUITS ACTIVATED BY LIGHT IN THE RETINA IN *FTL* MICE

Using the *FTL* mice, we started to investigate visual circuits in the retina and brain. The retina is part of the brain and represents a neuronal network, which accomplishes visual processing. Because of its simple and clear structure and spatially defined circuits, the retina is well suited for visualizing and tracing functionally activated circuitry. Some retinal circuits, for example the rod pathway (that is used for dim-light vision) are relatively well known due to electrophysiological studies as well as electron microscopy. However, knowledge about visual circuits/pathways processing other visual information is limited. The visual system can be selectively stimulated with light of different intensities or colour, moving objects etc., and these stimuli are processed in different, partly overlapping circuits or pathways. Our aim is to light up these circuits starting in the retina and tracing the processing pathways through the visual nuclei of the midbrain (lateral geniculate nucleus (LGN) and superior colliculus (SC)) to the visual cortex.

Our initial studies revealed that upon dark adaptation, *FTL* transgene expression is significantly suppressed in the retina (Figure 3B) as well as the superior colliculus but not in the LGN, compared to animals continually exposed to light (Figure 3A). In the visual cortex, the monocular region of area 17 (9) showed decreased beta-gal staining (Figure 3D). Light illumination strongly up-regulated transgene expression in the retina and visual cortex (Figure 3A and C). These data show that *FTL* expression is controlled by light stimulation in retina and area 17, the primary visual cortex and are consistent with the notion that *FTL* expression represents functional stimulation in the visual system.

The cellular organization of the retina is quite well known mainly due to immunocytochemical markers that recognize specific cell types (10). Using immunohistochemical double-labeling methods, we are in the process of classifying the retinal neurons, which express the *FTL* transgene in response to certain stimuli. Upon light illumination of the eyes, our initial studies identified cholinergic, dopaminergic and nitric oxide synthetase positive amacrine cells, as well as ganglion cells to express beta-gal (U Greferath, unpublished observations). These data support previous studies in the rabbit retina (11). We also find a subpopulation of bipolar cells expressing the transgene upon illumination. However, it seems that in the rod pathway, neither rod bipolar cells, nor AII amacrine cells express the transgene under dim light conditions (U Greferath, unpublished observations). Furthermore, with double-labeling immunohistochemistry, we have been able to localize synapses on functionally activated, transgene-expressing retinal neurons, by superimposing beta-gal immunolabeling with immunostainings specific to neurotransmitter receptors (U

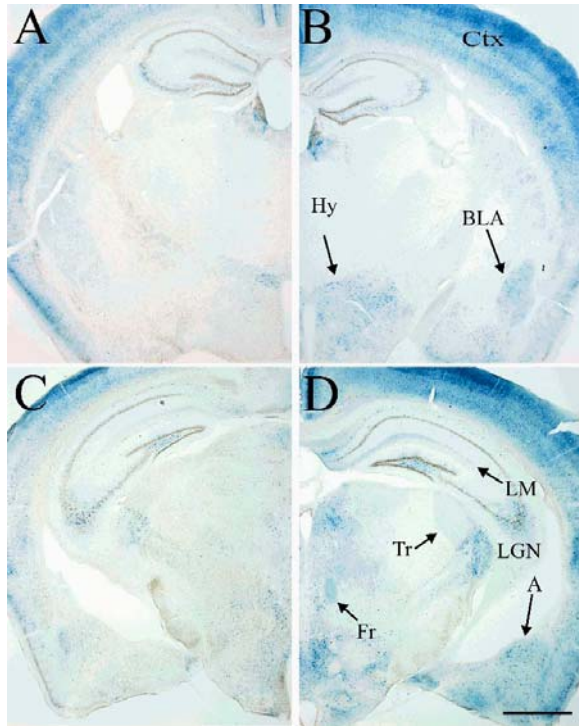


Figure 4. beta-gal expression in *FTL* mice following fear conditioning. Upper panels; sections approx Bregma – 1.7mm. Lower panels; Bregma –2.7mm. A, amygdaloid nuclei; BLA, basolateral amygdala; Ctx, cortex; Fr, fasciculus retroflexus; Hy, hypothalamus; LGN, lateral geniculate nucleus; LM, lacunosum moleculare; Tr, thalamic tracts. Scale bar = 1mm.

Greferath, unpublished observations). These data are already very encouraging and might enable us at a later stage to detect changes in synaptic connectivity upon activation, which represents synaptic plasticity.

6. STUDIES ON LEARNING AND MEMORY

In neurobiological terms, learning is typically identified with the establishment of associations between different stimuli, events, representations or other entities represented in the brain. This association process could be translated into an alteration of connectivity between any two or more neuronal groups or ensembles (12). Since neuronal connectivity occurs through synapses, learning could involve increased synaptic strength, synaptic response or synaptic number (13,14). It follows that there would exist particular circuitry for a learning experience, which would involve the associated neurons, their strengthened connections and other neural changes. This has been termed the memory trace or engram (15,16). The identification of such memory traces is central to understanding the neurobiology of learning and memory.

No studies have identified or visualized an engram in vertebrates, although there has been considerable effort to identify circuitry associated with particular

learning and memory models (17-19). One of the most concerted efforts has been in the study of Pavlovian fear conditioning. This involves associating a fairly strong aversive stimulus, such as a foot shock, with a neutral stimulus, such as a sound. After training, the animal will display fearful/anxious behaviour on being presented with the sound alone. This model is not only a good behavioural model for learning, but it may well involve the same brain systems as those which are involved in anxiety/fear related syndromes, such as posttraumatic stress disorders, phobias, generalized anxiety induced by environmental stress, and obsessive compulsive disorders (20). A scheme for a fear conditioning circuit has been described (18,19,21). The center of this circuit is the amygdala and the circuit extends through its afferent and efferent connections to many other parts of the brain.

Clearly, there has been enormous progress in understanding fear conditioning, but there are many questions still to be answered (22). Which neurons are directly involved in fear conditioning and what connections do they make? We know some of the gross pathways that are involved in fear conditioning, but we have no high-resolution picture of the neurons and their projections, within these pathways, which are activated and involved in conditioning. What about the synaptic changes which presumably accompany memory? What do we know about the number of these changes and their precise relationship with circuits? What precise circuit changes occur in the amygdala and other parts of the brain? These are questions of memory representation, and they need to be answered by using approaches that help to visualize this representation: the neurons, their processes and connections which actively undergo change during learning and which result in memory.

7. FEAR CONDITIONING STUDIES WITH *FTL* MICE

We have begun studies on fear conditioning in the *FTL* mice. While these studies are only at an early stage, they demonstrate that the *FTL* mouse will be useful to detect brain regions activated in this form of learning and to map the activated neuronal projections. We have established models for both auditory and contextual fear conditioning, in which mice learn to associate a foot shock with either a sound or a context. In our preliminary studies, we have used this model to condition *FTL* mice to the context of a chamber, and analyzed the expression of the *FTL* transgene following conditioning, using beta-gal histochemistry. We undertook a time course of expression, examining the brains of the mice from 6-24 hr after conditioning. There is a strong increase in beta-gal expression in several brain regions from 6 hr and extending up until 24 hr. Many amygdaloid nuclei, including the basolateral nuclei, showed increased expression of beta-gal compared to home cage control animals. We also found that fear conditioning induces expression in other brain regions, such as in medial regions of the thalamus and lateral geniculate nucleus, hippocampus and cortex. Some of these changes can be seen in Figure 4. This allows us to

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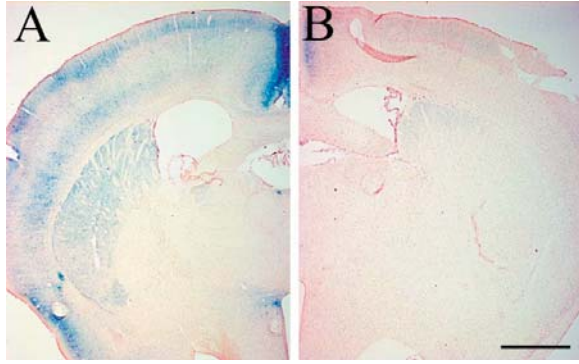


Figure 5. Effect of environmental enrichment on beta-gal expression in *FTL* mice. A. Coronal section through brain of A. standard housed *FTL* mouse; and B. environmentally enriched animal. Scale bar = 1mm.

easily identify and map regions of beta-gal induction in the *FTL* mice. In our experience, it is easier to see changes in expression in the *FTL* mice than with FOS immunohistochemistry, most likely because *FTL* expression fills the cell bodies and processes, whereas FOS is restricted to cell nuclei. This provides another advantage of the *FTL* mice compared to staining with FOS antibodies. Finally, and importantly, expression is not limited to cell bodies, but is also induced in neuronal processes, and is evident in axon tracts including anterior commissure, corpus callosum, thalamic tracts, and fasciculus retroflexus in conditioned animals (some of which are shown in Figure 4). In the hippocampus, expression is not only in the CA3 pyramidal cells and processes, but also in the CA1 region, in the lacunosum moleculare, representing CA3 axons, both in conditioned and control animals.

These studies demonstrate the usefulness of the *FTL* mice in mapping brain regions involved in fear conditioning and in the visualisation of tracts that are projecting from the activated brain regions. Ultimately, it should be possible to map the connectivity or circuitry associated with fear conditioning using the *FTL* mice and by following the beta-gal⁺ tracts through the brain.

8. ENVIRONMENTAL ENRICHMENT

Our preliminary studies above show the potential usefulness of *FTL* mice in mapping functional circuits involved in fear conditioning. However, the fairly widespread expression of beta-gal in these mice, even under control conditions is probably not due to the learning of a specific relationship (such as fear conditioning), but may reflect a generalized stressed/activity state of the mice. This generalized expression of beta-gal probably has functional significance for the mice, but for our purposes of identifying learning related circuitry, we need to subtract or eliminate it. One approach to do this is to expose the animals to an enriched environment, whereby the animals are housed in large cages with different objects that are changed on a regular basis. Environmental enrichment represents somatosensory experience for the animal

including the learning of new information from its enriched environment. In addition, it results in altered patterns of behaviour; such as enhancing learning and memory, and also decreases stress (23-26). Environmental enrichment results in decreased basal c-fos expression and shows increased learning-specific c-fos expression (27). It also results in improved performance in memory tests (27).

We found that environmental enrichment had effects on beta-gal expression in the *FTL* mice, resulting in elimination of much of the expression in the cortex and other brain regions that showed beta-gal in normally caged animals (Figure 5). This treatment of the animals should be very useful in giving discrete patterns of expression of beta-gal in the *FTL* animals in learning and memory tests.

9. DETECTION OF SYNAPTIC CHANGE

Synaptic plasticity, including changes in synapse number, and/or size, has been postulated to underlie the process of learning and memory (13,14). We plan to use our mapping studies with the *FTL* mice in fear conditioning to point to areas of potential synaptic change. Following several years of study, we have developed a rapid and sensitive method of determining if structural synaptic changes may have occurred within a particular area of the brain (28). This involves an enzyme linked immunosorbant assay (ELISA) to quantify levels of synaptic proteins within any brain region. While a variety of synapse-associated proteins show changes following learning, we have focused on three of these, which are of particular interest for studies of synaptic plasticity.

The first is the pre-synaptic marker, synaptophysin, which is a major synaptic vesicle protein and a very good marker for axon terminals (29,30). Changes in levels of synaptophysin are thus indicative of changes in synaptic number, size and/or vesicle density per synapse that may occur in specific parts of the brain following learning. In addition to showing altered patterns of behaviour, environmental enrichment has also been used as a model to look for experience-dependent synaptic change (31). Thus we have used the ELISA technique to detect highly significant changes in synaptophysin levels in many different parts of the brain following environmental enrichment (28).

In addition to detecting changes in synaptophysin levels following enrichment, we undertook analysis for changes in another marker of interest, the excitatory postsynaptic density protein, PSD-95. This scaffolding protein is thought to be involved in the clustering of glutamate receptors and has been shown to be important for learning and memory (32). We have developed a highly sensitive ELISA for PSD-95 and found that environmental enrichment also led to similar changes in levels of PSD-95 in many different parts of the brain, as seen with synaptophysin (28). This is in accordance with studies which have also shown increased gene expression for PSD-95 following enrichment and other forms of synaptic plasticity (33,34).

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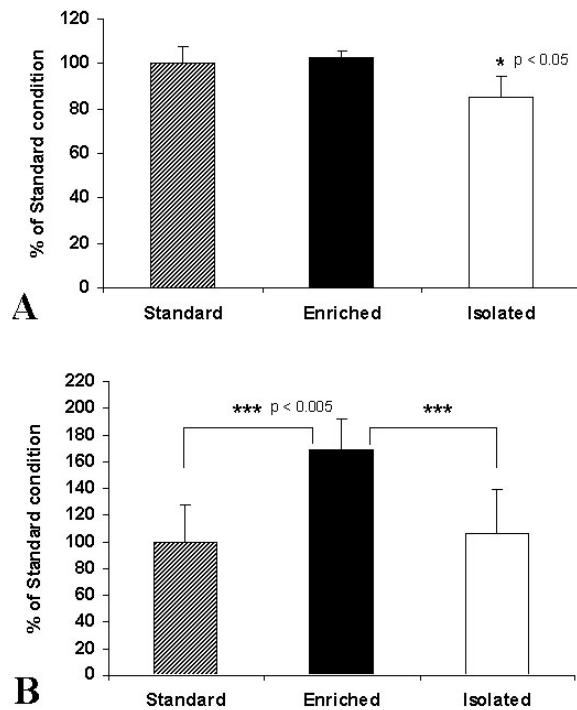


Figure 6. Effect of enrichment on relative PKC γ concentrations in hippocampus (A) and total PKC γ concentrations in visual cortex (B). Isolated animals were housed individually, standard animals housed in pairs and enriched animals housed in a group of 8 and provided with a variety of objects to interact with.

The third marker is the calcium-lipid dependent second messenger, Protein Kinase C γ (PKC γ), which is abundantly expressed in postsynaptic dendritic spines (35,36). Immunoreactivity for the PKC γ protein increases following learning (37-39), and additionally, PKC γ null mutant mice show impaired learning (40) which implicates this enzyme in the learning and memory process. We have established an ELISA to detect levels of activated PKC γ by using antibodies, which specifically detect epitopes on the activated form of the enzyme following cleavage of an inhibitor molecule. Levels of PKC γ also show changes following environmental enrichment. We found animals that were housed in an enriched environment for a month, had significantly higher concentrations of activated PKC γ in the hippocampus in comparison to isolated ($p < .05$), but not in comparison to standard housed animals (Figure 6a). In addition, standard housed animals also showed increased hippocampal PKC γ in comparison to isolated animals. Thus within the hippocampus, the changes in PKC γ activation appear to be primarily associated with the isolated condition. This may be indicative of lower steady state levels of PKC γ signaling in the hippocampus in the isolated animals. It is unclear why this is the case, however, it may reflect a general lower level of hippocampal activity in the isolated animals.

In addition to the hippocampus, we also found changes in PKC γ in the visual cortex. Enriched animals

showed higher concentrations of activated PKC γ per microgram protein in the visual cortex, in comparison to both standard and isolated conditions, however this was not significant (data not shown). However, we did find gross changes in the visual cortex, with enriched animals showing an increase in the mass of the dissected visual cortex in comparison to standard and isolated animals (28). This difference in mass was not found in the hippocampus. Thus, when taking into account this change in weight of the visual cortex, calculations of total PKC γ relative amounts revealed enrichment to have a significant 60% difference on total amount of activated PKC γ in comparison to both standard and isolated conditions (Figure 6b). This result indicates that environmental enrichment produces large changes in the mass of the visual cortex and concomitant changes in PKC γ . These changes probably underlie a major increase in storage of visual information in the enriched animals. The finding that there was no significant increase in concentration of the activated form of PKC γ is not surprising given that enrichment occurred over one month, and any changes in signaling which resulted in the increased information storage were thus probably complete at the time we sacrificed the mice.

10. PERSPECTIVE

The approaches we are developing will enable us to identify functional circuits for many different brain functions and to localize regions of synaptic plasticity. Our first approach, using the FTL transgenic mice, will allow us to directly visualize and map the connectivity of neurons that are activated in any functional stimulation that involves activation of *c-fos*. Since *c-fos* is the most widely studied of genes which are activated following functional stimulation (2,3), and is also expressed very widely, the use of the FTL mice may have a correspondingly wide application. For this approach to be of use, it is only required that *c-fos* be expressed following any particular stimulus. It needs to be noted that *c-fos* is not expressed in every neuron following stimulation, and thus its use will not be universal as an indicator of functional activation. For example, we have found that FTL expression is not induced in all neurons in the retina; in particular it is not induced in some of the primary receptive retinal neurons. In addition it is not expressed, or only expressed at low levels, in cortical motor neurons, which must be active very often in a moving animal. It is possible that *c-fos* expression is not induced in these neurons because this kind of activity is effectively constitutive for these neurons. Activation of *c-fos* may represent non-constitutive activation of the neuron, such as that which might be involved in plasticity, or in the translation of new proteins in response to a specific stimulus. In any case, this characteristic of *c-fos* is very useful, because it results in very low expression in unstimulated animals.

In our FTL transgenic mice, we found similar low levels of induction to that of endogenous *c-fos* (7). However, the FTL gene product is probably more stable compared to the FOS protein and therefore persists for

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longer times. Possibly for this reason, there are significant levels of the FTL protein in standard housed animals. We found that giving the mice environmental enrichment resulted in a significant decrease in expression of the FTL protein. We believe that the effects of environmental enrichment on FTL expression is a decrease in the levels of stress in the animals and lead to state of homeostasis. Thus, under these conditions, we would predict that there would be very little expression of c-fos. The observation that we find only very low levels of FTL protein is consistent with this prediction and with the view that c-fos expression indicates initiation of non-constitutive protein synthesis.

The effective use of these animals to map functionally activated circuits will require tracing beta-gal positive processes from cell body to termination, which in many cases may be a long distance through the brain. For these studies, it may be difficult to rely on staining and analysis of thin sections of the brain, because most processes will traverse through these sections very quickly. To trace processes, it may be optimal to generate 3D reconstructions of the brain, or to analyze thick sections for FTL expression. We are currently in the process of testing these approaches.

Our second approach is to look for synaptic change, using ELISA analysis of synaptic proteins. The advantages of this approach over other approaches are that it is rapid, sensitive and many samples can be analyzed. It should thus be possible to examine many regions of the brain where there is a possibility of synaptic plasticity in studies of learning and memory. It is then possible to identify within the circuits we have characterized with the FTL mice, key regions of structural synaptic change. In the future, we could use this information to look not only for areas of structural change, but also for functional changes in synapses. For example, if we develop an accurate picture of the locations of structural change in synapses, we could then look for functionally significant changes, such as in NMDA and AMPA receptors (34,41,42), in these same locations. Ultimately, these studies will lead to a closer definition of the engrams for memory.

11. ACKNOWLEDGEMENT

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