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INDUCTION OF EARLY GROWTH RESPONSE GENE 2 EXPRESSION IN THE FOREBRAIN OF MICE PERFORMING AN ATTENTION-SET-SHIFTING TASK

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Abstract

Early growth response (egr) genes encode transcription factors that are induced by stimuli that cause synaptic plasticity. Here we show that the expression of one member of this family, egr-2, is induced in the orbital frontal cortex (OFC) and medial prefrontal cortex (mPFC) of mice performing an attention-set-shifting task (ASST). The ASST is a series of two-choice perceptual discriminations between different odors and textures. Within the OFC and mPFC, different subregions exhibited egr-2 induction in response to different test-related features. In the medial OFC and the anterior cingulate subregion of the mPFC, egr-2 induction occurred in response to exposure to the novel odor stimulus. In the ventrolateral OFC and the pre- and infralimbic mPFC, additional egr-2 induction occurred during the associative learning phase of the ASST. In the infralimbic mPFC, further egr-2 induction occurred when mice performed set-shifting and reversal learning phases of the ASST. Mice with enhanced set-shifting performance exhibited decreased egr-2 induction in the mPFC indicating that the magnitude of egr-2 induction correlates with the magnitude of attentional demand. This decrease was largest in the infralimbic mPFC suggesting further that egr-2 induction in this region plays a role in the attentional control during set-shifting.

In contrast to egr-2, neither egr-1 nor egr-3 expression was altered in ASST-tested mice, and no egr-2 induction occurred in mice that performed a spatial working memory task. These findings suggest a specific role of egr-2-mediated transcriptional activation in cognitive functions associated with attention.

Keywords

early growth response gene; medial prefrontal cortex; orbital frontal cortex; attention; working memory; mice

The earliest genomic response to synaptic activity is the induction of expression of immediate early genes (IEGs) encoding transcription factors that participate in synaptic and structural neuronal responses to external stimulation. Although induction of expression of the IEG c-

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fos is a powerful tool to map the activation of neuronal pathways (Sagar et al, 1988), the identification of other IEG transcription factors is critical for determining whether specific gene expression programs are activated by specific stimuli.

Early growth response (EGR) genes are IEGs that encode transcription factors. The family of EGRs is composed of four members, *egr-1*, *egr-2*, *egr-3*, and *egr-4*. The four EGR proteins have a highly conserved DNA-binding domain composed of three Cys2His2 zinc-finger motifs that recognize a 9-base-pair consensus motif (GCG(G/T)GGGCG) in promotor regions of genes. However, the individual family members differ markedly in regions outside their common DNA-binding domain thus making it possible that they are regulated differentially by different signaling pathways (Beckmann and Wilce, 1997; Herdegen and Leah, 1998).

A defining feature of EGR genes is that they are activated by stimuli that cause synaptic plasticity (Beckmann and Wilce, 1997; O'Donovan et al., 1999). This is best documented for *egr-1* and *egr-3* proteins that have been shown to modulate synaptic plasticity by directly regulating the expression of a common effector, the activity-regulated cytoskeletal-related (*Arc*) gene (Li et al., 2005). *Egr-1* and *egr-3* are both implicated in learning and memory. For example, *egr-1* expression was shown to be induced in the anterior cingulate cortex, the CA1 region of the hippocampus, and the shell of the nucleus accumbens during retrieval of contextual fear memories, and in the amygdala and the nucleus accumbens core during retrieval of contextual and cued fear memories (Hall et al., 2001; Thomas et al., 2002). Studies on knockout mice further showed that *egr-1* was essential for the transition from short- to long-term plasticity and for the formation of long-term memories (Jones et al., 2001), and they confirmed a role for *egr-1* in the reconsolidation of memory after retrieval (Bozon et al., 2003; Lee et al., 2004).

Unlike *egr-1*-deficient mice that exhibit impairments in late-phase long-term potentiation (LTP) and long-term hippocampal- and amygdala-dependent memories, *egr-3*-deficient mice have deficits in both early- and late-phase hippocampal LTP as well as short- and long-term hippocampal- and amygdala-dependent learning and memory (Li et al., 2007).

In contrast to *egr-1* and *egr-3*, little is known about the specific roles of the related protein *egr-2* in synaptic-plasticity-dependent processes. *Egr-2* expression was shown to be robustly and sustainedly induced in the dentate gyrus following LTP-inducing perforant path stimulation suggesting that *egr-2* plays a role in the maintenance of LTP (Williams et al., 1995). However, a role of *egr-2* in memory-related or other cognitive functions remains to be established.

In a preliminary microarray-based screen, we found that *egr-2* mRNA expression was upregulated in the forebrain of mice performing an attention-set-shifting task (ASST). The ASST is a two-choice perceptual discrimination task involving a series of test phases that require associative learning, shifting between attentional sets, and reversal learning (Birrell and Brown, 2000). We have previously shown that exposure to the ASST also elicits robust induction of the IEG *c-fos* (Glickstein et al., 2005) just like other work has shown that *c-fos* expression is induced when animals perform a spatial working memory (WM) task (Vann et al., 2000). Because attention and WM are tightly interacting cognitive processes with overlapping anatomic activation profiles that are modulated by the same neurotransmitter systems (Awh et al., 2006; Brozowski et al., 1979; Aston-Jones et al., 1999; Lapid and Morilak, 2006; Goldman-Rakic, 1995; Raz, 2004; Arnsten and Li, 2005), we examined whether *egr-2* induction is specifically occurring in mice exposed to the ASST or whether WM-tested and ASST-tested mice utilize the same transcriptional regulatory mechanism that is initiated and orchestrated by *egr-2*.

EXPERIMENTAL PROCEDURES

Animals

All procedures involving animals were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at Columbia University and the New York State Psychiatric Institute. Adult male C57Bl/6J mice were group-housed in a standard animal care facility with a 12-hr day/night cycle where they had free access to food and water. The number of animals was kept to the minimum necessary to ensure statistical validity and no procedures were performed that caused pain or severe discomfort.

Cognitive tests

Prior to testing, food availability was restricted so that the body weights of the animals were gradually reduced (over a period of 7 to 10 days) to 85% of their starting weight. The animals remained group-housed during the period of food restriction and daily recordings of body weights revealed that all animals housed in the same cage lost weight to the same extent. The gradual food restriction also served as a habituation period to daily handling prior to testing, an essential prerequisite to minimize handling-associated stress responses.

All cognitive tests were conducted between 1 and 4 pm. One group of mice performed an *attention set-shifting test (ASST)* originally developed for the rat (Birrell and Brown, 2000). Details of the application of this test to mice are described in Glickstein *et al.* (2005). Briefly, terra cotta pots were used as digging bowls and their rims were scented with perfumed oil to produce a lasting odor. The bowls contained a small food pellet (Prolab Isopro RMH 3000; PMI Nutrition, Brendwood, MO) buried underneath different digging media. In each trial, two pots were placed adjacent to each other in the larger section of the test box. After a habituation to the test chamber, mice were first trained in a simple discrimination (SD) of either two odors or two different textures of digging media to a criterion of six consecutive correct trials. The use of odor or texture as the relevant stimulus dimension was randomized. After successful completion of the SD, a compound discrimination (CD) followed in which a new dimension that was not a reliable indicator of the food location was added to the stimuli presented in the initial SD. The next test phase required an intradimensional shift of attention (IDS). In this phase, both relevant and irrelevant stimuli were changed, but the previously relevant stimulus dimension (odor or digging medium) remained the same. After IDS completion, a new test phase required an extradimensional shift of attention (EDS) in which the formerly irrelevant dimension became relevant and the originally guiding dimension lost its predictive value. This EDS phase was then subjected to reversal rules (EDSRev), i.e., the previously negative stimulus became a positive one but the irrelevant stimulus dimension was still not predictive of the reward location.

In all phases of the ASST, animals had to reach a criterion of six consecutive correct trials and during the course of the test, each sensory stimulus was used only once. All animals performed the ASST in a single test session. For each test phase, the number of trials to criterion was computed, compared by a one-way analysis of variance (ANOVA; threshold of significance, $\alpha=0.05$), and significant differences were resolved *post hoc* using the Tukey-Kramer Multiple Comparisons test.

Control animals were also food restricted. In some experiments these controls were also exposed to the ASST test chamber where they actively foraged for food buried underneath familiar cage-bedding material in two unscented terra cotta pots. These controls did not exhibit induction of *egr-2* in response to test-box exposure. Thus, in the majority of the experiments, we used food-restricted animals as controls for basal *egr-2* expression. Food restriction was a

necessary manipulation to generate appropriate controls because we have previously shown that food restriction increases the locomotor activity of mice around feeding time (~1 pm) and also increases expression of Fos immunoreactivity in the medial prefrontal cortex (Glickstein et al., 2005). Additional control experiments, performed on mice exposed to odor-scented terra cotta pots located in the test chamber, are described in the results.

Another group of mice was subjected to a test of *spatial working memory* performed in a T-maze as described in Glickstein *et al.* (2002). Briefly, mice were first trained (over a period of 10 to 14 days) for alternate arm entries in the T-maze until they reached 70% correct arm entries (in 10 trials per day with no inter-trial delay) on two consecutive days. On the following day, mice performed the test once more, but with a 15 sec inter-trial delay. Control animals were also food restricted, and a subgroup of controls also participated in the first four days of training during which they were either forced to enter the arms in an alternating fashion or allowed to choose freely between both (baited) arms (see Results). None of the controls were exposed to inter-trial delays that engage working memory processes.

egr-2 mRNA and protein expression

To ensure reproducible sectioning, mouse brains were placed onto an acrylic brain matrice (Stoelting, Wood Dale, IL) that allows coronal sectioning at one mm intervals. From each brain, we sectioned the frontal five mm. Total RNA was isolated using guanidine/cesium chloride ultracentrifugation. Protein was extracted in a buffer containing 250 mM sodium chloride, 25 mM Tris-HCl (pH 7.5), 5 mM EDTA (pH 8), 1% Triton X-100, and protease inhibitors.

In initial experiments, total RNA was used in microarray studies that were performed by GenUs Biosystems (Northbrook, IL) using CodeLink UniSet™ Mouse Whole Genome Bioarrays (Amersham Bioscience; GE Healthcare, Piscataway, NJ). Their probe sequences were designed based on sequences selected from the NCBI UniGene build #139; RefSeq and dbEST databases. Codelink and GeneSpring software were used to identify transcripts with more than 2-fold changes in expression levels in mice exposed to the ASST. This study detected increased expression of *egr-2* mRNA in ASST-tested mice.

Additional RNA samples were used in real-time PCR experiments performed using the iQ5 Thermal Cycler (BioRad; Hercules, CA) in conjunction with iQSYBRgreen as previously described (Bhansali et al., 2007). Briefly, cDNA was synthesized from 10 µg of total RNA using murine moloney leukemia virus (M-MLV) reverse transcriptase (USB; Cleveland, OH). Amplification of *egr-2* cDNA was specified by the primer pair 5'-ATGAACGGAGTGGCGGGA-3'/5'-AGTAGAGGTGGTCCAGTT-3' which directs amplification of a 346 bp fragment of mouse *egr-2* (nucleotides 393 to 738). In all real-time PCR experiments, measurements were made of the number of cycles required to reach threshold fluorescence intensity (cycle threshold, Ct). Ct values for each reaction were normalized to those obtained for amplifications of the same cDNA samples using primers that amplified β -actin cDNA. Differences between normalized Ct values were determined using the $2^{\Delta Ct}$ method, and the results were expressed as fold changes over baseline.

Total protein (5 µg) was analyzed by Western blotting as previously described (Adlersberg et al., 2004) using rabbit polyclonal antibodies directed against *egr-2* (Covance; Berkeley, CA; PRB-236P; dilution: 1:1000), β -actin (Cell Signaling; Berkeley, MA; #4967; dilution: 1:1000), *egr-1* (Santa Cruz Biotechnology, Inc; Santa Cruz, CA; sc-189; dilution: 1:2000), and *egr-3* (Santa Cruz Biotechnology, Inc; sc-191; dilution: 1:1000). Bound antigens were visualized using a peroxidase-conjugated anti-rabbit IgG secondary antibody (Kirkegaard & Perry Laboratories, Inc.; Gaithersburg, MD) in conjunction with enhanced chemiluminescence

(ECL; Amersham), and optical densities of ECL signals were analyzed using NIH Image Analysis software.

Immunocytochemistry and stereology

Mice were deeply anesthetized with ketamine (100 mg/kg) and xylazine (15 mg/kg) and perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4). Brains were postfixed for 1 h (in the same fixative) and cryoprotected overnight (30% sucrose in 0.1 M PB).

The expression of *egr-2* immunoreactivity was analyzed in a series of adjacent, coronal 40- μ m-thick microtome sections that were collected in 200 μ m intervals from 6.5 to 1.5 rostral to bregma. In all experiments, sections obtained from ASST- or WM-tested mice were processed in parallel with their respective controls. A series of free-floating sections (blocked with 0.5% bovine serum albumin (BSA)) was incubated overnight at 4°C with a rabbit polyclonal antibody directed against *egr-2* (Covance, 1:1000) dissolved in PB containing 0.1% BSA and 0.25% Triton X-100. After incubation with primary antibody, sections were incubated with biotinylated goat anti-rabbit IgG (1:400 in PB containing 0.1% BSA; Vector Laboratories, Burlingame, CA), followed by a 30-min incubation in avidin-biotin-peroxidase complex (Vectastain Elite Kit; 1:100 in PB; Vector Laboratories). To visualize bound immunoperoxidase, sections were incubated for 4 min in 0.022% 3,3'-diaminobenzidine (DAB; Aldrich, Milwaukee, WI) and 0.003% hydrogen peroxide in PB. All sections were rinsed in PB and mounted onto gelatin-coated slides.

The densities of *egr-2*-labeled cells in the OFC and mPFC were determined using a two-dimensional stereological counting method in conjunction with defined counting frames that adequately covered the areas of basal *egr-2* expression. Sections were viewed at 20 $\times$ magnification using a Zeiss Axioskop 2 microscope (Oberkochen, Germany) interfaced with an Apple computer and Improvion Openlab 3.1 software (Coventry, UK). The densities of *egr-2*-labeled cells were determined in the medial and ventrolateral OFC and in layers II/III of the three subregions of the mPFC (infralimbic (IL), prelimbic (PrL), and anterior cingulate (AC)). Counting frames were created using the coordinates from the mouse brain atlas of Hof et al. (2000). For the medial OFC, a counting frame of 0.15 $\times$ 0.3 mm was placed over sections collected at bregma 2.0. The number of *egr-2*-labeled cells in the ventrolateral OFC was counted in the same sections, using a counting frame of 0.3 $\times$ 0.3 mm. For the mPFC, sections collected at bregma 2.0, 2.2, and 2.4 were analyzed using counting frames of 0.08 $\times$ 0.4 mm for layers II/III of the IL and PrL subregions, and 0.08 $\times$ 0.3 mm for layers II/III of the AC subregion. Cell counts were normalized by the area counted and expressed as number of *egr-2*-labeled cells/mm² (density).

Microscopic images were photodocumented using Open Lab (Improvion, Lexington, MA) and processed using Adobe Photoshop. Adjustments to image brightness and contrast were made for clarity of publication, but the resultant images reflect the experimental data.

RESULTS

Increased *egr-2* mRNA and protein expression in ASST-tested mice

We measured *egr-2* mRNA and protein levels in the forebrain of mice that performed either the ASST or the spatial working memory (WM) test. ASST-tested mice proceeded through the test in the order SD, CD, IDS, EDS, and EDSRev (see Experimental Procedures). For real-time PCR experiments, RNA was extracted from mice at completion of either the second set-shifting phase (EDS, labeled 'SS' in Fig. 1), the last reversal phase of the ASST (EDSRev, labeled 'Rev' in Fig. 1), or the spatial WM test. Control mice, like all tested animals, were food

restricted and exposed to the test box as described in the Experimental Procedures. The results shown in Fig. 1A illustrate that significant induction of *egr-2* mRNA expression occurred in ASST-tested mice regardless of whether they completed only the EDS or the last reversal phase (two-tailed *t* test; $p < 0.02$). In contrast, no *egr-2* induction was detected in WM-tested mice.

In ASST-tested mice, increased expression of *egr-2* mRNA also led to increased expression of *egr-2* protein measured 1 h post completion of either the first compound discrimination (CD), the set-shifting phases (SS), or the reversal phase of the ASST (Rev) (Fig. 1B). Measurements of optical densities of ECL signals (obtained from 7 independent experiments) revealed a ~50% increase in *egr-2* protein expression in the forebrain of ASST-tested animals that completed the entire test (1.56 ± 0.16 fold increase, two-tailed *t*-test; $p < 0.03$). *Egr-2* protein levels of WM-tested mice, however, were indistinguishable from controls (Fig. 1C).

Basal levels of *egr-1* and *egr-3* protein were substantially higher than those of *egr-2*, and these levels remained unaltered in ASST-tested animals (Fig. 1B). Hence, in ASST-tested mice, induction of *egr-2* expression was not accompanied by similar changes in expression of other members of EGR family of transcription factors.

Induction of *egr-2* expression in OFC and mPFC of ASST-tested mice

To identify the anatomic regions of increased *egr-2* expression in ASST-tested animals, immunocytochemical studies were performed. Because the results shown in Fig. 1B indicated that increased *egr-2* protein expression is already evident after CD-test completion, we examined mice that completed either only the CD phase (ASST-CD) or the entire test (ASST-Rev). In all experiments, tissue sections of tested animals were processed in parallel with their controls. In initial experiments, sections obtained throughout the entire rostro-caudal axis of the brain were examined. These experiments revealed two anatomic regions of increased *egr-2* expression, the OFC and mPFC.

Increased *egr-2* expression was detected in ASST-tested mice but not in mice that were placed into the same test box and allowed to dig for food in unscented terra cotta pots. However, in ASST-tested animals, induction of *egr-2* expression could still be due to exposure to the novel stimulus modalities (odor, texture). Since no *egr-2* induction was detected in the somatosensory cortex it is unlikely that tactile stimuli of the different bedding media used in the ASST were responsible for *egr-2* induction. However, *egr-2* expression was prominent in the OFC and it is known that neurons of the OFC respond to olfactory stimuli (Rolls and Baylis, 1994). Thus, we performed additional control experiments to discern whether induction of *egr-2* expression in the OFC occurred solely in response to odor exposure during the ASST. In these experiments, mice were exposed to the same scented digging pots that ASST-tested mice encountered during their tests. However, both pots were baited and no discrimination was required between them to receive food reward. Nevertheless, odor-exposed controls had to complete the same mean number of digging responses that tested animals needed to complete each of the phases of the ASST.

As shown in Fig. 2, a stereological count of *egr-2*-labeled cells revealed significant differences between non-odor exposed controls, odor-exposed controls, and ASST-tested animals in the medial (ANOVA; $F(3,30) = 18.573$; $p < 0.0001$) and ventrolateral OFC ($F(3,30) = 28.953$; $p < 0.0001$). In the medial OFC, odor-exposed animals exhibited significantly higher *egr-2* expression than non-odor exposed controls (*post hoc* Tukey Kramer Multiple Comparisons; $p < 0.001$). ASST-tested animals also exhibited significantly increased *egr-2* expression when compared to non-odor exposed controls ($p < 0.001$), but the difference between odor-exposed and ASST-tested animals was not significant.

This differs from results obtained from stereological counts of *egr-2*-labeled cells in the ventrolateral OFC. In this region, odor-exposed controls exhibited significantly increased *egr-2* expression ($p < 0.001$) when compared with non-odor exposed controls, but *egr-2* expression of ASST-tested mice that completed the entire test was still significantly higher compared with odor-exposed controls ($p < 0.05$) and also higher compared with mice that completed only the CD phase of the test, albeit this difference did not reach statistical significance. Thus, whereas in the medial OFC, induction of *egr-2* expression could be entirely attributed to exposure to the stimulus modality (odor) alone, *egr-2* induction in the ventrolateral OFC of ASST-tested mice had a significant task-evoked component that was stimulus-modality independent.

We next examined the induction of *egr-2* expression in the mPFC (Fig. 3). In control animals, expression of *egr-2* immunoreactivity was low and mainly confined to the superficial laminar territories (II/III) of all three subregions (IL, PrL, AC). ASST-tested animals exhibited robustly increased expression of *egr-2* immunoreactivity in these regions, and significant differences between controls, odor-exposed controls, and ASST-tested animals were found in all three subregions. In the IL (ANOVA, $F(3,35) = 32.329$; $p < 0.0001$), odor-exposed controls exhibited significantly higher *egr-2* expression compared with non-odor-exposed controls ($p < 0.01$) but significantly lower *egr-2* expression compared to ASST-tested animals that completed either the CD-phase of the test or the entire test (ASST-Rev) ($p < 0.05$). Moreover, *egr-2* expression was significantly higher in ASST-Rev-tested mice compared with ASST-CD-tested mice ($p < 0.05$) indicating that *egr-2* induction in the IL does not only occur during associative learning, but also during set-shifting and/or reversal learning.

In the PrL (ANOVA, $F(3,35) = 44.57$; $p < 0.0001$), stereological counts of *egr-2*-labeled cells revealed that odor-exposed controls expressed significantly more *egr-2* than non-odor-exposed controls ($p < 0.001$), but their density of *egr-2*-labeled cells was significantly lower compared with ASST-tested animals that completed the entire test (ASST-Rev; $p < 0.01$). Although the density of cells expressing *egr-2* was higher in mice that completed the entire ASST when compared with CD-tested mice (Fig. 3), this difference did not reach statistical significance suggesting that, in the PrL, *egr-2* induction occurs primarily during associative learning.

In the AC (ANOVA, $F(3,35) = 31.42$; $p < 0.0001$), odor-exposed controls also exhibited significantly increased *egr-2* expression compared with non-odor exposed controls ($p < 0.001$) and their density of *egr-2*-labeled cells did not differ from those counted for CD-tested animals and animals that completed the entire ASST (Fig. 3). Thus, in the cingulate subregion of the mPFC, like in the medial OFC, induction of *egr-2* expression could be entirely attributed to odor exposure alone.

In summary, in ASST-tested animals, a stimulus-modality-independent induction of *egr-2* expression occurred in the ventrolateral OFC and in the IL and PrL subregions of the mPFC during associative learning. In the IL subregion of the mPFC, however, further *egr-2* induction occurred during set-shifting and/or reversal learning.

Egr-2 expression in mice re-exposed to the ASST

Practice can enhance cognitive task performance. Indeed, compared with mice that performed the ASST only once, mice re-exposed to the ASST one day following initial test exposure exhibited significant improvement in response accuracy (ANOVA, $F(5,92) = 15.011$; $p < 0.0001$) that could be resolved *post hoc* specifically for the extradimensional set-shifting phase (EDS) of the ASST ($p < 0.01$) (Fig. 4). Mean response latencies, although decreased in re-tested mice, did not significantly differ between mice tested only on day 1 and mice re-tested on day 2.

We next examined whether this enhanced EDS performance and hence, decreased demand on attentional control, was also accompanied by quantitative differences in induction of *egr-2* expression. Figure 5 illustrates results obtained from stereological counts of the number of *egr-2*-labeled cells in the OFC of non-odor-exposed controls, controls exposed to odor either once (day 1) or re-exposed to odors on day 2, ASST-tested mice (tested on day 1), and ASST-tested mice re-exposed to the test on day 2.

In the medial OFC, significant differences in the densities of *egr-2*-labeled cells indicated by ANOVA ($F(4,34) = 21.886$; $p < 0.0001$) were resolved *post hoc* as follows: Re-exposure to odors on day 2 did not lead to significant *egr-2* induction, but ASST-re-tested animals expressed significantly more *egr-2* compared with their odor-exposed controls (day 2) ($p < 0.001$). The densities of *egr-2*-labeled cells, however, did not significantly differ between mice re-exposed to the ASST and mice that performed the ASST only once.

Similar differences were found in the ventrolateral OFC (ANOVA, $F(4,34) = 20.091$; $p < 0.0001$). Odor exposure on day 2 led to significantly lower densities of *egr-2*-labeled cells compared with odor exposure on day 1 ($p < 0.05$). Mice re-exposed to the ASST expressed significantly more *egr-2* compared with their odor-exposed controls (day 2; $p < 0.001$), but the densities of *egr-2*-labeled cells did not significantly differ between mice re-exposed to the ASST and mice that performed the ASST only once.

These results illustrate that, in the OFC, re-exposure to the ASST led to significantly lower *egr-2* expression in response to odors alone. As a result, the ratio of test-induced *egr-2* induction over odor-evoked induction is 2.1-fold (ventrolateral OFC) and 3.5-fold (medial OFC) higher in re-tested animals compared with animals tested only once. However, the magnitude of *egr-2* induction in re-tested animals was similar to that measured for mice exposed to the ASST only once.

Re-exposure to the ASST also led to changes in *egr-2* induction in the mPFC (Fig. 6). In the IL (ANOVA, $F(4,37) = 21.399$; $p < 0.0001$), odor exposure on day 2 did not lead to *egr-2* induction. Thus, the density of *egr-2*-labeled cells in mice exposed to odor only was significantly lower compared with the corresponding density measured after odor exposure on day 1 ($p < 0.01$). Mice re-exposed to the ASST on day 2 exhibited robust induction of *egr-2* expression, and the density of *egr-2*-labeled cells was significantly higher compared with their odor-exposed controls ($p < 0.01$). However, the density of *egr-2*-labeled cells detected in re-tested mice was lower compared with mice tested only once ($p < 0.01$).

In the PrL (ANOVA, $F(3,37) = 17.496$; $p < 0.0001$), odor re-exposure did not increase the density of *egr-2*-labeled cells above baseline and re-tested mice exhibited significantly increased *egr-2* expression compared with their odor-exposed controls ($p < 0.01$). The density of *egr-2*-labeled cells in re-tested animals was lower compared to the corresponding density measured in mice exposed to the ASST only once. This difference, however, did not reach significance.

In the AC (ANOVA, $F(4,37) = 20.994$; $p < 0.0001$), re-exposure to odor did not lead to induction of *egr-2* expression. Thus, compared to the density of *egr-2*-labeled cells measured after odor exposure on day 1, the density measured after odor re-exposure was significantly lower ($p < 0.01$). The density of *egr-2*-expressing cells measured after re-exposure to odor did also not differ significantly from the density measured in re-tested mice and, similar to results obtained for the IL and PrL, the density of *egr-2*-labeled cells detected in re-tested animals was lower ($p < 0.05$) when compared with corresponding densities calculated from mice tested only once.

In summary, similar to results obtained for the OFC, re-exposure to odor led to no induction of *egr-2* expression in all three subregions of the mPFC. Therefore, the ratio of test-induced *egr-2* induction over odor-evoked induction was 1.6-fold (IL), 2.2-fold (PrL), and 3.6-fold (AC) higher in re-tested animals compared with animals tested only once. However, the magnitude of *egr-2* induction in re-tested animals was lower compared with mice exposed to the ASST only once, and this was most significant for the IL subregion of the mPFC.

Egr-2 expression in mice performing a spatial WM task

As previously shown, the ASST and tests of spatial WM both elicit induction of the IEG *c-fos* although these tests use completely different stimuli and require different motor acts (Vann et al., 2000; Glickstein et al., 2005). Since it is known that WM and attentional control functions are tightly interacting cognitive processes (see Introduction) we tested whether the ASST and the spatial WM test also utilize *egr-2* as a common regulatory transcription factor. Results from real-time PCR experiments and quantitative Western blots shown in Fig. 1 indicate that neither *egr-2* mRNA nor *egr-2* protein expression were significantly increased in WM-tested mice. These results are consistent with the results shown in Table 1 illustrating that there were no significant differences in the densities of *egr-2*-labeled cells in WM-tested mice in the medial OFC (ANOVA; $F(2,24) = 2.158$; $p = 0.14$) and ventrolateral OFC (ANOVA; $F(2, 24) = 3.312$; $p = 0.1$) when compared with basal *egr-2* expression. A similar result was obtained for the mPFC (ANOVA; IL: $F(2,27) = 0.9715$; $p = 0.39$; PrL: $F(2,26) = 2.570$; $p = 0.1$; AC: $F(2,27) = 2.772$; $p = 0.083$). Thus, no significant induction of *egr-2* expression occurred in the OFC and mPFC of WM-tested mice although these mice exhibited significant differences in the percentages of correct arm entries when they performed the test without inter-trial delay ($82 \pm 4\%$) and with the 15-sec inter-trial delay ($60 \pm 7\%$) (two-tailed t test; $p < 0.01$). Such decline in performance is typical for delay periods that require engagement of WM.

In summary, unlike *c-fos* induction, *egr-2* induction occurs only in response to a cognitive task that requires perceptual discrimination between different stimulus dimensions and stimulus properties, and not in tests that require spatial WM.

The spatial WM test necessitated prior training during which mice learned the rules of the test (alternate arm entry) just like mice learned the rules of the ASST during their test exposure. Since *egr-2* induction in ASST-tested mice was observed during the associative learning phase, we examined whether *egr-2* induction also occurs during the initial training that forced mice to enter the arms of the T-maze in an alternating fashion. Interestingly, the number of *egr-2* expressing nuclei counted for mice that participated in this training was even lower compared to basal *egr-2* expression (see Table 1), and this was especially prominent in the medial and ventrolateral OFC ($p < 0.03$; t -test). However, immunocytochemical studies revealed striking differences in the cellular distribution of *egr-2* immunoreactivity in the OFC (Fig. 7). At baseline and in WM-tested mice, *egr-2* immunoreactivity was almost exclusively localized in nuclei. In mice that were forced to enter the arms of the T-maze in an alternating fashion during training, but not in mice that were allowed to choose these arms freely and in a non-alternating fashion, extranuclear *egr-2* immunoreactivity was prominent. By comparison, such cytoplasmic expression was not detected for the IEG *c-fos* (Fig. 7).

DISCUSSION

The present study shows that the expression of one member of the EGR family of regulatory transcription factors, *egr-2*, is induced in the forebrain of mice exposed to an attention-set-shifting task. At the anatomic level, this induction occurred in the OFC and mPFC and, by comparison, it was far more restricted than the induction of *c-fos* expression previously reported for ASST-tested mice (Glickstein et al., 2005). In addition, although mice exposed to a spatial WM test were found to exhibit induction of *c-fos* expression just like ASST-tested mice (Vann

et al., 2000), no induction of *egr-2* expression was found in WM-tested mice. Finally, in ASST-tested mice, only *egr-2* (and not the related transcription factors *egr-1* and *egr-3*) expression was induced.

Regulatory transcription factors may respond to specific stimulus modalities used in cognitive tasks rather than the cognitive challenge *per se*. The OFC, for example, receives olfactory input from the piriform cortex, and neurons of the OFC detect and discriminate among odors (Rolls and Baylis, 1994; Lipton et al., 1999), represent learned associations between odors and location (Lipton et al., 1999), and encode rewards and punishments associated with odors (Schoenbaum et al., 1998). These findings indicate that the OFC acts both as a secondary olfactory cortex and a higher order association cortex (Lipton et al., 1999; Feierstein et al., 2006; Roesch et al., 2006). It was therefore critical to discriminate between *egr-2* induction that occurred in response to odor exposure alone and induction that occurred in response to ASST-test exposure (i.e., stimulus-modality-independent). The results shown in Figs. 2 and 3 illustrate that induction of *egr-2* in the medial OFC and the AC subregion of the mPFC of ASST-tested mice could be attributed to odor exposure alone.

However, our measures of the densities of *egr-2* expressing cells revealed that additional *egr-2*-expressing cells are recruited when mice perform the ASST. This occurred in a subregion-specific manner and involved only the infra- and pre-limbic subregions of the mPFC and the ventrolateral OFC. Whereas the IL and PrL subregions of the mPFC are thought to be involved in preserving attentional selectivity to relevant stimuli during learning and to mediate shifts between rules that govern the task, the ventrolateral OFC is thought to represent lower order rules, such as reversal learning that does not require changes in perceptual processing (Dalley et al., 2004). Indeed, additional *egr-2*-expressing cells are recruited in the IL and PrL of the mPFC and the ventrolateral OFC when mice completed the set-shifting and reversal learning phases of the ASST. However, only in the IL, the density of *egr-2*-labeled cells was significantly higher in mice that completed the last reversal phase of the ASST when compared with mice that completed the CD phase. This suggests that, in the PrL and IL of the mPFC and the ventrolateral OFC, additional *egr-2*-expressing cells are recruited primarily during associative learning, but that even more *egr-2* expressing cells are needed in the IL to support either set-shifting or reversal learning (or both).

A specific role for *egr-2* induction in the IL during set-shifting is further suggested by results of our studies on mice re-exposed to the ASST. In these mice, re-exposure to odor alone did no longer induce *egr-2* expression suggesting that *egr-2* induction in response to odor is a non-specific response to “novelty”. Moreover, *egr-2* induction was lower in the mPFC of re-tested mice compared with mice tested only once, and this difference was largest for the IL subregion of the mPFC. Since re-testing decreased the demand on attentional control in the extradimensional set-shifting phase of the ASST (see Fig. 4), the data suggest that transcriptional activation mediated by *egr-2* in the IL may play a specific role in the control of set-shifting. This is consistent with findings that lesions involving the IL of the rat mPFC led to specific impairment in the EDS phase of the ASST (Birrell and Brown, 2000) and lends further support for the view that the IL is specifically involved in the selection of higher order rules that govern set-shifting (Dalley et al., 2004). It remains to be examined whether cells that exhibit task-induced *egr-2* induction exert an excitatory effect on their targets or whether they mediate inhibitory response control.

In contrast to the robust induction of *egr-2* expression in ASST-tested animals, no induction was detected in mice that performed a spatial WM task. Although attentional control systems involve WM processes (Awh et al., 2006), the two cognitive tests used in the present study differ substantially in the load of WM. In a single test session, ASST-tested mice have to acquire, maintain, and shift attentional sets, and react flexibly under reversal conditions. Thus,

they must continuously attend to the (changing) perceptual stimuli to accurately discriminate between them. Hence, throughout the test, the demand for attention is high and, in each phase of the ASST, attention is drawn to new stimulus properties that do not match those stored in WM. In contrast, WM-tested mice have already completed training (lasting 10 to 14 days with no inter-trial delays). On their final test day, the mice are familiar with the maze, have learned the rule of the test (alternate arm entries), do not need to attend to novel or changing stimuli, but perform the test for the first time with a 15 sec delay period. Hence, successful task performance depends primarily on spatial WM.

The different effects of ASST- versus WM-test exposure on *egr-2* expression are not simply explained by differences in stimulus modalities used in both tests. Although we have shown that odor exposure alone elicits robust *egr-2* induction, our results obtained from mice re-tested in the ASST have shown that *egr-2* induction also occurs in the absence of odor-evoked induction (Figs. 5 and 6). Moreover, differences in times of test exposure can also not explain the lack of *egr-2* induction in WM-tested mice. WM-tested mice needed ~10 min to complete the test. In the same time, mice have completed the CD-phase of the ASST, and this short test exposure already elicited induction of *egr-2* expression that, in the IL subregion of the mPFC, for example, was already significantly higher ($p < 0.05$) compared with odor-exposed controls. Finally, the different motor demands of the two tests can also not explain the different *egr-2* responses since neither control mice digging for food in ASST-test box nor mice running in the T-maze exhibited *egr-2* induction.

WM operations depend upon the transient activation of microcircuits and have only a limited storage capacity (Baddeley, 2003). This situation may be incompatible with long-lasting changes in gene expression resulting from WM-test exposure. However, although *egr-2* induction in ASST-tested mice occurred during the associative learning phase, there was also no *egr-2* induction in mice during the learning phase of the T-maze training. Strikingly, however, there were differences in the cellular distribution of *egr-2* expression in mice that were forced to alternate their arm entries during training. In these mice, but not in mice that chose their arms freely and in a non-alternating fashion during training, *egr-2* immunoreactivity appeared in the cytoplasm of cells in the OFC. Although the reason for the extranuclear localization of *egr-2* protein in mice forced to perform alternations is still not known, these findings suggest that shuttling of *egr-2* protein between the cytosol and nucleus may be yet another mechanism by which *egr-2* is recruited to or excluded from the transcriptional machinery.

In summary, the expression of individual members of the EGR family is differentially induced during different cognitive processes. In subregions of the OFC and mPFC, *egr-2* expression is induced during associative learning and attentional set-shifting, but not during spatial WM operations. Thus, although WM and attention are tightly interacting processes, they do not share a unitary transcriptional activation mediated through the regulatory transcription factor *egr-2*. The present study suggests that the plasticity-inducible transcription factor *egr-2* is a highly sensitive molecular tool to identify specific neuronal populations that support distinct domains of attentional control. *Egr-2* recognizes EGR response elements in promotor regions of a number of neuronal genes that are likely to play a role in mediating plastic changes that occur in response to cognitive demand. These genes encode, for example, the synaptic vesicle proteins synapsin I and II (Thiel et al., 1994; Petersohn et al., 1995), synaptobrevin 2 (Archer et al., 1990), and synaptophysin (Özcelik et al., 1990) that are involved in regulating neurotransmitter release, the nerve growth factor receptor p75 (Nikam et al., 1995), and the enzyme acetylcholinesterase (Li et al., 1993) that degrades a neurotransmitter essential for the orienting control function of attention (Witte et al., 1997). Thus, future studies must not only identify the types of neurons that exhibit induced *egr-2* expression in response to ASST

exposure, but also identify the individual downstream genes that are activated (or even repressed; O'Donovan et al., 1999) by *egr-2* expression in the OFC and mPFC.

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ABBREVIATIONS

IEG	immediate early gene
EGR	early growth response gene
LTP	long-term potentiation
WM	working memory
ASST	attention-set-shifting task
mPFC	medial prefrontal cortex
OFC	orbital frontal cortex
IL	infralimbic
PrL	prelimbic
AC	anterior cingulate
SD	simple discrimination
CD	compound discrimination
IDS	intradimensional set-shift
EDS	extradimensional set-shift
EDS-Rev	EDS-reversal

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Fig. 1.

Egr-2 mRNA and protein expression in ASST- and WM-tested mice. **A**, Egr-2 mRNA expression in controls and ASST- and WM-tested mice determined with real-time RT-PCR experiments. Data are means $\pm$ sem of determinations made from six animals per group and are expressed as fold change over control (C) values. ASST-tested animals either completed the set-shifting phases (SS) or the entire test ending with a reversal of the last set-shifting phase (Rev). In contrast to significantly increased egr-2 mRNA expression in ASST-tested animals, egr-2 mRNA levels of WM-tested mice did not differ from controls. **B**, Representative examples of Western blots of total protein extracted from control mice (C) and mice that completed either the compound discrimination (CD), the IDS and EDS set-shifting phases (SS), or the reversal (Rev) phase of the ASST. Blots were probed with an antibody raised against egr-2 and re-probed with an antibody raised against β -actin. The same protein samples were also probed with antibodies raised against egr-1 and egr-3. The ASST led to a rapid rise in

egr-2 protein levels but egr-1 and egr-3 protein levels remained unaltered. C, Ratio of optical densities (OD) of egr-2 protein signals detected on Western blots of protein extracted from ASST- or WM-tested animals and controls. OD measures of each sample were normalized using corresponding OD measures of β actin. Data are means $\pm$ sem of seven independent experiments, and indicate a ~1.5-fold increased expression of egr-2 protein that occurred only in ASST-tested mice.

Fig. 2.

Egr-2 expression in the OFC of odor-exposed and ASST-tested mice. *Top:* Representative examples of DAB-stained sections of the medial OFC labeled with an antibody directed against egr-2 and viewed at 20 $\times$ magnification. Basal: food-restricted control; odor: odor-exposed control; ASST-Rev: egr-2 expression after completion of the entire ASST. Scale bar, 0.05mm. *Bottom:* Comparison of the densities of egr-2-labeled cells in the medial (mOFC) and ventrolateral OFC (vOFC) of controls (basal), odor-exposed controls (odor) and animals that completed either the compound discrimination (ASST-CD) or reversal phase of the ASST (ASST-Rev). Data are means $\pm$ sem (basal: $n = 10$; odor-exposed: $n = 7$; ASST-CD: $n = 9$; ASST-Rev: $n = 8$) of the densities of egr-2-labeled cells located within the boundaries of defined counting frames (see Experimental Procedures). Data were compared by ANOVA followed *post hoc* with Tukey Kramer Multiple Comparisons tests.

Fig. 3.

Egr-2 expression in the mPFC (layers II/III) of odor-exposed and ASST-tested mice. *Top left:* Representative examples of DAB-stained sections of the IL subregion of the mPFC labeled with an antibody directed against egr-2. Basal: food-restricted control; odor: odor-exposed control; ASST-Rev: egr-2 expression after completion of the entire ASST. Scale bar, 0.05mm. *Top Right and Bottom:* Comparison of the density of egr-2-labeled cells in the IL, PrL and AC subregions of the mPFC of controls, odor-exposed controls, and animals that completed either the compound discrimination (ASST-CD) or reversal phase of the ASST (ASST-Rev). Data are means $\pm$ sem (basal: n = 12; odor-exposed: n = 7; ASST-CD: n = 11; ASST-Rev: n = 9) of the density of egr-2-labeled cells located within the boundaries of defined counting frames of three sections (see Experimental Procedures). Statistical differences revealed by ANOVA were resolved *post hoc* using Tukey Kramer Multiple Comparisons tests.

Fig 4.

Number of trials to criterion and mean response latencies per trial determined for mice that performed the ASST once and mice that were re-exposed to the ASST. White bars: performance on day 1, black bars: performance on day 2 (re-exposure). The individual set-shifting (IDS, EDS) and reversal phases (EDS-Rev) are indicated. Data represent means $\pm$ sem (IDS: $n = 18$; EDS: $n = 18$; EDS-Rev (day 1): $n = 10$; EDS-Rev (day 2): $n = 16$) and were compared by ANOVA followed *post hoc* by Tukey Kramer Multiple Comparisons tests.

Fig. 5.

Egr-2 expression in the OFC of mice re-exposed to odor or the ASST. *Top:* Representative examples of DAB-stained sections of the medial OFC labeled with an antibody directed against egr-2. Basal: food-restricted control; odor (1): odor-exposed control; odor (2): odor-re-exposure control; ASST (1): egr-2 expression after completion of the entire ASST; ASST (2): egr-2 expression after re-exposure to the entire ASST. Scale bar, 0.05mm. *Bottom:* Comparison of the density of egr-2-labeled cells in the medial (mOFC) and ventrolateral OFC (vlOFC) of controls, odor-exposed and odor re-exposure controls, and animals that completed the entire ASST once or twice. Data represent means $\pm$ sem (basal: $n = 10$; odor (1): $n = 7$; odor (2): $n = 6$; ASST (1): $n = 8$; ASST (2): $n = 8$) of densities of egr-2-labeled cells.

Fig. 6.

Egr-2 expression in the mPFC of mice re-exposed to odor alone or the ASST. *Top:* Representative examples of DAB-stained sections of the IL subregion of the mPFC labeled with an antibody directed against egr-2. Basal: food-restricted control; odor (1): odor-exposed control; odor (2): odor-re-exposure control; ASST (1): egr-2 expression after completion of the entire ASST; ASST (2): egr-2 expression after re-exposure to the entire ASST. Scale bar, 0.05mm. *Bottom:* Comparison of the density of egr-2-labeled cells in the IL, PrL and AC subregions of the mPFC of controls, odor-exposed and odor re-exposure controls and animals that completed the ASST once or twice. Data represent means $\pm$ sem (basal: $n = 12$; odor (1): $n = 7$; odor (2): $n = 6$; ASST (1): $n = 9$; ASST (2): $n = 8$) of the densities of egr-2-labeled cells.

Fig. 7.

Cellular distribution of *egr-2* immunoreactivity in WM-tested mice and their controls. Representative examples of immunolabeled sections of the medial OFC viewed at 40× magnification. In WM-tested mice, *egr-2* immunoreactivity is predominantly localized in nuclei and indistinguishable from basal *egr-2* expression. In mice that were forced to enter both arms of the T-maze in an alternating fashion during initial training, *egr-2* immunoreactivity appears prominently in the cytoplasm. Expression of FOS immunoreactivity (labeled with the rabbit polyclonal anti-*c-fos* antibody (AB-5, Calbiochem; #PC38; dilution: 1:10,000) remained nuclear in these mice. Scale bar: 100 μm.

TABLE 1

Density of egr-2-labeled cells at baseline compared with corresponding densities measured in mice during T-maze training (WM-training), WM-tested (WM-test) and ASST-tested mice (ASST).*

	basal	WM-training	WM-test	ASST
mOFC	300.0 ± 63.9	97.0 ± 51.4	348.1 ± 125.9	1275.0 ± 150.7
vlOFC	279.0 ± 51.1	53.1 ± 33.4	388.8 ± 143.6	1519.3 ± 141.2
mPFC; IL	608.5 ± 107.0	693.1 ± 100.7	856.0 ± 170.9	1856.8 ± 118.5
mPFC; PrL	525.1 ± 84.4	795.0 ± 157.4	985.4 ± 226.10	2007.7 ± 127.5
mPFC; AC	231.7 ± 43.5	310.6 ± 60.5	626.1 ± 209.9	1125.4 ± 83.5

* Data represent mean ± sem of densities/mm² measured in 10 (basal), eight (WM-training, ASST-tested), and nine (WM-tested) animals.