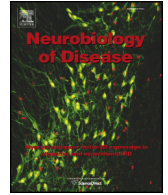




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Genome-wide approaches reveal EGR1-controlled regulatory networks associated with neurodegeneration

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ABSTRACT

Early growth response gene 1 (*Egr1*) is a member of the immediate early gene (IEG) family of transcription factors and plays a role in memory formation. To identify EGR1 target genes in brain of Alzheimer's disease (AD) model mice — APP23, we applied chromatin immunoprecipitation (ChIP) followed by high-throughput DNA sequencing (ChIP-seq). Functional annotation of genes associated with EGR1 binding revealed a set of related networks including synaptic vesicle transport, clathrin-mediated endocytosis (CME), intracellular membrane fusion and transmission of signals elicited by Ca^{2+} influx. EGR1 binding is associated with significant enrichment of activating chromatin marks and appears enriched near genes that are up-regulated in the brains of APP23 mice. Among the putative EGR1 targets identified and validated in this study are genes related to synaptic plasticity and transport of proteins, such as *Arc*, *Grin1*, *Syn2*, *Vamp2* and *Stx6*, and genes implicated in AD such as *Picalm*, *Psen2* and *App*. We also demonstrate a potential regulatory link between EGR1 and its newly identified targets *in vivo*, since conditions that up-regulate *Egr1* levels in brain, such as a spatial memory test, also lead to increased expression of the targets. On the other hand, protein levels of EGR1 and ARC, SYN2, STX6 and PICALM are significantly lower in the brain of adult APP mice than in age-matched wild type animals. The results of this study suggest that EGR1 regulates the expression of genes involved in CME, vesicular transport and synaptic transmission that may be critical for AD pathogenesis.

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Introduction

Early growth response gene-1 (*Egr1* in mice, *EGR1* in humans) (Beckmann and Wilce, 1997) is considered a key transcriptional regulator linking injurious stimuli to the induction of effector molecules. With sustained or increased chronic EGR1 activation the response may ultimately result in cardiovascular pathology, or sclerosis that is either systemic (scleroderma) or organ specific (e.g. in liver or lung) (Bhattacharyya et al., 2013; Silverman and Collins, 1999). EGR1 is an activity-dependent IEG transcription factor, which is up-regulated within minutes following a variety of memory tests (Miyashita et al., 2009; Miyashita et al., 2008). In the hippocampal CA3-established ensembles of place cells, increased mRNA synthesis of *Egr1* and a small number of known gene targets, including Synapsin I and Synapsin II (*Syn1*,

Syn2) and activity-regulated cytoskeleton associated protein (*Arc*), provides a mean for fast encoding of experiences (Knapska and Kaczmarek, 2004; Kubik et al., 2007). Until now, research related to the biology of EGR1 in CNS has been concentrated either on behavioral paradigms that lead to *Egr1* up-regulation (Davis et al., 2010; Knapska and Kaczmarek, 2004; Poirier et al., 2008), or identification of regulatory pathways involved in transcriptional up-regulation of *Egr1* (Ritchie et al., 2011; Thiel and Cibelli, 2002; Thiel et al., 2010). Down-regulation of *Egr1* has been reported in AD model mice — APP/PS1, at a time when these mice exhibit cognitive deficits (Dickey et al., 2004; Dickey et al., 2003). It has been found, however, that in conditions of environmental enrichment, APP/PS1 mice respond by elevated expression of IEGs, including *Egr1* (Lazarov et al., 2005). So far EGR1 target genes and regulatory networks in the brain of model mice in the context of neurodegeneration have not been systematically explored. Using ChIP-seq we mapped genome-wide EGR1 binding in brain tissue of APP-expressing mice. We demonstrate that binding of EGR1 in close proximity to the transcription start sites of putative target genes correlates to enhanced histone acetylation and methylation indicative of transcriptional activity. Remarkably, the annotated

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targets are highly enriched for functions related to vesicular transport, synaptic vesicle transport, CME and intracellular protein transport, as well as mRNA translation, protein phosphorylation and catabolic processes. We also provide the results of functional in vitro and in vivo validation of novel EGR1 targets, with known or suspected roles in risk and pathogenesis of AD and psychotic disorders. Thus, the findings in this report provide a molecular explanation for the role of EGR1 in synaptic plasticity and reveal possible molecular relationships between extrinsic signals and regulatory networks involved in neurodegeneration.

Materials and methods

All animal experiments were approved by the University of Pittsburgh Institutional Animal Care and Use Committee. APP23 mice (Sturchler-Pierrat et al., 1997) and WT littermates were used at 6–8 months of age. C57BL/6 were purchased from Hilltop Laboratory Animals and examined at 2 months of age (Scottsdale, PA).

Chromatin immunoprecipitation and high-throughput sequencing

Mice were anesthetized with Avertin (250 mg/kg of body weight, i.p.) and blood was drawn from the heart (Fitz et al., 2012). The mice were perfused transcardially with 25 ml of cold 0.1 M PBS, pH 7.4. Brains were rapidly removed and divided into hemispheres, and olfactory bulbs and subcortical areas were removed. Small pieces of cortex and hippocampus and cortex, including parts of the parasubiculum, postsubiculum and primary visual cortex, were removed for RNA isolation, snap-frozen on dry ice and stored at -80°C . The remaining tissue was processed for ChIP. The Chromatin for ChIP from a pool of six to eight mouse cortices (the entire posterior part) and hippocampi was prepared as previously described (Cronican et al., 2013; Lefterova et al., 2010; Steger et al., 2010). The antibodies used for immunoprecipitation are provided in the Supplementary Information. ChIP-Seq and control input libraries were sequenced to between 36 and 40 bases on an Illumina GA-II. Reads were aligned to mouse genome mm8 (NCBI Build 37) using ELAND. Regions of enrichment in the sequencing datasets were identified using GLTR algorithm (Tuteja et al., 2009).

Plasmids, lentiviral vectors and in vitro experiments

All sources of DNA, parent constructs, standard cloning and procedures for lentiviral amplifications are provided in the Supplementary Information. Electroporation of Cos7 cells using Amaxa Nucleofector System (Lonza, Basel, Switzerland) and reporter assays was performed following the manufacturer's protocols. For Western blotting (WB) protein extracts from cells and frozen hippocampi were processed as in Fitz et al. (2013) and the relative intensities of the bands quantified by densitometry.

Quantitative real-time PCR

First strand cDNA was synthesized using Sprint RT Complete Random Hexamer strips (Clontech, Mountain View, CA) from 300 to 500 ng of total RNA. RT-QPCR was performed as before (Cronican et al., 2010). All Taqman assays were from Life Technologies (Grand Island, NY). Amplification plots were analyzed by comparative $\Delta\Delta\text{Ct}$ method.

Statistical analysis

All results are reported as mean \pm S.E.M. All statistical analyses were performed in GraphPad Prism, version 4.0 (LA Jolla, CA) and differences considered significant where $p < 0.05$.

Results

Genome-wide mapping of EGR1 binding

ChIP-seq was performed to explore EGR1 binding on a genome-wide scale. GLTR peak-calling algorithm (Tuteja et al., 2009) was used to identify 18,584 peaks of EGR1 enrichment at $\text{FDR} = 0.5\%$. EGR1 binding sites were connected to 11,103 genes with a transcription start site (TSS) within 100 kb of an EGR1 site. Tabulation of the position of EGR1 sites relative to parts of genes revealed that most of the sites were in the proximal promoter or introns, and relatively few were far from their putative targets (Fig. 1A). Half of the sites (51%) are concentrated within 5 kb upstream of the TSS (Supplemental Fig. 1), in contrast to most other transcription factors that appear to bind preferentially distal enhancers rather than proximal promoters (Farnham, 2009). The sequences of the 1000 EGR1-binding sites with strongest GLTR scores together with the surrounding 1000 bp were analyzed to identify enriched transcription factor binding motifs. The top consensus motif that was identified (Fig. 1B) is nearly identical to the most overrepresented sequence identified in a recent study reporting EGR1 ChIP-chip in monocytic cells (Kubosaki et al., 2009) and is a close match to the canonical EGR1 DNA response element previously characterized (Christy and Nathans, 1989; Gniazdowski and Czyz, 1999; Pasumarthi et al., 1997; Rebar, 2004). Additional transcription factor motifs that were identified in the vicinity of EGR1 binding include E2F, SP1, CREB, and ATF6 among others (Fig. 1C), although their binding in proximity of EGR1 and functional collaboration will require further validation.

Colocalization of EGR1 binding sites to TSS, activating and repressive histone marks

To assess the functionality of EGR1 binding regions, we examined the chromatin context of EGR1 binding and evaluated enrichment of histone modifications correlated with active or repressive transcriptional states (Barski et al., 2007; Heintzman et al., 2009; Lee and Mahadevan, 2009; Trojer and Reinberg, 2007). ChIP was performed for acetylated histone 3 Lys9 (H3K9ac) and for histone 3 Lys27 trimethylation (H3K27me3), and the ChIP-seq signal for these marks was quantified at EGR1 binding regions. As shown on Fig. 1D the mark of active chromatin H3K9ac is strongly enriched in the vicinity of EGR1 binding, while H3K27me3 – a repressive mark, appears depleted. Out of 8493 genes with EGR1 binding within 1 kb of the TSS, only 228 genes had detectable H3K27me3 and the majority of those also showed H3K9ac. Since most EGR1 binding sites occur within 5 kb of TSS (Supplemental Fig. 1), we also examined a histone mark that is frequently found at the TSS of actively transcribed genes, H3K4me3 (Barski et al., 2007; Heintzman et al., 2007; Lee and Mahadevan, 2009). This histone modification also appears highly enriched, suggesting that genes with nearby EGR1 binding are likely to be transcribed. Indeed, when we correlated the occupancy of EGR1 on genes according to their expression level as determined in a previous study (Lefterova et al., 2007), we found that genes that are up-regulated in the brain of APP23 mice are more likely to have EGR1 binding near their TSS (Fig. 1E). Examples of genes with nearby EGR1 binding and enriched H3K9ac and H3K4me3 (Grin1, Vamp2, Picalm, Psen2) or H3K27me3 (Vax1) are shown in Supplemental Fig. 2. Taken together, these findings indicate that EGR1 binding occurs in proximity of thousands of genes in the brain of APP23 mice and may play an active role in activating their transcription.

Functional pathway analysis

For pathway analysis we selected the strongest EGR1 binding sites, with a GLTR score of ≥ 14 and a TSS of a gene located within 10 kb from the binding site. We identified 1949 sites that fit these criteria

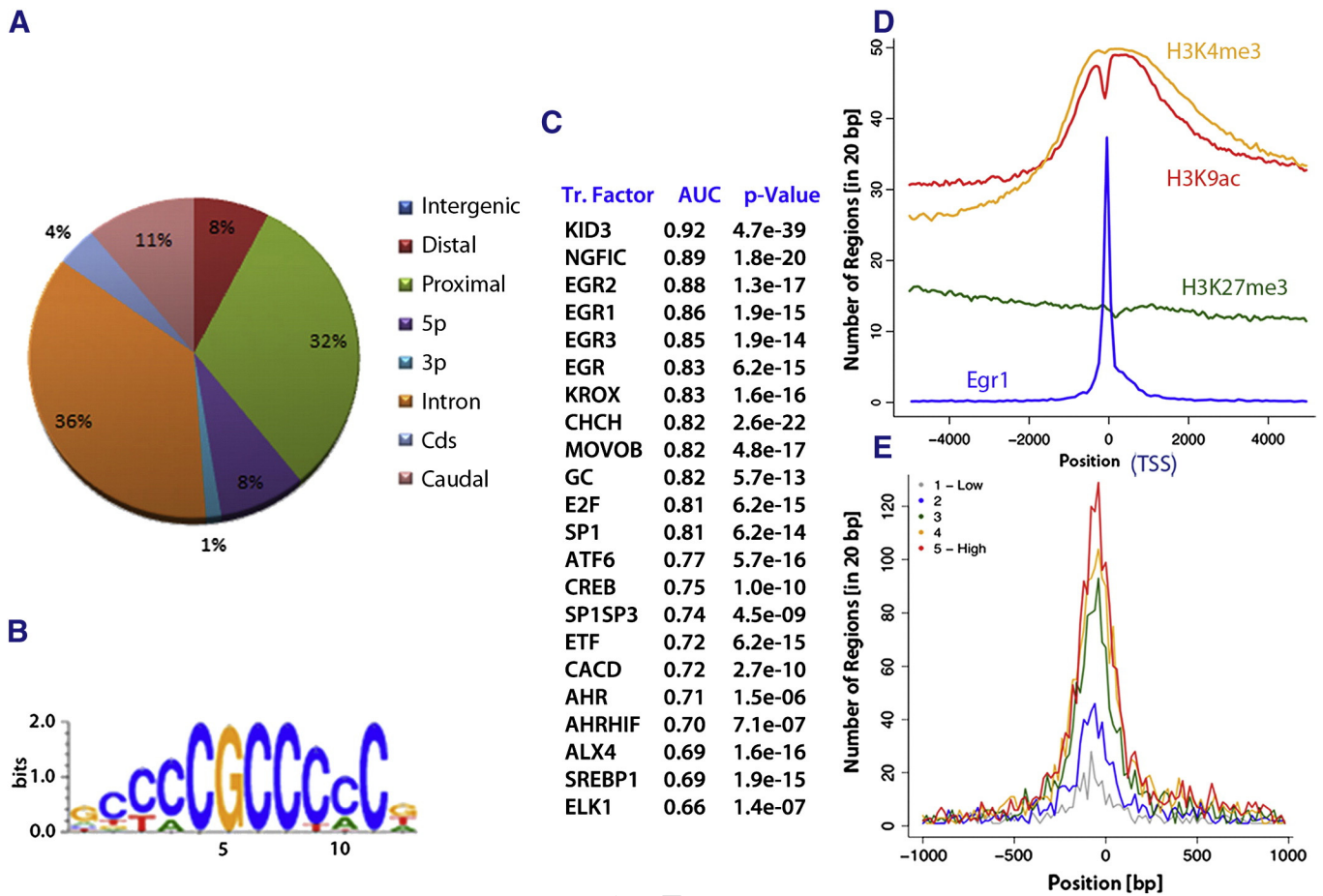


Fig. 1. Location of binding sites relative to genes. **A.** EGR1 sites were connected to 11,103 genes with a TSS within 100 kb of an EGR1 site. For these connections, the closest TSS as well as any genes that were no more than 50% further than the closest TSS were analyzed. **B.** Web logo of the EGR1 binding motif identified by de novo motif search within the 1000 EGR1 ChIP-seq peaks with strongest GLTR scores together with the surrounding 1000 bp. **C.** Area under the curve (AUC) approach was used to identify known motifs that were enriched in the area of the EGR1 binding. **D.** Average profiles of the activating and repressive histone marks around EGR1 binding regions. All profiles are centered at the middle 1 bp of the binding regions. The average signal represents the average number of reads across a category of binding regions. **E.** Occupancy of EGR1 on genes grouped by their expression level. Number of regions in 20 bp vs distance in bp of significant EGR1 binding at FDR 0.5% are tabulated relative to TSS of genes identified as significantly up-regulated in a previous study using APP23 mice. (Lefterov et al., 2007).

and selected the nearest genes for further analysis using functional annotation clustering in DAVID (Huang et al., 2009a; Huang et al., 2009b). The most highly significant GO terms for Biological Process (BP), Molecular Function (MF) and Cellular Component (CC) are listed in Table 1: a significant number of EGR1 target genes are strongly associated with i) protein targeting, localization and transport; ii) membrane organization, invagination, endocytosis, intracellular and vesicular/synaptic vesicle, transport, iii) protein phosphorylation and intracellular signaling cascades. (The full list of GO terms with a significant Fold enrichment is presented on Supplemental Table 1). These findings were confirmed using PANTHER pathway maps in DAVID (Lefterova et al., 2010; Lefterova et al., 2008) (Supplemental Table 2). We used Ingenuity Pathway Analysis (IPA) web tool to gain further insight into the role of EGR1 in CNS signaling and metabolic pathways, and to provide biological interpretation of gene interactions in a network context. We submitted all 106 genes from the non-redundant GO terms of the PANTHER pathway maps generated by DAVID (Supplemental Table 3). The top 5 entries of each IPA category generated with that list are summarized on Table 2. Importantly, the canonical pathways within each of the networks with highly significant scores were at high level of agreement with the results of the GO analysis performed in DAVID. On Supplemental Fig. 3 we present the associated network functions of Network #1, with the highest p-score: Molecular transport, Protein Trafficking, Cellular Functions and Maintenance.

Validation of EGR1 target genes in cell culture

In our validation studies we focused on EGR1 targets associated with APP processing, protein trafficking, CME/vesicle transport and fusion, protein phosphorylation triggered by Ca^{2+} influx dependent neurotransmission, and members of the Soluble NSF Attachment Protein Receptor (SNARE) complex (Supplemental Table 5). First, in N2A cells we confirmed EGR1 binding and examined the effects of over-expressed full-length EGR1 (fEgr1). EGR1 binding to its targets was significantly increased in cells transduced with fEgr1 but not in cells infected with the control GFP virus. In contrast, there was no increase in EGR1 enrichment for the negative, non-target control genes (Fig. 2A). We next assessed whether EGR1 overexpression affects mRNA levels. We tested 12 putative targets (Supplemental Fig. 4) and found that *Grin1*, *CamkV*, *Snap91*, *Stx6*, *Psen2* and *Vps18*, as well as the known EGR1 target *Arc* were significantly increased, although not all of the tested genes were similarly affected. Next we compared the expression of fEgr1 and truncated, dominant-negative variant of EGR1 (trEgr1) (Mayer et al., 2009; Stefano et al., 2006). Fig. 2B shows the infection with fEgr1 increases mRNA expression level of EGR1 targets, while trEgr1 significantly decreased the expression of *Arc*, *Grin1*, *Nab2* and *Syn2*, although it did not affect the expression levels of *Stx6*, *Psen2* and *Foxo3*. Since EGR1 binds to the proximal promoters of the latter three genes, sustained transcriptional activation by other factors could explain the failure of the truncated dominant negative construct to down-regulate expression of *Stx6*,

Table 1Gene ontology term analysis for genes identified by GLTR as putative EGR1 targets with a score of ≥ 14 and within 10 kb distance from the TSS.

GO category	Description	GO term	Fold enrichment	Benjamini factor	No. of genes
Biological process	Endocytosis	GO:0006897/0010324	2.254	1.13E-2	30
	Membrane organization	GO:0016044	1.973	1.73E-2	38
	Protein transport	GO:0015231/0045184	1.953	2.40E-6	90
	Vesicle-mediated transport	GO:0016192	1.940	2.88E-4	64
	Intracellular transport	GO:0046907	1.835	2.97E-3	56
	Protein catabolic process	GO:0030163	1.804	7.76E-4	71
Molecular function	Microtubule binding	GO:0008017	3.359	1.19E-2	14
	Phosphoprotein phosphatase act.	GO:0004721	2.407	6.80E-3	25
	Transcription cofactor activity	GO:0003712	2.195	1.26E-2	27
	Phosphatase activity	GO:0016791	2.152	3.46E-3	35
	Enzyme binding	GO:0019899	2.045	1.27E-2	32
	Transcription factor binding	GO:0008134	2.033	4.32E-3	40
Cellular component	Clathrin coat	GO:0030118	4.436	5.20E-3	10
	Postsynaptic density	GO:0014069	3.253	2.41E-2	11
	Ruffle	GO:0001726	3.209	4.50E-2	10
	Synaptosome	GO:0019717	3.199	5.62E-3	14
	Clathrin-coated vesicle	GO:0030136	2.901	.28E-3	20
	Melanosome	GO:0042470/0048770	2.839	6.61E-3	16
	Cell leading edge	GO:0031252	2.828	1.22E-3	21
	Microtubule assoc, complex	GO:0005875	2.778	2.04E-2	14
	Coated vesicle	GO:0030135	2.676	1.58E-3	22
	Membrane-bound vesicle	GO:0031988	2.227	5.04E-7	62
	Cytopl. membrane-bound vesicle	GO:0016023	2.222	5.89E-7	61
	Cytoplasmic vesicle	GO:0031410	2.108	6.84E-7	71
	Vesicle	GO:0031982	2.092	4.78E-7	72
	Endomembrane system	GO:0012525	2.086	9.57E-7	74

Foxo3 and *Psen2*. Another possibility is that the dominant negative construct does not block EGR binding completely and residual EGR activity is sufficient to maintain target expression.

Table 2

Ingenuity pathway analysis — networks, diseases and biological functions.

Top networks		
ID	Associated network function	Score
1	Molecular transport, protein trafficking, cellular functions and maintenance	53
2	Neurological disease, skeletal and muscular disorders, cellular assembly and organization	42
3	Cellular assembly and organization, molecular transport, protein trafficking	29
4	Cellular assembly and organization, cellular functions and maintenance, molecular transport	29
List of diseases		
Diseases and disorders (p-value)		#molecules
1	Developmental disorder (2.6E-06–4.69E-02)	7
2	Endocrine system disorder (2.6E-06–1.19E-02)	4
3	Hereditary disorder (2.6E-06–3.61E-02)	13
4	Metabolic disease (2.6E-06–3.54E-02)	5
5	Neurological disease (2.6E-06–4.84E-02)	26
Top biological functions		
Molecular and cellular functions (p-value)		#molecules
1	Molecular transport (2.63E-18–4.82E-02)	41
2	Protein trafficking (2.63E-18–2.96E-02)	26
3	Cellular function and maintenance (8.02E-14–4.69E-02)	52
4	Cellular assembly and organization (2.96E-11–4.69E-02)	56
5	Cellular compromise (1.04E-08–4.69E-02)	11
Physiological system development and function (p-value)		#molecules
1	Nervous system development and function (7.96E-07–4.69E-02)	35
2	Tissue development (1.16E-04–4.29E-02)	13
3	9 cardiovascular system development and function (1.91E-04–10.4.69E-02)	5
4	Organ morphology (5.47E-04–3.54E-02)	12
5	Tissue morphology (2.6E-06–4.69E-02)	10

We also examined protein levels of a subset of putative EGR1 targets in N2A cells expressing fEgr1, trEgr1, or GFP. While fEgr1 transduction led to increased EGR1 protein compared to the endogenous levels detected in GFP-transduced cells, there was a significant reduction in cells transduced with trEgr1 (Fig. 2C). A decreased expression level reflects successful, although partial, down-regulation presumably due to binding of the dominant-negative construct to EGR1 response elements in its own proximal promoter (Tur et al., 2010). Our ChIP-seq data confirm that there is a direct binding of EGR1 at sites within 600 bp upstream of the *Egr1* TSS as previously identified (Tur et al., 2010) and also at newly identified sites — 293 bp and +469 bp relative to the TSS. Transcriptional control by other factors whose binding sites remain accessible in conditions of trEgr1 expression, apparently provides detectable amount of EGR1. Therefore, it is not surprising that protein levels of only some EGR1 targets are significantly affected when EGR1 binding is blocked (Syn2 in Fig. 2C), while other targets such as *Arc*, *Grin1* and *Stx6* are not affected, even though they respond by increased mRNA and protein levels in conditions of fEgr1 over-expression.

To further confirm that EGR1 binding near newly identified putative targets is functional, we performed reporter assays (Lefterova et al., 2008) in Cos7 cells using the proximal promoters of human *PICALM* and mouse *Grin1*. These genes were chosen based on their relevance to psychiatric disorders and neurodegeneration. GRIN1 is a principal component of the NMDA receptor complex (Danysz and Parsons, 2003; Parsons et al., 2013) and there is evidence suggesting NMDAR dysfunction in schizophrenia (Javitt, 2004; Javitt et al., 2012) whereas a sequence variant of *PICALM* (rs3851179) has been identified as associated with a high risk for AD (Harold et al., 2009; Sleegers et al., 2010). Fig. 2D (left panel) demonstrates that human EGR1 can drive the transcription of a luciferase reporter gene from the mouse *Grin1* promoter (see EGR1 binding to *Grin1* on Supplemental Fig. 2). Furthermore, we found that trEgr1 reduced the basal activity of the *Grin1* reporter and this effect was rescued to the level of the GFP control when fEgr1 was expressed in addition to trEgr1 (Supplemental Fig. 5). These results demonstrate that *Grin1* is an EGR1 target with a binding site mapped at –273 bp upstream of the TSS (Supplemental Fig. 2). Similarly, as shown on Fig. 2D (right panel), human EGR1 could drive activity of

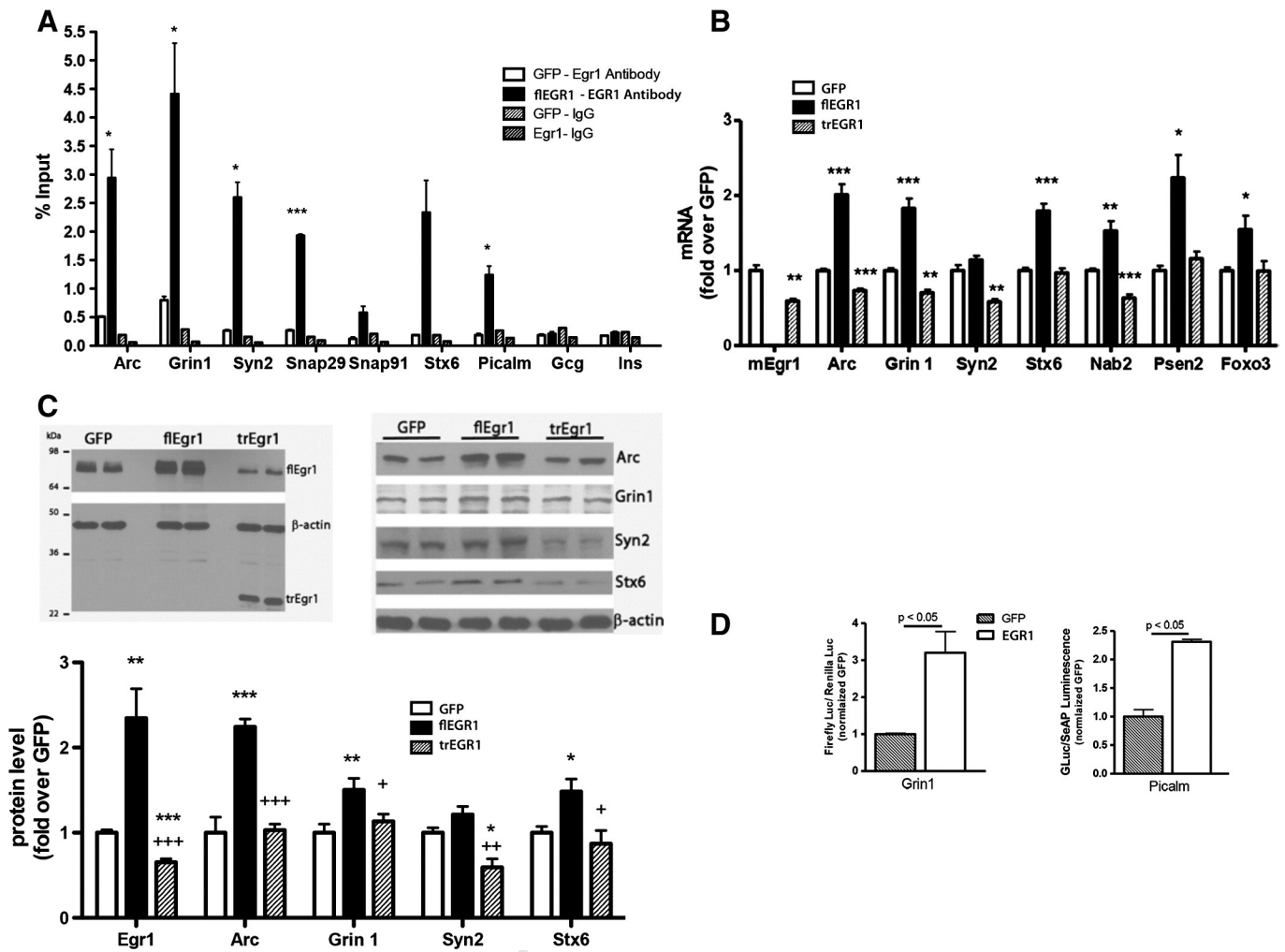


Fig. 2. Full-length and truncated Egr1 differentially affect the expression of Egr1 target genes. N2A cells were infected with lentiviral vectors expressing full length human EGR1 (flEgr1), functionally defective truncated (dominant-negative) construct (trEgr1) or control lentivirus (GFP). **A.** Over-expression of flEgr1 increases its binding at the promoters of newly identified target genes. ChIP was performed on EGR1 and GFP expressing cells either with rabbit EGR1 antibody or rabbit IgG followed by QPCR to determine EGR1 binding. Results are from 2 independent experiments. **B.** Infection with flEgr1 increases mRNA expression level of EGR1 targets, while infection with trEgr1 decreases mRNA expression of mouse Egr1 (mEgr1) and some of its targets except Stx6, Psen2 and Foxo3. Data from one experiment ($n = 4$). ***, $p < 0.001$, **, $p < 0.01$ and *, $p < 0.05$ compared to GFP. **C.** Protein level of EGR1 target genes is affected by expression of flEgr1. The bands of the respective proteins were normalized to β -actin and the differences in protein levels are represented as fold of GFP. Data are the average of at least two experiments in triplicate. Analysis is by t -test. ***, $p < 0.001$, **, $p < 0.01$ and *, $p < 0.05$ compared to GFP. + + +, $p < 0.001$; + +, $p < 0.01$ and +, $p < 0.05$ compared to flEgr1. **D.** Luciferase reporter assays using *Grin1* and *PICALM* proximal promoters. For *Grin1* reporter assay, Cos-7 cells were co-transfected with reporter (pGrin1-Fluc), expression (pCMV6-hEgr1 or pCMV6-mEgr1) and control (Renilla) vectors. Ratios of luminescence induced by Egr1 and Renilla plasmids were normalized to the corresponding ratio of luminescence induced by GFP and Renilla plasmids 48 h post transfection. For *PICALM*, Cos-7 cells were co-transfected with *PICALM* reporter, human EGR1 (pCMV6-hEgr1 or GFP?) expression constructs and a reporter plasmid as described in methods. Ratios of dual luminescence/alkaline phosphatase induced by Egr1 were normalized to the corresponding ratio of luminescence/alkaline phosphatase induced by GFP 48 h post transfection. For both promoter assays, 3 independent experiments, 3–4 wells per experiment, analysis by t -test).

the human *PICALM* proximal promoter, indicating that this gene is also a direct target of EGR1.

In vivo validation of EGR1 target genes

To establish that EGR1 plays a role in vivo in the transcriptional regulation of the putative targets identified above we first examined EGR1 binding in brain of 2 month old WT C57BL/6 mice. The genes chosen for validation included those examined in vitro, and others from the highly enriched GO categories (Table 1). As can be seen on Fig. 3A, EGR1 binding is significantly enriched in the promoters of the putative targets, associated with biological processes, molecular functions and cellular components critical for synaptic vesicle formation, protein phosphorylation and NMDAR mediated signaling in brain. In contrast, no EGR1 occupancy was detected in the promoters of genes used as negative controls.

It has been shown that long-lasting synaptic plasticity, consolidation, and reconsolidation of long-term memory require gene transcription programs and protein translation (Davis et al., 2003; Davis et al., 2010; Gräff and Mansuy, 2008; Knapska and Kaczmarek, 2004). EGR1 is considered one of the key mediators of the rapid activation of those downstream programs. Therefore, we examined if there are activity-induced changes in the expression of known and newly identified EGR1 targets in the hippocampus and cortex of wild type mice. We chose a modification of the Morris Water Maze (MWM) paradigm that is widely used to study activity-dependent EGR1 mediated up-regulation of genes (D'Hooge and De Deyn, 2001; Fordyce et al., 1994; Guzowski et al., 2001; Owen and Brenner, 2012; Porte et al., 2008). As shown on Fig. 3B, following a single-day MWM test, mRNA levels of the majority of genes chosen for validation in the hippocampus (including *Egr1*) were increased significantly as compared to caged controls, as were the positive controls *Nab2* and *Arc*. In contrast,

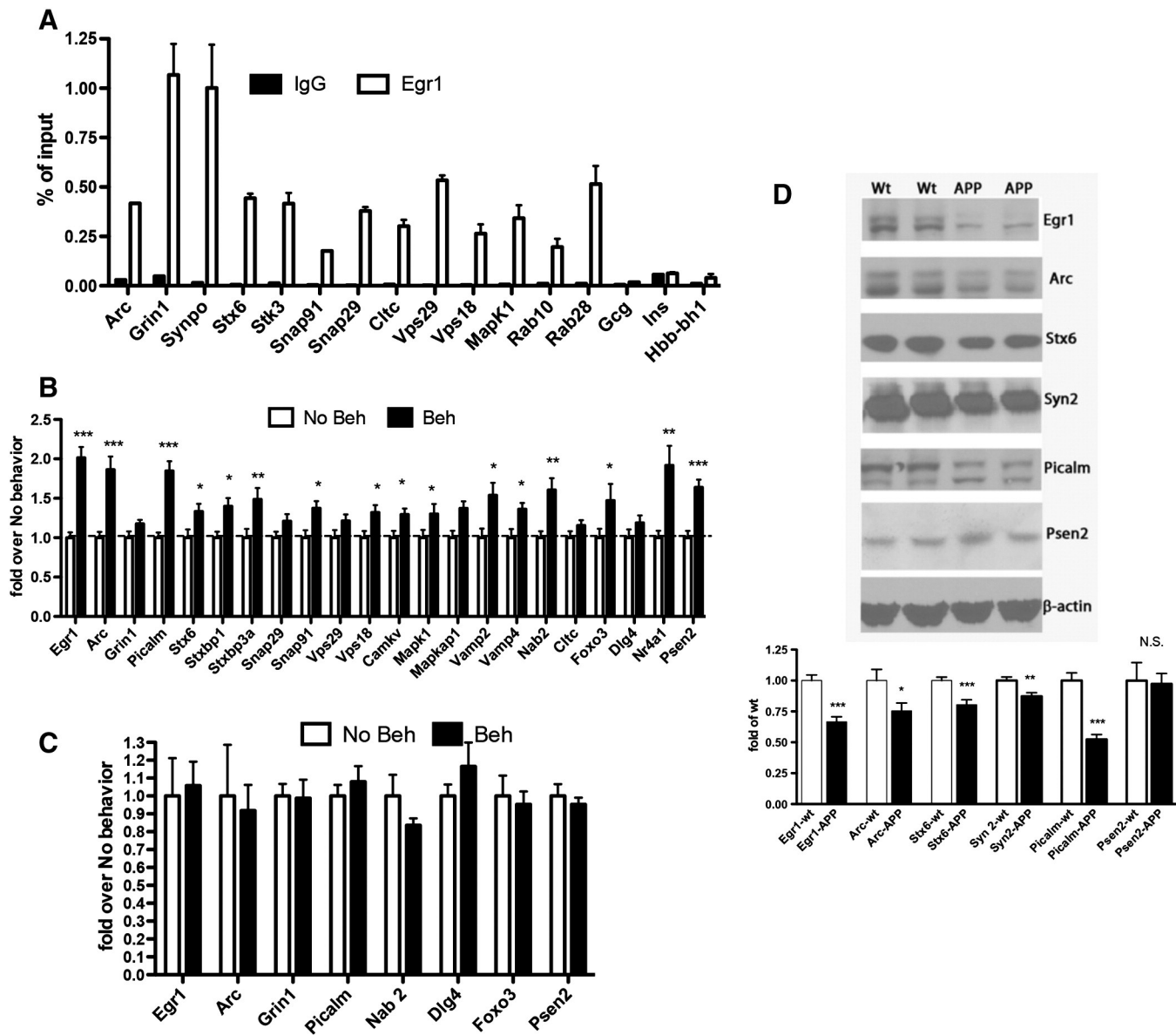


Fig. 3. Activity- and disease-dependent alterations in EGR1-regulated genes in brain of WT and APP mice. **A.** ChIP was performed on homogenates from cortices and hippocampi of 2-month old WT mice (male and female) using rabbit EGR1 antibody or control rabbit IgG followed by RT-QPCR. Ins, Gcg and Hbb-bh-1 are used as negative controls. Data are average from two independent experiments. **B** and **C.** Two-month old WT mice were subjected to a single day spatial test in MWM as described in the methods and mRNA expression of EGR1 and its targets was examined in hippocampus (**B**) and cortex (**C**). Analysis is by *t*-test. ***, $p < 0.001$, **, $p < 0.01$ and *, $p < 0.05$ mice in the behavior paradigm (Beh) compared to mice not subjected to behavior training (No Beh). $N = 8$ –16 mice per group. **D.** Protein expression of EGR1 and selected target genes is decreased in hippocampus of APP expressing mice in comparison to their WT controls. Protein level of EGR1 and its targets was examined in APP23 mice (6–8 months old, mean age = 7.5 months, male and female) and compared to their age- and gender-matched WT littermates. Note that all mice were naïve i.e. not subjected to behavior training. The bands of corresponding proteins were normalized to the level of β -actin and the differences of protein level are presented as fold of the level in WT mice. Analysis is by *t*-test. ***, $p < 0.001$, **, $p < 0.01$ and *, $p < 0.05$. $N = 6$ –12 mice per group.

there was no increase in expression levels of the same genes in the cortex (Fig. 3C).

Disease dependent changes in protein level of EGR1 and its target genes

It has been suggested that EGR1 is important for learning and memory, and in conditions of neurodegeneration, the cognitive impairment might be at least partially related to decreased amounts of EGR1 available for orchestration of immediate responses to external signals (Bozon et al., 2002; Davis et al., 2003; Jones et al., 2001; Miyashita et al., 2009). Previously we have demonstrated that APP23 mice have significant memory deficits compared to age matched wild type mice (Fitz et al., 2010; Lefterov et al., 2009). In this regard we compared protein levels of EGR1 and a subset of its targets in brain

of age-matched adult WT and APP-expressing mice. Mice were examined at 6–8 months of age (mean = 7.5 months), corresponding to the onset of amyloid phenotype for this model when amyloid plaques can be first detected. As shown in Fig. 3D, protein levels of EGR1 and several of its targets are expressed at significantly lower levels in AD mice compared to age and gender matched controls. The WB data also demonstrate that protein levels of some EGR1 targets, such as Psen2, remain unchanged in adult WT and APP23 mice.

Discussion

EGR1 has established roles in synaptic transmission, plasticity, learning and long-term memory (Davis et al., 2003; Jones et al., 2001), although the specific genes mediating these effects have remained

largely unknown. We have used ChIP-seq to map EGR1 binding to thousands of genomic regions, the majority of which is located near TSS of potential gene targets. We show that many of these binding events are likely to be functional given the association with active chromatin marks and the correlation with gene expression levels. Gene ontology and pathway analyses revealed that putative EGR1 gene targets are enriched in biological processes highly relevant to synaptic activity and plasticity: endocytosis, membrane organization and vesicle mediated transport. We have confirmed that such genes are likely to be direct EGR1 targets through a series of in vitro validation experiments, including reporter assays and manipulation of EGR1 levels with correlation to the expression of putative targets. Additionally we have shown that EGR1 and several of its newly identified targets are up-regulated in vivo in an established behavioral paradigm for *Egr1* activation while a subset of the same genes is down-regulated in APP23 Alzheimer's disease model mice.

Recent studies have interrogated EGR1 binding in different cellular contexts such as the response of the erythroleukemia cell line K562 to Phorbol-12- myristate 13- acetate (Tang et al., 2010) or monocytic differentiation using THP-1 cells and chromatin immunoprecipitation with promoter array (ChIP-chip) (Kubosaki et al., 2009). Importantly, the study by Kubosaki et al. (Kubosaki et al., 2009) found that there is enrichment of EGR1 binding in CpG islands, and co-localization with histone acetylation at TSS. In addition, in their list of GO biological process terms, they find enrichment of protein transport, intracellular protein transport and protein localization. In the CNS, a variety of stress conditions, learning tests and induction of Long Term Potentiation elicit changes in EGR1 expression (Davis et al., 2010; Jones et al., 2001). Sustained binding of EGR1 to its targets has long been hypothesized to play an important role in the transcriptional control and constitutive supply of proteins critical for synaptic transmission, plasticity, learning and long-term memory (Kubosaki et al., 2009). Nevertheless, the overwhelming number of reports on the roles of EGR1 in brain has focused on changes in *Egr1* expression, while the number of its known direct targets has remained low.

Our study is the first to comprehensively explore EGR1 target genes and regulatory networks in brain under endogenous EGR1 control. Using genome-wide sequencing we have identified thousands of new EGR1 targets in the brain of APP23 mice and have outlined EGR1-regulated networks of genes that appear responsive to behavioral stimuli. The list of 1381 genes, selected for analysis based on strong EGR1 binding and proximity of EGR1 binding and gene TSS, contains a number of genes with biological functions critical for synaptic plasticity, memory, protein transport, synaptic vesicle transport, membrane fusion and Ca^{2+} -mediated signaling. Among these, members of the SNARE complex are of particular interest given results from a recent epidemiological report on AD patients (Honer et al., 2012) and previous studies in other pathological and genetic conditions (Begemann et al., 2010; Sawada et al., 2005) implicating these proteins in human cognition. The study by Honer et al. identified specific presynaptic proteins and protein–protein interactions that together make up a cognitive reserve that at least partially reduces the risk of dementia with aging (Honer et al., 2012). Our study identified EGR1 binding sites at genes assayed in the Honer et al. study in addition to several other members of the SNARE complex, including Synaptobrevin (*Vamp2*), Syntaxin- 6, Complexin-1, and Syntaxin-binding proteins. Additionally, we have validated *Picalm* as an EGR1 target, which has been shown to interact with members of the SNARE complex and to participate in CME and vesicular transport (Harel et al., 2008; Miller et al., 2011). Furthermore, numerous studies have provided compelling evidence supporting the role of receptor trafficking and CME abnormalities in neurological and neuropsychiatric disorders (McMahon and Boucrot, 2011; Schubert et al., 2012). Thus, the results of our study add valuable information regarding the possible role of EGR1 in cognition and memory formation. Interestingly, the *Psen2* gene, which is associated with early onset AD in humans, was identified as a putative EGR1 target in our study. Previous

reports showed transcriptional control of human but not mouse *Psen2* by EGR1 in neuronal and non-neuronal cell lines (Ounallah-Saad et al., 2009; Renbaum et al., 2003). Our ChIP-seq experiments found an EGR1 peak approximately 50 bp upstream of the longer *Psen2* transcript (Supplemental Fig. 2) which presumably could allow transcriptional control of both the ubiquitous and neuronal-specific transcripts (Renbaum et al., 2003). We also show that *Psen2* was up-regulated with ectopic EGR1 expression in N2A cells and in an activity-dependent manner in the hippocampus following an MWM task.

Our data are also in line with a recent report (Naert and Rivest, 2012) in which APP/PS1 mice were used to demonstrate a decrease of in situ mRNA signal of *Egr1* and *Arc* in the hippocampus of 6-month old AD model animals compared to WT mice at the same age, while there was no difference between 3-month-old WT and APP/PS1 mice. Importantly, the decrease in expression levels of *Egr1* and *Arc* correlated to the occurrence of cognitive impairment with aging, although a molecular mechanism was not provided. We hypothesize that the disease-dependent decrease in EGR1 expression leads to a relative decrease in expression of EGR1-regulated genes involved in the regulation of synaptic activity, and this contributes to cognitive decline. A recent study with postmortem brains from AD patients at different Braak stages found a significant decrease in *EGR1* expression level at a time when cognitive impairment is dominating the clinical picture (Bossers et al., 2010). Similarly, changes in EGR1 expression in brain of AD model mice, as found in our study, may significantly contribute to their AD-like phenotype. What is the cause for the changes in EGR1 expression, however, remains an unanswered question. While it is difficult to directly extrapolate data from mice studies to human, age-dependent epigenetic alterations instigated by environmental factors are one possible explanation. The availability of advanced sequencing technologies now allows investigation with postmortem brain tissue, so these questions can be addressed in human and AD animal studies aiming to determine the dysfunction of EGR1 controlled gene networks at different stages of the disease progression. In this regard, the list of EGR1 targets and functional networks in APP23 mice as provided by this study establish a model suitable to further reveal the molecular basis for learning and memory as well as age and disease-dependent changes in cognition.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.nbd.2013.11.005>.

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References

- Barski, A., et al., 2007. High-resolution profiling of histone methylations in the human genome. *Cell* 129, 823–837.
- Beckmann, A.M., Wilce, P.A., 1997. Egr transcription factors in the nervous system. *Neurochem. Int.* 31, 477–510 (discussion 517–6477).
- Begemann, M., et al., 2010. Modification of cognitive performance in schizophrenia by complexin 2 gene polymorphisms. *Arch. Gen. Psychiatry* 67, 879–888.
- Bhattacharyya, S., et al., 2013. Egr-1: new conductor for the tissue repair orchestra directs harmony (regeneration) or cacophony (fibrosis). *J. Pathol.* 229, 286–297.
- Bossers, K., et al., 2010. Concerted changes in transcripts in the prefrontal cortex precede neuropathology in Alzheimer's disease. *Brain* 133, 3699–3723.
- Bozon, B., et al., 2002. Regulated transcription of the immediate-early gene *Zif268*: mechanisms and gene dosage-dependent function in synaptic plasticity and memory formation. *Hippocampus* 12, 570–577.
- Christy, B., Nathans, D., 1989. DNA binding site of the growth factor-inducible protein *Zif268*. *Proc. Natl. Acad. Sci. U. S. A.* 86, 8737–8741.
- Cronican, A.A., et al., 2010. Proton pump inhibitor lansoprazole is a nuclear liver X receptor agonist. *Biochem. Pharmacol.* 79, 1310–1316.
- Cronican, A.A., et al., 2013. Genome-wide alteration of histone H3K9 acetylation pattern in mouse offspring prenatally exposed to arsenic. *PLoS One* 8, e53478–e.
- Danysz, W., Parsons, C.G., 2003. The NMDA receptor antagonist memantine as a symptomatological and neuroprotective treatment for Alzheimer's disease: preclinical evidence. *Int. J. Geriatr. Psychiatry* 18, S23–S32.

- 470 Davis, S., et al., 2003. How necessary is the activation of the immediate early gene *zif268*
471 in synaptic plasticity and learning? *Behav. Brain Res.* 142, 17–30.
- 472 Davis, S., et al., 2010. The formation and stability of recognition memory: what happens
473 upon recall? *Front. Behav. Neurosci.* 4, 177–177.
- 474 D'Hooze, R., De Deyn, P.P., 2001. Applications of the Morris water maze in the study of
475 learning and memory. *Brain Res. Brain Res. Rev.* 36, 60–90.
- 476 Dickey, C.A., et al., 2003. Selectively reduced expression of synaptic plasticity-related
477 genes in amyloid precursor protein + presenilin-1 transgenic mice. *J. Neurosci.* 23,
478 5219–5226.
- 479 Dickey, C.A., et al., 2004. Induction of memory-associated immediate early genes by nerve
480 growth factor in rat primary cortical neurons and differentiated mouse Neuro2A cells.
481 *Neurosci. Lett.* 366, 10–14.
- 482 Farnham, P.J., 2009. Insights from genomic profiling of transcription factors. *Nat. Rev.*
483 *Genet.* 10, 605–616.
- 484 Fitz, N.F., et al., 2010. Liver X receptor agonist treatment ameliorates amyloid
485 pathology and memory deficits caused by high-fat diet in APP23 mice. *J. Neurosci.*
486 30, 6862–6872.
- 487 Fitz, N.F., et al., 2012. Abca1 deficiency affects Alzheimer's disease-like phenotype in
488 human ApoE4 but not in ApoE3-targeted replacement mice. *J. Neurosci.* 32,
489 13125–13136.
- 490 Fitz, N.F., et al., 2013. Comment on "ApoE-directed therapeutics rapidly clear β -amyloid
491 and reverse deficits in AD mouse models". *Science* 340, 924–92c.
- 492 Fordyce, D.E., et al., 1994. Genetic and activity-dependent regulation of *zif268* expression:
493 association with spatial learning. *Hippocampus* 4, 559–568.
- 494 Gniazdowski, M., Czyz, M., 1999. Transcription factors as targets of anticancer drugs. *Acta*
495 *Biochim. Pol.* 46, 255–262.
- 496 Gräff, J., Mansuy, I.M., 2008. Epigenetic codes in cognition and behaviour. *Behav. Brain*
497 *Res.* 192, 70–87.
- 498 Guzowski, J.F., et al., 2001. Experience-dependent gene expression in the rat hippocampus
499 after spatial learning: a comparison of the immediate-early genes *Arc*, *c-fos*, and
500 *zif268*. *J. Neurosci.* 21, 5089–5098.
- 501 Harel, A., et al., 2008. Evidence for CALM in directing VAMP2 trafficking. *Traffic* 9,
502 417–429.
- 503 Harold, D., et al., 2009. Genome-wide association study identifies variants at *CLU* and
504 *PICALM* associated with Alzheimer's disease. *Nat. Genet.* 41, 1088–1093.
- 505 Heintzman, N.D., et al., 2007. Distinct and predictive chromatin signatures of transcrip-
506 tional promoters and enhancers in the human genome. *Nat. Genet.* 39, 311–318.
- 507 Heintzman, N.D., et al., 2009. Histone modifications at human enhancers reflect global
508 cell-type-specific gene expression. *Nature* 459, 108–112.
- 509 Honer, W.G., et al., 2012. Cognitive reserve, presynaptic proteins and dementia in the
510 elderly. *Transl. Psychiatry* 2, e114–e.
- 511 Huang, D.W., et al., 2009a. Bioinformatics enrichment tools: paths toward the compre-
512 hensive functional analysis of large gene lists. *Nucleic Acids Res.* 37, 1–13.
- 513 Huang, D.W., et al., 2009b. Systematic and integrative analysis of large gene lists using
514 DAVID bioinformatics resources. *Nat. Protoc.* 4, 44–57.
- 515 Javitt, D.C., 2004. Glutamate as a therapeutic target in psychiatric disorders. *Mol. Psychiatry* 9,
516 984–97 (979–97, 979).
- 517 Javitt, D.C., et al., 2012. Has an angel shown the way? Etiological and therapeutic implica-
518 tions of the PCP/NMDA model of schizophrenia. *Schizophr. Bull.* 38, 958–966.
- 519 Jones, M.W., et al., 2001. A requirement for the immediate early gene *Zif268* in the
520 expression of late LTP and long-term memories. *Nat. Neurosci.* 4, 289–296.
- 521 Knapska, E., Kaczmarek, L., 2004. A gene for neuronal plasticity in the mammalian brain:
522 *Zif268/Egr-1/NGF-A/Krox-24/TIS8/ZENK*? *Prog. Neurobiol.* 74, 183–211.
- 523 Kubik, S., et al., 2007. Using immediate-early genes to map hippocampal subregional
524 functions. *Learn. Mem.* 14, 758–770.
- 525 Kubosaki, A., et al., 2009. Genome-wide investigation of in vivo EGR-1 binding sites in
526 monocytic differentiation. *Genome Biol.* 10, R41–R.
- 527 Lazarov, O., et al., 2005. Environmental enrichment reduces Abeta levels and amyloid
528 deposition in transgenic mice. *Cell* 120, 701–713.
- 529 Lee, B.M., Mahadevan, L.C., 2009. Stability of histone modifications across mammalian
530 genomes: implications for 'epigenetic' marking. *J. Cell. Biochem.* 108, 22–34.
- 531 Lefterov, I., et al., 2007. Expression profiling in APP23 mouse brain: inhibition of Abeta
532 amyloidosis and inflammation in response to LXR agonist treatment. *Mol.*
533 *Neurodegener.* 2, 20–20.
- 534 Lefterov, I., et al., 2009. Memory deficits in APP23/Abca1 +/- mice correlate with the level
535 of Abeta oligomers. *ASN Neurol.* 1.
- Lefterova, M.I., et al., 2008. PPARgamma and C/EBP factors orchestrate adipocyte biology
via adjacent binding on a genome-wide scale. *Genes Dev.* 22, 2941–2952.
- Lefterova, M.I., et al., 2010. Cell-specific determinants of peroxisome proliferator-
activated receptor gamma function in adipocytes and macrophages. *Mol. Cell. Biol.*
30, 2078–2089.
- Mayer, S.L., et al., 2009. Epidermal-growth-factor-induced proliferation of astrocytes
requires Egr transcription factors. *J. Cell Sci.* 122, 3340–3350.
- McMahon, H.T., Boucrot, E., 2011. Molecular mechanism and physiological functions of
clathrin-mediated endocytosis. *Nat. Rev. Mol. Cell Biol.* 12, 517–533.
- Miller, S.E., et al., 2011. The molecular basis for the endocytosis of small R-SNAREs by the
clathrin adaptor CALM. *Cell* 147, 1118–1131.
- Miyashita, T., et al., 2008. Networks of neurons, networks of genes: an integrated view of
memory consolidation. *Neurobiol. Learn. Mem.* 89, 269–284.
- Miyashita, T., et al., 2009. Rapid activation of plasticity-associated gene transcription in
hippocampal neurons provides a mechanism for encoding of one-trial experience. *J. Neurosci.* 29, 898–906.
- Naert, G., Rivest, S., 2012. Age-related changes in synaptic markers and monocyte subsets
link the cognitive decline of APP(Swe)/PS1 mice. *Front. Cell. Neurosci.* 6, 51–51.
- Ounallah-Saad, H., et al., 2009. Transcriptional regulation of the murine Presenilin-2 gene
reveals similarities and differences to its human orthologue. *Gene* 446, 81–89.
- Owen, G.R., Brenner, E.A., 2012. Mapping molecular memory: navigating the cellular
pathways of learning. *Cell. Mol. Neurobiol.* 32, 919–941.
- Parsons, C.G., et al., 2013. Memantine and cholinesterase inhibitors: complementary
mechanisms in the treatment of Alzheimer's disease. *Neurotox. Res.* Q6
- Pasumarthi, K.B., et al., 1997. Cloning of the rat fibroblast growth factor-2 promoter region
and its response to mitogenic stimuli in glioma C6 cells. *J. Neurochem.* 68, 898–908.
- Poirier, R., et al., 2008. Distinct functions of egr gene family members in cognitive processes.
Front. Neurosci. 2, 47–55.
- Porte, Y., et al., 2008. Spatial memory in the Morris water maze and activation of cyclic
AMP response element-binding (CREB) protein within the mouse hippocampus. *Learn. Mem.* 15, 885–894.
- Rebar, E.J., 2004. Development of pro-angiogenic engineered transcription factors for the
treatment of cardiovascular disease. *Expert Opin. Investig. Drugs* 13, 829–839.
- Renbaum, P., et al., 2003. Egr-1 upregulates the Alzheimer's disease presenilin-2 gene in
neuronal cells. *Gene* 318, 113–124.
- Ritchie, M.F., et al., 2011. Transcriptional mechanisms regulating Ca(2+) homeostasis. *Cell Calcium* 49, 314–321.
- Sawada, K., et al., 2005. Hippocampal complexin proteins and cognitive dysfunction in
schizophrenia. *Arch. Gen. Psychiatry* 62, 263–272.
- Schubert, K.O., et al., 2012. Hypothesis review: are clathrin-mediated endocytosis
and clathrin-dependent membrane and protein trafficking core pathophysiological
processes in schizophrenia and bipolar disorder? *Mol. Psychiatry* 17, 669–681.
- Silverman, E.S., Collins, T., 1999. Pathways of Egr-1-mediated gene transcription in
vascular biology. *Am. J. Pathol.* 154, 665–670.
- Sleegers, K., et al., 2010. The pursuit of susceptibility genes for Alzheimer's disease:
progress and prospects. *Trends Genet.* 26, 84–93.
- Stefano, L., et al., 2006. Up-regulation of tyrosine hydroxylase gene transcription by
tetradecanoylphorbol acetate is mediated by the transcription factors Ets-like
protein-1 (Elk-1) and Egr-1. *J. Neurochem.* 97, 92–104.
- Steger, D.J., et al., 2010. Propagation of adipogenic signals through an epigenomic transition
state. *Genes Dev.* 24, 1035–1044.
- Sturchler-Pierrat, C., et al., 1997. Two amyloid precursor protein transgenic mouse models
with Alzheimer disease-like pathology. *Proc. Natl. Acad. Sci. U. S. A.* 94, 13287–13292.
- Tang, C., et al., 2010. Global analysis of in vivo EGR1-binding sites in erythroleukemia
cell using chromatin immunoprecipitation and massively parallel sequencing. *Electrophoresis* 31, 2936–2943.
- Thiel, G., Cibelli, G., 2002. Regulation of life and death by the zinc finger transcription
factor Egr-1. *J. Cell. Physiol.* 193, 287–292.
- Thiel, G., et al., 2010. Egr-1-A Ca(2+) -regulated transcription factor. *Cell Calcium* 47,
397–403.
- Trojer, P., Reinberg, D., 2007. Facultative heterochromatin: is there a distinctive molecular
signature? *Mol. Cell* 28, 1–13.
- Tur, G., et al., 2010. Factor binding and chromatin modification in the promoter of murine
Egr1 gene upon induction. *Cell. Mol. Life Sci.* 67, 4065–4077.
- Tuteja, G., et al., 2009. Extracting transcription factor targets from ChIP-Seq data. *Nucleic*
Acids Res. 37, e113–e.