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# AP-1 TARGETS IN THE BRAIN

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## 1. ABSTRACT

Activator protein-1 (AP-1) is a transcription factor involved in many aspects of the brain physiology and pathophysiology. In spite of strong engagement in a transcriptional regulation of the brain gene expression, only a few, if any, downstream AP-1 targets have unequivocally been identified so far. In the review we discuss only the best characterized AP-1 target genes in the brain, and we highlight the shortages of our understanding of AP-1 action in the central nervous system as well as indicate what could be done to ameliorate the situation.

## 2. INTRODUCTION

Understanding of neuronal and glial gene expression is of utmost importance to approach brain physiology and pathology. During the recent years one may witness an eruption of studies on gene expression patterns in the brain. Especially abundant are data on transcription factors encoded by the immediate early genes. However, despite the fact that these results are very important in suggesting possible processes which are controlled by the transcription factors, the thorough understanding of their role in brain functions and dysfunction requires application of other experimental approaches, such as intervening ones allowing to modify the levels and/or protein function. Unfortunately, direct means to affect genes in the brain *in vivo*, such as homologous recombination, application of antisense oligos, etc., all are flawed with numerous technical difficulties (see 1, 2). On the other hand, it is fortunate that in the case of transcription factors we may follow their well defined biological functions, and thus to identify their target genes, what in turn should help to elucidate the biological roles of transcription factors themselves. In this review we are focusing on activator protein-1 (AP-1), one of the most often studied transcription factors in the central nervous system.

## 3. AP-1 AS A TRANSCRIPTION FACTOR

AP-1 is a dimeric transcription factor (TF) composed of c-Jun and its homologs JunB, and JunD complexed to c-Fos or its related proteins FosB, DeltaFosB, Fra-1, and Fra-2. All of them are encoded by immediate early genes belonging to the bZIP superfamily, what means that they dimerize via their leucine zipper domains and bind DNA by N-terminally located basic region. Jun

proteins can form stable homodimers, but Fos family members apparently cannot (3). c-Jun was a first oncogenic product for which transcription factor activity was defined (4). *c-fos* is an immediate early gene, coding for proto-oncogenic transcription factor protein c-Fos heterodimerizing with Jun to form AP-1. The other members of Jun and Fos families that have been discovered later were named because of their homologies to the originally discovered prototypes.

Various AP-1 components show non-uniform stimulus responses and the dimers formed display a diverse stability, DNA binding specificity and affinity (5, 6, 7, 8). Moreover, transcriptional activity of AP-1 can be affected by different transcriptional coactivators such as cAMP response element binding protein (CREB)-binding protein (CBP) and Jun activation domain binding protein 1 (JAB 1) (9, 10). The AP-1 can either induce or inhibit the expression of a given gene (11, 12, 13, 14). JunD/c-Fos and JunD/FosB have the highest transactivational and DNA binding activity *in vitro* of all complexes (15), whereas JunD/ATF-2 have apparently a repressive action (16). Probably for technical reasons, the evidence for AP-1 dependent increases in gene expression is much more abundant. Interestingly, however, in the brain, the major inducible AP-1 components are c-Fos and JunB (see, e.g. 17, 18, 19, 20) and it has been elegantly shown that JunB appears to display a gene silencing, rather than gene activating properties (21). On the other hand, phosphorylated c-Jun seems to act as a transcriptional activator. In adult brain an upregulation of AP-1 components occurs in response to seizures, lesions, sensory stimulations and behavioral trainings of various kinds, etc. (17, 20, 22, 23, 24, 25, 26, 27). In this context, it might be worth mentioning e.g. that in the rat visual cortex, under basal conditions FosB and JunD comprise the AP-1 components (17). Sensory stimulation of the visual cortex results in an AP-1 activation with a dynamically changing composition, containing P-c-Jun as well as c-Fos, and JunB, detectable only at two hours after the stimulation, whereas JunB persists at least up to 6 hrs (17, 22).

Jun/Fos type AP-1 complexes bind predominantly to its cognate sequence TGA(C/G)TCA, named either AP-1 binding site or TRE (12-O-tetradecanoyl phorbol 13-acetate [TPA] response element), in many gene promoters and enhancers to regulate the gene transcription. The binding is markedly influenced by the TRE flanking sequences (5, 13) and can be regulated by tissue-specific repressive elements as evidenced by neuromodulin transcriptional regulation (28). Notably, it appears that there is a crosstalk in the regulation of the promoter activity between TRE and cAMP-responsive element (CRE) (15, 29). Furthermore, Jun and Fos proteins can form heterodimers with some members of activating transcription factor (ATF) family, and e.g., c-Jun/ATF2 dimers preferentially bind CRE (TGACGTCA) in gene promoters and show low binding activity to TRE (30), whereas CREB can be a component of TRE binding complexes (31). Unfortunately, these data have been collected in the *in vitro* experiments often relying on recombinant proteins. Hence, their biological significance remains largely unknown.

## 4. TECHNICAL CONSIDERATIONS CRITICAL FOR DEFINING AP-1-DRIVEN GENE EXPRESSION IN THE BRAIN

There is a number of methods that have been applied to suggest that a specific gene is possibly AP-1 dependent in the brain. The most popular is to show a presence of TRE/AP-1 sequence in the vicinity of the coding region of the gene under study. Unfortunately, this is not a very revealing information as AP-1/TRE-like sequences are quite abundant in the genome. Thus it appears to be of a critical importance to show that under the experimental conditions analyzed, the gene under study contains the apparent regulatory sequence(s) capable of binding the appropriate proteins in the electrophoretic mobility shift assay (EMSA) aided by a supershift approach with specific antibodies directed against defined AP-1 proteins. It would be especially useful to show that the apparent AP-1 binding site is occupied indeed by Fos and/or Jun proteins in the brain. Such an analysis could involve DNA footprinting approach as well as application of DNA-protein cross-linking methods.

Another, repeatedly raised argument relies on spatial and temporal coincidence of the expression of AP-1 proteins and the mRNA under question in response to specific stimulation. This is, however, a very indirect, although pivotal, support for the notion under the investigation. Such colocalization studies can be misleading. Especially so, if one uses methods that do not allow to investigate both the mRNA and protein at a single cell resolution.

As it has been mentioned above, lack of appropriate technologies, allowing to affect the gene expression in the brain prevents from providing a definitive evidence in this regard. However, neuronal tissue cultures can be useful in this context providing at least circumstantial functional evidence. Especially, useful are the studies involving primary neuronal or glia cultures transfected with gene constructs containing mutated gene promoters controlling expression of the reporters, such as luciferase, chloramphenicol acetyltransfrease (CAT), and LacZ, coding for beta-galactosidase. However, it is important to recall results of the seminal *in vivo* study employing transgenic mice with various variants of *c-fos* promoter which only partially confirmed the *in vitro* data showing specific response of defined regulatory elements to various treatments (32). Hence, the use of transgenic animals carrying the reporter gene under control of various promoter variants of the investigated gene, as well as the implementation of the studies on down-regulation of AP-1 function *in vivo* by means of dominant negative mutants should always be considered. In addition, the consequences of AP-1 overexpression in transgenic animal could also be important to investigate.

## 5. PUTATIVE AP-1 TARGET GENES

### 5.1. Genes encoding the transmembrane and extracellular proteins

#### 5.1.1. Tissue inhibitor of metalloproteinases-1 (*timp-1*)

An extensive evidence for AP-1-driven gene in the brain is provided for *timp-1* (33). TIMP-1 is an essential component of the extracellular matrix remodeling system, counter-balancing activity of matrix metalloproteinases. Components of the system in the brain participate in the regulation of neuronal plasticity and cell death, and when deregulated, in pathophysiology of Alzheimer disease, stroke, ischemia and epilepsy (34, 35). Nedivi *et al.* (36) and Rivera *et al.* (37) reported that *timp-1* expression raises in response to neuronal excitation after peripheral treatment with kainic acid (KA). Various studies implicated the AP-1 in control of *timp-1* expression in non-neuronal cells (38, 39). Following that Jaworski *et al.* (33) analyzed *timp-1* expression in rodent hippocampus in response to neuronal excitation produced by either kainate (KA) or pentylenetetrazole (PTZ)-evoked seizures. Using Northern blot the authors detected marked increases in the levels of *timp-1* mRNA following the seizures. Furthermore, these changes were dependent on *de novo* protein synthesis and were following increases in *c-fos* mRNA. *In situ* hybridization showed that a pattern of *timp-1* mRNA expression in the brains of KA or PTZ treated rats overlapped spatially with c-Fos protein expression detected by immunocytochemistry. In EMSA experiments using nuclear extracts from hippocampi of either KA- or PTZ-treated rats there was a dramatic increase of *timp-1*-TRE DNA-binding activity that contained various AP-1 proteins. *timp-1* promoter was also activated *in vivo* by KA- or PTZ-induced excitatory activation in hippocampi of *timp-LacZ* transgenic mice. Finally, in primary rat dentate gyrus granule cell cultures, the wild type *timp-1* promoter responded to L-glutamate stimulation, whereas AP-1 site mutated *timp-1* promoter had attenuated responsiveness (33).

To further support the hypothesis that AP-1 indeed regulates *timp-1* expression in the brain, it would be useful to show that AP-1 components occupy the putative DNA binding site within the proximal gene promoter as well as to show functional evidence *in vivo*, such as use of transgenic animals with the transgene containing reporter gene under control of various promoter variants, as well as to employ studies on up-, and down-regulation of AP-1 function *in vivo* and its effect on endogenous *timp-1* expression.

#### 5.1.2. Growth-associated protein 43 (*GAP-43, B50, neuromodulin, F1, PP46*)

*GAP-43* encodes for a membrane, axonal growth cone protein, also known either as neuromodulin or F1 or B50. The GAP-43 expression is mainly limited to the nervous system. It is widely expressed in developing neurons during axonogenesis and in glial cells under some conditions. In neurons, after establishment of synaptic contacts, GAP-43 expression is downregulated and can be restored by axonal injury. GAP-43 induction after axotomy correlates with c-Jun activation (44). In regenerating neurons which are prevented from reinnervating their target tissue, c-Jun and GAP-43 expression can be elevated even for months (45). *GAP-43* contains AP-1 motif in the promoter (46), which appears to contribute to the basal *GAP-43* promoter activity in primary cultures of rat cerebral cortex neurons from 18 day old embryos, and in neuronal cell line - CAD (17). In reporter assay in neurons, mutation of TRE diminished GAP-43 promoter activity to ca. 40% (17). Interestingly, the GAP-43 AP-1 site drives also *GAP-43* promoter activity in a wide range of non-neuronal cells that express no endogenous GAP-43, but this happens only in the absence of a tissue-specific *GAP-43* repressive element (TSGRE) located between TATA box and TRE element (17). TSGRE ensures neuron-specific *GAP-43* activation by AP-1. Data from reporter assays show that TSGRE mutations cannot influence AP-1-driven *GAP-43* promoter activity in neuronal cells (17). In primary cultures of embryonic rat cerebral cortex neurons the *GAP-43* promoter activity and specificity of expression is controlled by regulatory elements known as Cx1 and Cx2 (17). In reporter assay in neurons the mutations of Cx1 and Cx2 sites diminished *GAP-43* promoter activity to ca. 45%, and additional mutation of TRE element lowers the value to ca. 20% (17). Thus, in the case of *GAP-43* the evidence for AP-1 role in its regulation is suggestive, however still rather limited.

#### 5.1.3. Angiotensin II type 1 receptor (*AT1R, AGTR1, AT1*)

Activation of the arterial baroreceptors is transferred by glutamatergic neurotransmission into the principal recipient of primary baroreceptor afferent fibers in the brainstem - the nucleus tractus solitarii (NTS), where it stimulates c-Fos expression (40). Basal and induced c-Fos protein expression in NTS is an early step in the cascade of intracellular events that lead to long-term inhibitory modulation of the baroreceptor reflex response. In NTS there are expressed genes for AT1R and angiotensin II type 2 receptor (AT2R). Both of them have in their promoters TRE motifs (41, 42), but only *AT1R* seems to be the AP-1 target gene (43). There is a positive temporal correlation between the induction of *c-fos* mRNA (60 min) and AT1R re-expression (90 min) after sustained hypertension. c-Fos seems to co-localize with AT1R, but not AT2R. Furthermore, AT1R re-expression and restoration of pressor response to angiotensin II (ANG II) at 90 min after baroreceptor activation is significantly attenuated by pretreating Sprague-Dawley rats with the antisense *c-fos* oligonucleotides (43). In aggregate, the data supporting the direct role of AP-1 in regulation of the *AT1R* expression are very limited, and either circumstantial, or involving unreliable antisense approach (see 2).

#### 5.1.4. Glutamate receptor 2 (*GRIA2, GluR2, GluRB*)

GluR2 is a a subunit of the α-amino-3-hydroxy-5-methyl-4-isoxazole (AMPA) glutamate receptor, which is a ligand-activated cation channel. Genes encoding for the specific glutamate receptor subunits, including *GluR2*, contain AP-1 sites in their promoters (47). Using transgenic mice inducibly overexpressing DeltaFosB throughout the striatum (including nucleus accumbens, NA), it has been shown that *GluR2* appears to be regulated by DeltaFosB in

NA (48). In the transgenic mice, DeltaFosB upregulation leads to induction of active AP-1 (confirmed by EMSA) and 50% increase of GluR2 protein in NA. EMSA with anti-DeltaFosB antibody has revealed that *GluR2* promoter is bound by DeltaFosB. Despite lack of other evidence, AP-1 role in control of *GluR2* expression seems to be rather persuasive.

#### 5.1.5. Gonadotropin-releasing hormone receptor (*GnRHR*)

GnRHR is G protein-coupled, heptahelical receptor on the surface of pituitary gonadotropes. Amplitude and frequency of pulsatile GnRH release from the hypothalamus regulates *GnRHR* expression and therefore GnRHR concentration on the cell surface. A response of the pituitary gonadotropes to GnRH correlates directly with the concentration of GnRHR. GnRHR activation increases synthesis and release of the pituitary gonadotropins – luteinizing hormone and follicle-stimulating hormone as well as transcription of its own gene. Transgenic mice carrying 1.9 kb 5'-flanking region of the *GnRHR* linked to luciferase gene had undisturbed tissue-specificity and GnRH responsiveness of the promoter (49). Similar mice carrying mutated canonical AP-1 motif at a position –336/–330 of the *GnRHR* promoter lost GnRH-dependent *GnRHR* responsiveness, but had intact tissue expression pattern (50). Using EMSA with supershifts it has been shown that GnRH enhances AP-1 (JunD, FosB and c-Fos) binding to the –336/–330 bp AP-1 site in murine gonadotrope-derived alphaT3-1 cell line (50). In alphaT3-1 cells, *c-fos* is transcriptionally activated, whereas JunD and FosB are stimulated posttranslationally (50). *GnRHR* expression in mouse pituitary gonadotropes is upregulated synergistically by GnRH and activin. For this effect, region –387/–308 of *GnRHR* promoter is required. This promoter segment in alphaT3-1 cell line is composed of two overlapping cis-regulatory elements: GnRHR activating sequence (GRAS) at position –329/–318 and a SMAD-binding element (SBE) at –331/–324. Competition EMSA experiments using –335/–312 probe and alphaT3-1 cell nuclear extract together with SMAD, Jun and Fos, FosB, Fra-1, Fra-2 antibodies demonstrated direct binding of AP-1 (c-Fos/c-Jun and c-Jun/c-Jun) protein complexes to non-consensus AP-1 binding site at position –327/–322 (AGTCAC) and SMAD proteins (SMAD3 and SMAD4 but not SMAD2) to –329/–328 sequence. Interestingly, the interaction between AP-1 and SMAD has also been described in the *c-jun* promoter (51). SMAD proteins exert their effects only after binding to at least one transcriptional partner to form a multifactorial complex known as activin-responsive factor (ARF) that includes the *GnRHR* promoter AP-1 complexes. Mutations of either AP-1 or SMAD binding sequences abrogated GnRH- and activin-responsiveness of *GhRHR* promoter and reduced its basal activity (52). Additionally, GnRH-responsiveness of mouse *GnRHR* is mediated by protein kinase C and has been localized to an enhancer element known as the sequence underlying responsiveness to GnRH-1 (SURG-1) at position –292/–285 and the –276/–269 sequence underlying responsiveness to GnRH-2 (SURG-2), containing AP-1 binding site at –274/–268 (53, 54). EMSA using alphaT3-1 nuclear extracts confirmed that AP-1 protein is rapidly bound to SURG-2 after GnRH stimulation. In reporter assay it has been shown that mutation of SURG-2 abolished *GnRHR* promoter activity completely, whereas mutated SURG-1 lowered the activity ca. 7-fold. This indicates that SURG-2 is a critical element for the activity of *GnRHR* promoter, nevertheless SURG-1 is also very important (53). There is also the –1000/–994bp located, inhibitory, atypical AP-1 binding site in the *GnRHR* promoter which is activated by PKC pathway induced by GnRH agonists (55). The AP-1-related functionality of this site was confirmed by competitive EMSA with supershifts and site-directed mutagenesis combined with a reporter assay in alphaT3-1 cells.

### 5.2. Genes encoding the neurotrasmitters and and neuropeptides

#### 5.2.1. Follicle-stimulating hormone-beta (*FSHbeta*)

*FSHbeta* has two putative AP-1 sites at –120 bp and –83 bp in the proximal promoter. Both sites have been demonstrated to be important for transcriptional stimulation of *FSHbeta* in HeLa cells (56). In HeLa cells cotransfected with GnRH receptors and *FSHbeta* promoter linked to reporter gene, it was found that GnRH upregulates *FSHbeta* expression via the two AP-1 motifs (57). *FSHbeta* regulation was studied in transgenic mice containing either wild type or mutated at both AP-1 sites –4741/+759bp *FSHbeta* promoter (58). Using pituitary cell culture from the mice an importance of the two AP-1 motifs for GnRH-mediated *FSHbeta* induction in gonadotropes has been further supported (58). Cells with mutated transgene did not respond with luciferase activation for GnRH and combined GnRH/activin treatment, whereas in wild type transgene caring cells luciferase was activated by the GnRH/activin stimulation. However, *in vivo* GnRH-mediated *FSHbeta* induction has been shown to be AP-1-independent during estrous cycle, what was also observed in other model systems as gonadectomy, chronic GnRH treatment with Lupron (a long-acting GnRH agonist), injection of GnRH antiserum (58). In the absence of the two AP-1 sites, basal expression levels of the transgene were comparable to those observed with wild type ones, suggesting that these motifs may not be important for *FSHbeta* expression in the pituitary. *In vivo* studies with the transgenic mice revealed that activin, a potent FSHbeta?activator, regulates *FSHbeta* transcription by AP-1 independent mechanism (58). Thus, the role of AP-1 in control of *FSHbeta* transcription in the brain still remains poorly defined.

#### 5.2.2. Corticotrophin-releasing hormone (*CRH*, *CRF*)

CRH is a component of hypothalamic-pituitary-adrenal (HPA) axis synthesized mainly in the hypothalamus. It acts on the pituitary to stimulate the adrenocorticotropic hormone (ACTH) release, which is turn induces secretion of the glucocorticoids (GC) from the adrenal cortex. Alterations in CRH synthesis can result in depression, abnormal stress response, behavioral changes, and immunotoxicity. *CRH* transcription in the hypothalamus correlates with increases in AP-1 in response to emotional stress. This apparent link is aided by data obtained with JNK1 (c-Jun N-terminal kinase) knockout mouse study which showed severely reduced stress-related

*CRH* induction in the hypothalamus leading to attenuated GC release (59). The other example of stress stimulus inducing HPA axis is a bacterial infection. Released during the infection, blood-brain barrier penetrating, pro-inflammatory cytokines such as IL-1, activate JNK in the hypothalamus and periphery leading to appearance of AP-1-driven gene transcription. *CRF* has inhibitory glucocorticoid response-binding element (nGRE) at position –278/–249 in its promoter (60, 61). nGRE contains two AP-1 binding sites. Proximal AP-1 site seems to confer positive *CRH* promoter responsiveness to cAMP and AP-1 proteins, as well as negative response to GC. Interestingly, also human CRF-binding protein gene has putative TRE in its promoter (62). nGRE functions probably as a composite regulatory element. Mutations in GR and AP-1 motives of nGRE abolished GC-dependent *CRH* repression in mouse corticotroph AtT-20 cells (61). Thus, AP-1 proteins appear to cooperate with GR to downregulate *CRH* expression. This effect is tissue-specific and limited to hypothalamus. Brain expression of CRH outside of this structure is unaffected by GC (63), and in the human placental trophoblast GC even upregulate CRH (64). Using hypothalamus samples dissected from the rat brains and incubated *in vitro* with acephate, methamidophos and IL-1 Singh (65) showed that these factors upregulated *CRF* mRNA expression and increased c-Fos binding to nGRE of *CRF* promoter. Unfortunately, most of the aforementioned studies only indirectly have approached the link between AP-1 and *CRH* expression in the brain.

### 5.2.3. Arginine vasopressin (*AVP, ADH*)

*AVP* has a single AP-1 element which binds to the AP-1 complex containing c-Fos or FRAs in HeLa cells (66). After ANG II administration into the rat lateral brain ventricle, EMSA using protein extracts from hypothalamic paraventricular nucleus (PVN) and supraoptic nucleus (SON) showed that CRE-like binding site for Fos/Jun dimers from *AVP* promoter bound more proteins as compared to the samples from control rats (67). The specificity of the binding was confirmed by EMSA competition assay. The same lysates showed enhanced c-Jun and c-Fos expression in PVN and SON after ANG II administration. Thus, *AVP* seems to be the AP-1 target gene in PVN and SON. Using adrenalectomized rats replaced with different levels of substitutive corticosterone it has been noticed that in the parvocellular division of the PVN during the stress, glucocorticoid negative feedback was exerted on *AVP*, but not *CRF*, expression through mechanism that probably involved GR interactions with AP-1 proteins (68). Stress accelerated induction of Fos, and increased binding of proteins from thalamus extracts of adrenalectomized rats to AP-1 site. Taking into account limited approach just described, additional studies on this subject are very desirable to test a role of AP-1 in *AVP* expression.

### 5.2.4. Preproenkephalin (*PENK*)

*PENK* was the first reported AP-1 target in the brain (69). Unfortunately, our understanding of the *PENK* transcriptional regulation is still rather superficial, and only partially supportive for the suggested essential role of AP-1. PENK is expressed in neurons as well as in the glial cells. AP-1 regulates *PENK* transcription after binding to ENKCRE-2 domain in the promoter (69, 70, 71), which has atypical consensus for AP-1 (TGCGTCA). Arachidonic acid (AA) stimulates AP-1-driven PENK expression in primary rat astrocyte-enriched cultures (72). It has been described that after AA administration c-Fos, c-Jun, Fra-1, Fra-2, JunB and JunD proteins were induced and ENKCRE-2 as well as AP-1 DNA-binding activities were elevated. Cycloheximide treatment inhibited AA effects on ENKCRE-2 and AP-1 DNA-binding activities, suggesting that newly synthesized proteins are responsible for increased DNA binding. Tax1, transcription regulatory protein of human T-cell lymphotropic virus type I is *PENK* transactivator in glial cells (73). Using EMSA with supershifts, increased c-Fos/c-Jun DNA binding activity has been shown in stable glial cell lines expressing Tax1. AP-1 transcription factors are not the only ones essential for *PENK* expression. Prostaglandin E2 upregulates PENK in primary astrocyte-enriched cultures (74). Nevertheless, this effect is probably dependent on phosphorylation of CREB rather than on AP-1 activation. AP-1 is probably also not essential for cholera-toxin induced and cycloheximide-dependent pertussis toxin induced stimulation of *PENK* transcription in primary astrocyte-enriched cultures (75).

Single injection of KA induces PENK expression after 6 hr, then after 3 weeks there is a second increase in the steady-state levels of PENK expression in the rat hippocampus which lasts for at least one year (76). Originally, spatio-temporal evidence suggested that for both waves of PENK expression after KA administration AP-1 was responsible, with c-Fos, Fra-1, Fra-2, c-Jun and JunB for first peak, and JunD and 35-kDa FRA for the second (76, 77). After that it has been shown by EMSA with supershifts that AP-1 is responsible for short-term *PENK* activation after KA (77), and Sp-1 transcription factor for the long-lasting PENK expression (78). Using EMSA Won *et al.* (79) showed that melatonin inhibited KA-induced PENK and PDYN expression in rat hippocampi by blocking AP-1 or ENKCRE-2 DNA binding activity. ENKCRE-2 is found both in *PENK* promoter and in *PDYN* promoter. Interestingly, in the granular cells of the rat hippocampal dentate gyrus KA treatment upregulated *PENK* and *PDYN* transcription, whereas pentylenetetrazole injection increased PENK mRNA, but slightly decreased PDYN message, despite of increased AP-1 DNA binding activity which has been shown by EMSA (80).

Pituitary adenyl cyclase-activating peptides (PACAP) 27 and 38 stimulate *PENK* transcription in PC12 cells due to cooperative effects of AP-1 and CREB on the promoter (81). AP-1 is not sufficient to stimulate PACAP-induced *PENK* expression by itself because insulin-like growth factor 1 (AP-1 activator) could not upregulate *PENK* transcription, while introduction of c-Fos antisense RNA reduced PACAP-induced *PENK* expression by 80%. Caffeine administration into male rats stimulates PENK expression in striatum (82). In these rats there was an upregulation of mRNA for c-Fos, c-Jun and JunB, but no JunD in the striatum confirmed by *in situ* hybridization.

Shortly after that PENK mRNA was induced in the same brain region. Furthermore, increased AP-1 DNA-binding activity was found after caffeine injection in rat striatum in EMSA experiment with AP-1 consensus, and a presence of AP-1 proteins (c-Fos, c-Jun and JunB) in AP-1 motif was confirmed by supershifts. Surprisingly, Svenningsson *et al.* (82) could not detect altered binding of AP-1 to ENKCRE-2 by EMSA which questions the link between caffeine-driven AP-1 induction and *PENK* transcriptional upregulation.

Analyses of the supershifted EMSA patterns revealed that CREB, and not AP-1, plays an important role in the *PENK* stimulation by haloperidol in the rat striatum (83). Studies of transgenic mice carrying *PENK* regulatory sequences (3 kb fragment of 5'-flanking region, the first exon and intron and 1.2 kb fragment of 3'-flanking region) fused to the beta-galactosidase showed that after hyperosmotic stress c-Fos, phosphorylated CREB and transgene expression colocalized in the PVN (84). Furthermore, implementation of EMSA with supershifts showed increased AP-1 DNA binding activity in hypothalamus, nevertheless Fos failed to bind to ENKCRE-2 enhancer, unless phosphorylated CREB was present. It suggests that P-CREB, and not AP-1, regulates *PENK* expression in PVN of hypertonic stressed mice. Using EMSA and supershifts Pennypacker *et al.* (85) could not find AP-1 proteins in ENKCRE-2 element obtained from hippocampal neuronal and mixed neuronal/glial cell cultures despite of *PENK* transcriptional induction. After peripheral nociceptive stimulation of rats by formalin injection into the hindpaw *PENK* transcription is activated in the brainstem parabrachial nucleus (86). Using *in situ* hybridization and immunohistochemistry it has been shown that almost all PENK expressing neurons displayed phosphorylated CREB protein, while only a small fraction of the neurons expressed Fos immunoreactivity.

### 5.2.5. Preprodynorphin (*PDYN*)

PDYN is a member of opioid neuropeptide precursor protein family. It gives a rise to secondary peptides prodynorphin A, prodynorphin B and α-neoendorphin. These peptides bind kappa-opioid receptors and inhibit neurotransmission. PDYN has been involved in pathophysiology of drug abuse (morphine, cocaine, nicotine, ethanol), cocaine abstinence, ethanol withdrawal, epilepsy, pain and mood disorders. *PDYN* induction occurs in a tissue-specific manner following different stimuli. There are many papers that reported AP-1-dependence of *PDYN* expression in the spinal cord (87-96), but much less is known about this relationship in the brain. AP-1-driven stimulation of *PDYN* has been suggested to be dependent on the non-canonical AP-1 binding site (ncDynAP-1; TGAGAAACA) and combined CRE/AP-1 motifs (DYNCRE2 and DYNCRE3) in *PDYN* promoter (90, 91, 97, 98, 99). A reporter assay in PC12 cells indicated that DYNCRE3 element mediated CREB-driven repression of *PDYN* (91). Stimulus-specific changes in nuclear protein composition establish a functional hierarchy among the regulatory sites (87). ncDynAP-1 is the most important motif for *PDYN* induction in neurons of supraoptic nucleus after acute osmotic stress (shown by EMSA with supershifts), and is activated there by c-Fos and c-Jun (87). A relationship between AP-1 activation and *PDYN* induction in supraoptic nucleus is strengthened by colocalization of c-Fos protein with *PDYN* mRNA. ncDynAP-1 is also important for *PDYN* activation in NCB20 neuroblastoma cell line after treatment with phorbol esters (90). Acute or chronic amphetamine administration induces *PDYN* in the rat striatum and in primary neuronal cultures of striatal neurons (100). Using EMSA it has been shown that phospho-CREB bound CRE/AP-1 motifs from *PDYN* promoter are mediators of this effect. Not all AP-1 complexes stimulate *PDYN* transcription. DeltaFosB decreases dynorphin expression (101). $D_1$ receptor agonists induce *PDYN* expression in the striatum (102). This effect is multiplied several fold by removing the dopaminergic innervation to the striatum. Using adult male rats with neurons in the substantia nigra injected unilaterally with 6-hydroxydopamine and then after recovery with another dopamine agonist – apomorphine it was shown that apomorphine induced strongly *PDYN* mRNA and protein in the striatum ipsilateral to the side of the 6-hydroxydopamine injection (102). *PDYN* induction coincided with upregulated expression of Fos proteins, but not Jun proteins, and increased AP-1 DNA binding activity in the striatum. It has also been shown that chronic pain increases PDYN expression in the sensory neurons that could be mediated by AP-1 motif activation in the promoter (91).

A common polymorphism has been described in the *PDYN* core promoter region which influences the *PDYN* expression. The polymorphism is a 68 bp sequence containing an atypical AP-1 site and repeated from 1 to 4 times in particular alleles. EMSA experiments indicated biological activity of atypical AP-1 site (TGACTTA) of 68 bp sequence (103). Chloramphenicol acetyltransferase (CAT) assays using neuroblastoma NG108-15 cells showed no differences in basal promoter activity between particular promoter polymorphs. On the other hand, TPA stimulation activated promoters containing triplicate and quadruplicate 68 bp sequences by 47% and 53% respectively, but not the other two promoter variants (103). A population-based association study of 118 heroin addicts and 111 unaffected individuals showed no significant differences in gene frequencies and genotype distribution between both groups (103). Another study examining allelic frequencies and genotype distribution of the polymorphism involved 61 individuals with cocaine dependence, 21 with cocaine abuse and 91 with no history of any substance dependence or abuse. It showed that the subjects with *PDYN* alleles composed of promoter regions containing triplicate or quadruplicate 68 bp polymorphic sequences have lower susceptibility to develop cocaine dependence or abuse (104). Considering the importance of a link between dopamine, drugs of abuse and PDYN it would be of great interest to gather more definitive evidence for a role of AP-1 in driving the *PDYN* expression.

### 5.2.6. Tyrosine hydroxylase (*TH, TYH*)

TH is the catecholamine biosynthesis rate-limiting enzyme expressed in many brain structures. AP-1 binds to the TH-"Fat Specific Element" (TH-FSE)

TGATTCAGAGGCA in the *TH* promoter. TH-FSE contains homology to TRE (105, 106). TH-FSE mediates both induction and repression of the *TH* promoter by Fos proteins in PC12 cells (107). In the rat pheochromocytoma PC12 and PC8b cell lines, TH-FSE participates strongly in basal *TH* promoter transcriptional activity and is necessary for NGF-induced *TH* expression (105, 108). Adult transgenic mice bearing lacZ or HSV1-tk reporter linked to the containing mutated AP-1 site 5.3kb fragment of 5' *TH* flanking sequence have abolished TH expression in catecholaminergic structures such as olfactory bulbs (periglomerular cells), hypothalamus (arcuate nucleus, paraventricular and periventricular nuclei), substantia nigra and ventral tegmental area, locus coeruleus, and adrenal medulla (109). On the other hand, the TH expression in the transgenic mouse embryos was maintained. Thus, AP-1 motif appears to be essential for basal *TH* expression in adult tissues *in vivo*, whereas different TF binding elements are engaged in *TH* regulation during development.

*TH* expression in the rodent olfactory bulb displays activity dependence. After nasal closure, TH expression is downregulated. At the same time there is a decrease in FosB levels showed by Western blotting. Olfactory bulb immunohistochemical data show that after odor-deprivation c-Fos and FosB protein downregulations parallel decrease in TH expression. It have also been showed that *c-fos* mRNA and protein partially colocalized with TH mRNA and protein in the glomerular layer of olfactory bulb (18). Furthermore, a subpopulation of FosB immunoreactive cells in the glomerular layer also express TH (18). In contrast, nasal closure did not influence CREB and JunD expression in the olfactory bulb. In EMSA experiments odor-deprived mice had reduced TH-AP-1 binding in comparison with the control animals (18). Supershift assays revealed strong presence of FosB and JunD (the only Jun protein studied) in AP-1 complexes of control mice and reduced presence in odor-deprived mice. c-Fos, CREB and CREM were absent in TH-AP-1 site of both studied groups. In contrast, TH-CRE binding activity was composed of CREB, CREM, FosB, and to lesser extend JunD, but not c-Fos. The data suggests that CRE motif plays minor role as compared to TRE motif in regulation of *TH* expression in the mouse olfactory bulb. Study using three lines of transgenic mice carrying a construct with 8.9kb fragment of *TH* promoter driving a *lacZ* reporter gene expression with a mutant TH-AP-1 site demonstrated that this element confers olfactory bulb dopamine-specific *TH* expression regulation (110). β-gal activity measured in olfactory bulb homogenates from mutant mice was from 4- to 15-fold lower. The mice had no transgene expression in the mitral cell layer and significantly reduced in the periglomerular region of the olfactory bulb, while similar transgenic mice with wild type promoter showed there strong transgene expression.

Lithium cation is one of the drugs used for the treatment of manic-depressive illness (MDI). There is a bulk of evidence for the involvement of the noradrenergic and dopaminergic systems in the pathophysiology of MDI (111). At therapeutic concentrations, lithium regulates gene expression by induction of AP-1 DNA binding activity (112, 113). Lithium treatment of male Wistar rats leads to the increase of TH expression in frontal cortex, striatum and hippocampus shown by western blot analysis (111).

During an *in vitro* development of striatal neurons there is a stage at which they can be transdifferentiated from GABAergic to TH expressing /dopamine-producing cells by synergistic action of acidic fibroblast growth factor (aFGF), and dopamine or protein kinase A (e.g. isobutylmethylxanthine (IBMX) plus forskolin) or protein kinase C (e.g. TPA) activators. The signals merge mainly onto mitogen-activated protein kinase (MAPK) and activate it. Combined aFGF *TH* induction by addition of aFGF, dopamine, IBMX, forskolin and TPA to cultures of E14 rat striatal neurons changes a composition of the AP-1 complexes bound to TH-AP-1 site (14). EMSA supershift analysis revealed that TH-AP-1 site is occupied by c-Jun, JunD and FosB in uninduced E14 rat striatal neurons. There is no c-Fos present in TH-AP-1 despite noticeable c-Fos expression confirmed by Western blot analysis. One hour after the stimulation, JunD and FosB binding strongly increased whereas c-Jun remained constant, furthermore two additional AP-1 complexes were formed which supershifted with the anti-c-Fos antibody. These additional complexes were almost absent at 6h after stimulation. At this time point JunD and FosB bindings were still elevated and c-Jun one was constant. AP-1 at 1h and 6h and in unstimulated cells did not contain Fra-1, Fra-2 and JunB. c-Jun and JunD western blot expression levels were constant during time course, so changes in phosphorylation status possibly are responsible for enhanced JunD binding to TH-AP-1 site. Neither aFGF nor dopamine nor IBMX/forskolin nor TPA treatment were capable of inducing the *TH* expression, but the action of particular stimuli alone did change AP-1 composition in the characteristic way. Changes in the composition of AP-1 complexes at 1h during aFGF treatment mimics the ones during the combined treatment (aFGF+dopamine+ IBMX+forskolin+TPA) qualitatively. The amount of c-Fos, FosB and JunD in AP-1 during the former stimulation was considerably less than during the combined one. At 1h after dopamine treatment only JunD binding to TH-AP-1 increased. IBMX plus forskolin treatment augmented binding of FosB at 1h, whereas TPA stimulation led to decreased FosB and JunD bindings. Thus aFGF plays central but insufficient role in initiating *TH* expression in striatal neurons in culture. The other stimuli amplify activating effects of aFGF or reverse repressive changes induced by aFGF during *TH* activation. EMSA competition experiment showed that during combined aFGF/dopamine/IBMX/forskolin/TPA treatment of striatal neurons TH-CRE oligonucleotide could partially deplete the TH-AP-1 complexes (14). Then supershift experiments confirmed that CREB/ATF family members contribute to formation of AP-1 complexes in TH-AP-1 site. Unstimulated striatal neurons had ATF-2 and CREM-1 binding activities in TH-AP-1 site which were reduced 1h after combined stimulation. ATF-2 and CREM-1 levels in western blotting were unchanged. There were no detectable CREB-1, CREB-2, ATF-1, ATF-3 and CBP in TH-AP-1 site in stimulated and unstimulated cells. Dopamine or TPA treatment had no effect on CREB/ATF members. aFGF

treatment stimulated ATF-2 and CREM-1 binding, whereas IBMX/forskolin inhibited it. Pretreatment the striatal neurons with PD 98059 (a specific inhibitor of MAPK and its upstream kinase - MAPK kinase) and then stimulation with aFGF/dopamine/IBMX/ forskolin/TPA prevented all changes induced in TH-AP-1 by the stimulation, moreover FosB, JunD and c-Jun bindings observed under unstimulated condition were absent after incubation of the neurons with PD 98059. Taken the above data together, in unstimulated striatal neurons AP-1 in TH-AP-1 site consists mainly of c-Jun/c-Jun, JunD/JunD, c-Jun/JunD which show low binding activity and of c-Jun/FosB and JunD/FosB with high binding activity. After treatment with aFGF/dopamine/IBMX/forskolin/TPA and *TH* induction the predominant complexes are potent transactivators and DNA binders JunD/c-Fos and JunD/FosB, and abundance of repressive complexes JunD/ATF-2 and CREM-1/CREM-1 is reduced.

The multiple studies showing *TH* expression to be AP-1 dependent in the brain as well as other tissues and cells in culture appear to provide particularly compelling evidence for a role for AP-1 in this case. However, the variety of methods employed is still far from exhaustive, especially as far as the brain is concerned.

### 5.3. Genes encoding the neurotrophins, and cytokines

#### 5.3.1. Fas Ligand

AP-1 regulated transcription of Fas ligand (*FasL, CD95-L, APO-1*) and Fas genes is well documented outside CNS. Recently, AP-1-driven regulation of *FasL* has been shown in the mouse brain cortex (114). Using extracts from mice cortex and oligonucleotides corresponding to AP-1 consensus as well as to three fragments of *FasL* promoter containing computer-indicated, potential TRE elements (–282/–252, –233/–215 and –119/–103 fragments) Ishibashi *et al.* (114) performed EMSA assay. EMSA patterns revealed strongly increased protein complex formation with all 4 sequences in extracts derived from cortex of mice subjected to focal cerebral ischemia/reperfusion using intraluminal suture method, whereas only slight increase in extracts from transgenic mice overexpressing human intracellular glutathione peroxidase (GPx1). These results suggested that AP-1 was regulated by GPx1-sensitive reactive oxygen species and after activation by free radicals stimulates FasL expression. EMSA supershift assay showed presence of c-Fos and c-Jun in complexes formed with AP-1 consensus, –282/–252 and –119/–103 fragments, and ATF-2 together with c-Jun on the –233/–215 fragment. The specificity of the binding was verified in the presence of competitive and noncompetitive oligonucleotides. To obtain more definitive support for the role of AP-1 in control of *FasL* in the brain it would be very important to follow other experimental approaches as described above.

Spatio-temporal evidence suggested that tumor necrosis factor-related apoptosis-inducing ligand (*TRAIL*) and *Fas-L* may be AP-1 targets in the brain during ischemia induced apoptosis. Ischemic injury stimulates a cellular stress response which leads to activation of c-Jun N-terminal kinases/stress-activated protein kinases (JNK/SAPKs). The kinases translocate into the nucleus and phosphorylate c-Jun at Ser63 and Ser73. This results in activation of c-Jun which may upregulate transcription of *TRAIL*, *Fas-L* and *TNF-alpha* in the neurons of the adult brain (115). After ischemia TUNEL-positive brain neurons showed colocalization of phosphorylated c-Jun (but not non-phosphorylated c-Jun) with Fas-L and TRAIL mRNA and protein. Administration of FK506 (immunosuppressant blocking c-Jun phosphorylation), parallel to induction of the ischemia, inhibited Fas-L and TRAIL expression and prevented occurrence of the apoptosis. Similar results were obtained in cultures of neuron-derived neuroblastomas. It would be interesting to see whether these data are matched by the others obtained with more extended scope of methods allowing to study a role of AP-1 in a regulation of the gene expression in the brain.

#### 5.3.2. Nerve growth factor (*NGF*)

Brain NGF expression occurs in an area-specific manner related to inducer (116). Activation of β-adrenergic receptors induces NGF synthesis in the rat cerebral cortex but not in the other brain areas. Excitatory neurotransmitters upregulate NGF mostly in limbic system, i.e., in hippocampus and in the cortex and IL-1 in hippocampus. NGF is apparently involved in development and maintenance of epileptic patterns of neuronal activity and functional neuroplasticity after seizures. It induces axonal sprouting, neurotransmitter synthesis, kindling and potentiation synaptic transmission. *NGF* AP-1 binding site is situated in the first intron of the gene (22, 117). It has been suggested that *NGF* can be AP-1 target during global ischemia in gebrils (118). Solution hybridization technique quantification showed that *NGF* transcription is markedly upregulated at 4 and 24 h after hilus-lesion (HL)-induced limbic seizures in rats, with a intervening return to control animal values at 10hr (13). EMSA studies of dentate gyrus nuclear extracts from rats after HL-induced limbic seizures revealed that binding to *NGF* AP-1 was significantly elevated by 4hr, and remained such to at least 24hr time point (13). Additionally, EMSA supershift analysis of the AP-1 composition at 4, 10 and 24hr after HL-induced lesions with antibodies against c-Jun, JunB, JunD, c-Fos, FosB, Fra2 showed that JunB presence in AP-1 complexes was correlated with decreased *NGF* expression, whereas JunD with *NGF* upregulation. c-Jun was not detected, but because the first studied time point was 4 h, so it is possible that c-Jun (P-c-Jun?) composes AP-1 complexes bound to the *NGF* TRE earlier. Furthermore, Gall and Elliott did not detect Fra-2 presence, but they observed AP-1 containing c-Fos and FosB (13). c-Fos was abundant in AP-1 at early time point and then subsided, FosB acted inversely.

Induction of NGF expression in the rat cerebral cortex by stimulation of β-adrenergic receptors with their agonist clenbuterol is AP-1 independent process despite of observed c-Fos and c-Jun mRNA upregulation (116). EMSA using *NGF*-AP-1 motif showed no changes in AP-1 binding in the hippocampus, cerebellum and cerebral cortex after clenbuterol treatment. In reporter assay the clenbuterol-driven NGF induction was not affected by deletion of the AP-1 element of *NGF* promoter in C6-2B glioma cells. Similar experiment performed with PC12 cells showed strongly decreased basal promoter activity.

PC12 cells contain C/EBPbeta but not essential for *NGF* promoter activity C/EBPdelta. It suggests that AP-1 may regulate basic *NGF* transcription when C/EBPdelta is not active.

#### 5.3.3. Tumor necrosis factor alpha (*TNF-alpha*)

TNF-alpha is one of the main inflammatory cytokines in the central nervous system. TNF-alpha increases the blood-brain barrier permeability, sometimes even leading to its breakdown. Furthermore, TNF-alpha contributes to inflammation-related neurotoxicity, brain injury and degeneration, for instance during methamphetamine (METH) (119) or cocaine abuse (120). METH-dependent alterations of human brain microvascular endothelial cell (BMEC) redox status lead to activation of redox-responsive TFs, AP-1 and NF-kappaB. It has been shown by EMSA and luciferase assays with wild type as well as AP-1 site and NF-kappaB site mutated *TNF-alpha* promoters that *TNF-alpha* transcription in human BMECs is upregulated by coordinated action of AP-1 and NF-kappaB. Neither AP-1 site-mutated TNF-alpha promoter luciferase construct nor NF-kappaB site mutated one could be transactivated in human BMECs. After METH administration into mice increased AP-1 DNA binding activity was observed in corpus striatum, frontal cortex and hippocampus (the other brain regions were not investigated) (121), and then an upregulation of *TNF-alpha* mRNA. Augmented TNF-alpha protein expression was detected only in the frontal cortex.

#### 5.3.4. Monocyte chemoattractant protein-1 (*MCP-1*)

MCP-1 is a chemokine implicated in the pathogenesis of HIV-associated dementia. It stimulates chemotaxis and transmigration of inflammatory cells as well as upregulates cytokines and adhesion molecules. *MCP-1* promoter contains TRE (122), and AP-1-driven transcriptional regulation of *MCP-1* has been reported for IL-1beta-stimulated human endothelial cells (123). Tat is a *trans*-activating non-structural HIV nuclear regulatory protein which markedly increases cellular oxidative stress and can break down the blood-brain barrier. $Tat_{1-72}$ injected into mouse hippocampus *in vivo* strongly increased MCP-1 expression in BMECs. It was showed by EMSA and reporter gene assay that $Tat_{1-72}$ stimulates AP-1 site DNA binding activity and AP-1 transactivation in cultures of the BMEC. EMSA supershifts identified c-Fos and c-Jun in AP-1 site of the $Tat_{1-72}$ stimulated *MCP-1* in BMECs (124).

### 5.4. Other genes

Cyclin-dependent kinase 5 gene (*Cdk5*) was pulled down as the AP-1 target in DNA array analysis of striatum mRNA from transgenic mice inducibly expressing DeltaFosB (125). In the striatum of the transgenic mice and chronically cocaine treated rats there was an increase in *Cdk5* and *p35* (neuron-specific Cdk5-activating cofactor; another putative AP-1 target gene) message, protein and activity. Moreover, striatal injection of Cdk5 inhibitors (roscovitine and olomoucine) potentiated behavioral effects of chronic cocaine administration. This has indicated that observed Cdk5 upregulation was a compensatory adaptation for chronic cocaine exposure constituting negative feedback loop trying to restore homeostasis.

## 6. PERSPECTIVE

In this review we have attempted to collect examples of genes for which evidence of AP-1 regulation in the brain has been particularly strong or just strongly debated. The careful survey of the literature shows that despite claims of a number of AP-1 dependent genes, the supporting data for such a regulation in the brain has been in fact very limited. We have included in our review only those whose AP-1 regulation was supported by meaningful evidence derived from the *in vivo* studies. Purposely, we have left out a number of very interesting genes, whose AP-1-driven expression in the brain was shown mainly, if not exclusively in *in vitro* experiments. To name just some of them we can list: glial fibrillary acidic protein (*GFAP*) (85, 126, 127), urokinase-type plasminogen activator (*uPA*) (128, 129), plasminogen activator inhibitor-1 (*PAI-1*) (128), $alpha_1$-antichymotrypsin (*ACT*) (130), amyloid precursor protein (*APP*) (131), proopiomelanocortin (*POMC*) (132-135), thyrotropin-releasing hormone (*TRH*) (136, 137), neuropeptide Y (*NPY*) (138, 139, 140), glutamate receptor 6 (*GluR6*) (141), galanin (*GAL*) (142, 143, 144), inducible nitric oxide synthase (*iNOS*) (145, 146, 147), tyrosine kinase receptor for scatter factor/hepatocyte growth factor (*c-met, HGFR*) (148), brain-derived neurotrophic factor (*BDNF*) (141), vascular endothelial growth factor (*VEGF*) (149), neurotensin/neuromedin N (*NT/N*) (150), Bcl-2-interacting mediator of cell death *(Bim)* (118, 151), *c-jun* (67), *fosB* (141), nuclear factor-kappaB p105 (*NF-kappaB p105*) (152).

Interestingly, the potentially AP-1-driven genes could be grouped in just a handful of categories. Thus, it remains as an attractive possibility that AP-1 controls a defined subset of neuronal and glial responses in the brain. It has, however, to be noted that elaborated evidence of AP-1 dependence is available only for a very limited number of genes in the brain.

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