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## Transcriptional control of maladaptive and protective responses in alcoholics: a role of the NF- $\kappa$ B system

Tatjana Yakovleva<sup>1</sup>, Igor Bazov<sup>1</sup>, Hiroyuki Watanabe<sup>1</sup>, Kurt F. Hauser<sup>2</sup>, and Georgy Bakalkin<sup>1,3</sup>

<sup>1</sup>Division of Biological Research on Drug Dependence, Department of Pharmaceutical Biosciences, Uppsala University, Uppsala, Sweden

<sup>2</sup>Department of Pharmacology & Toxicology, and Institute for Drug and Alcohol Studies, Virginia Commonwealth University School of Medicine, Richmond, Virginia 23298, USA

### Abstract

Alcohol dependence and associated cognitive impairment appear to result from maladaptive neuroplasticity in response to chronic alcohol consumption, neuroinflammation and neurodegeneration. The inherent stability of behavioral alterations associated with the addicted state suggests that transcriptional and epigenetic mechanisms are operative. NF- $\kappa$ B transcription factors are regulators of synaptic plasticity and inflammation, and responsive to a variety of stimuli including alcohol. These factors are abundant in the brain where they have diverse functions that depend on the composition of the NF- $\kappa$ B complex and cellular context. In neuron cell bodies, NF- $\kappa$ B is constitutively active, and involved in neuronal injury and neuroprotection. However, at the synapse, NF- $\kappa$ B is present in a latent form and upon activation is transported to the cell nucleus. In glia, NF- $\kappa$ B is inducible and regulates inflammatory processes that exacerbate alcohol-induced neurodegeneration. Animal studies demonstrate that acute alcohol exposure transiently activates NF- $\kappa$ B, which induces neuroinflammatory responses and neurodegeneration. Postmortem studies of brains of human alcoholics suggest that repeated cycles of alcohol consumption and withdrawal cause adaptive changes in the NF- $\kappa$ B system that may permit the system to better tolerate excessive stimulation. This type of tolerance, ensuring a low degree of responsiveness to applied stimuli, apparently differs from that in the immune system, and may represent a compensatory response that protects brain cells against alcohol neurotoxicity. This view is supported by findings showing preferential downregulation of pro-apoptotic gene expression in the affected brain areas in human alcoholics. Although further verification is needed, we speculate that NF- $\kappa$ B-driven neuroinflammation and disruption to neuroplasticity play a significant role in regulating alcohol dependence and cognitive impairment.

### Keywords

alcoholism; dependence; addiction; neuroinflammation; neuroplasticity; gene transcription; transcription factors; NF- $\kappa$ B; p50; human brain

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<sup>3</sup>To whom correspondence may be addressed: Georgy Bakalkin, Division of Biological Research on Drug Dependence, Department of Pharmaceutical Biosciences, Uppsala University, Box 591, 751 24 Uppsala, Sweden, Georgy.Bakalkin@farmbio.uu.se, Phone: (+46) 18 471 5050, Fax: (+046) 18-50 19 20.

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## 1. Introduction

Although the mechanisms underlying alcoholism remain to be elucidated, the molecular hypothesis postulates that alcohol abuse and dependence result from alterations in gene expression underlying neuroadaptations to chronic alcohol consumption. Molecular adaptations in the nucleus accumbens (NAc), ventral tegmental area, amygdala and dorsolateral prefrontal cortex (dl-PFC) (Di Chiara et al., 2004; Fadda and Rossetti, 1998; Koob, 2003; Nestler, 2005) have been implicated in the behavioral changes such as craving and relapse. Alcohol abuse also causes deficits in perceptual-motor skills, visual-spatial functions, abstraction and problem solving (Bowden and McCarter, 1993; Parsons and Nixon, 1993; Ratti et al., 2002; Schmidt et al., 2005). These impairments may be related to alcohol-induced damage to the dl-PFC and hippocampus (Beatty et al., 1996; Crews et al., 2005; Pfefferbaum et al., 2001; Sullivan and Pfefferbaum, 2005). White matter and cell loss in the dl-PFC and orbitofrontal cortex (OFC), and the loss of hippocampal volume and shrinkage of hippocampal neurons are characteristic of the maladaptive response (Agartz et al., 1999; Aschner and Allen, 2000; Harper et al., 1985; Jensen and Pakkenberg, 1993; Kril et al., 1997; Kril and Harper, 1989; Miguel-Hidalgo et al., 2006).

Work over the past 20 years has provided evidence for a role of several transcription factors (TFs) in regulation of gene expression underlying substance addiction. Prominent examples include cAMP-response element-binding protein, the glucocorticoid receptor,  $\Delta$ FosB (a Fos family protein), and NF- $\kappa$ B (Nuclear Factor  $\kappa$ -light-chain-enhancer of activated B cells) (Ang et al., 2001; Carlezon et al., 2005; Deroche-Gamonet et al., 2003; Green et al., 2006; Green et al., 2008; Mackler et al., 2000; Russo et al., 2009).

The NF- $\kappa$ B family of TFs (Fig. 1) is best studied for their critical role in the immune function and inflammation (Chen and Greene, 2004; Lin and Karin, 2007). In the brain, NF- $\kappa$ B regulates synaptic plasticity and memory, neuroinflammation, neuronal survival and death (Mattson, 2005; Meffert and Baltimore, 2005; Meffert et al., 2003; O'Neill and Kaltschmidt, 1997). Several findings have implicated NF- $\kappa$ B as mediator of neurotoxic or neuroplastic effects induced by addictive drugs. For example, the NF- $\kappa$ B system has been suggested to mediate the neurotoxic effects of amphetamine derivatives in striatum (Asanuma and Cadet, 1998), and levels of individual NF- $\kappa$ B subunits in the nucleus accumbens (NAc) were found to be altered by chronic cocaine exposure (Ang et al., 2001). A role for NF- $\kappa$ B-induced transcriptional changes has been proposed in the regulation of structural and behavioral plasticity to cocaine in the NAc (Russo et al., 2009). Cocaine-induced NF- $\kappa$ B-dependent gene transcription is speculated to mediate the rewarding effects of cocaine, since inhibiting NF- $\kappa$ B blocks its rewarding effects. Activation of NF- $\kappa$ B is accompanied by elevations in the number of dendritic spines on NAc neurons (Russo et al., 2009).

The NF- $\kappa$ B family of transcription factors may play a unique role in alcoholism due to their polyfunctionality, which is associated with their ability to respond to a variety of physiological and pathogenic extracellular signals including stimulation by growth factors, inflammatory intermediates, and glutamate excitotoxicity. In turn, the NF- $\kappa$ B-mediated response is highly contextual depending on the cell type in which this factor is expressed, the constellation of NF- $\kappa$ B/Rel homo- and heterodimers, and on co-factors involved in the epigenetic and/or transcriptional control of NF- $\kappa$ B function. This review focuses on the role of NF- $\kappa$ B in neuronal plasticity, neuroinflammation and neurodegeneration; all of which are relevant for alcohol dependence, abuse, and associated cognitive impairment. Special emphasis is given to the molecular analysis of the NF- $\kappa$ B system in postmortem brains of human alcoholics. This approach is especially important since no single transcription factor from the human CNS has been systematically studied in relation to an addictive disorder.

Molecular findings from human autopsy samples support the hypothesis that the NF- $\kappa$ B system is downregulated in brain areas affected by alcohol consumption and withdrawal. Moreover, the downregulation of the NF- $\kappa$ B system appears to be an adaptive mechanism that protects the remaining neurons against alcohol neurotoxicity and neuroinflammation.

## 2. The NF- $\kappa$ B/Rel family of transcription factors in the brain

Transcription factors of the NF- $\kappa$ B/Rel family are inducible proteins that regulate the expression of genes involved in inflammation, immune responses, and cell survival (Kaltschmidt et al., 2005; Li and Verma, 2002; Mattson, 2005; Yamamoto and Gaynor, 2001). These factors are homo- or heterodimers of p65 (Rel A), p50 and other proteins of the NF- $\kappa$ B/Rel family (Fig. 1). The p65/p50 heterodimer (NF- $\kappa$ B) generally activates gene transcription, while the p50 homodimer represses it (Guan et al., 2005; Li et al., 1994). In most cell types, NF- $\kappa$ B is sequestered in the cytoplasm as part of a complex with inhibitor I $\kappa$ B proteins. Nuclear translocation of NF- $\kappa$ B is induced by multiple extracellular stimuli that trigger activation of an I $\kappa$ B kinase (IKK) complex, which phosphorylates the I $\kappa$ Bs leading to their ubiquitination and proteasomal degradation. The released NF- $\kappa$ B migrates to the nucleus to act as a transcription factor. The IKK complex contains the two kinases IKK $\alpha$  and IKK $\beta$  and the regulatory subunit NEMO/IKK $\gamma$ , and functions as integrator of signals regulating NF- $\kappa$ B activity. Transactivating capacity is also regulated in the cell nuclei through phosphorylation of p65 and p50 by IKK $\beta$  and other kinases, and p65 by lysine (37/218/221) methylation (Ea and Baltimore, 2009; Ghosh and Karin, 2002; Lu et al., 2010; Moynagh, 2005; Yamamoto and Gaynor, 2004).

The NF- $\kappa$ B TFs had been also identified in the brain where their basal expression levels are higher compared to most peripheral tissues (Bakalkin et al., 1993). The p50/p65 heterodimeric variant of NF- $\kappa$ B is the major  $\kappa$ B-binding complex detected in the adult rodent brain (Table 1) (Bakalkin et al., 1993; Kaltschmidt et al., 1993; Meffert et al., 2003; Schmidt-Ullrich et al., 1996) (Fig. 2). In contrast, three complexes, cRel/p65, p50/p65, and p50 homodimers are present in the developing rat brain (Bakalkin et al., 1993). In the adult brain, p65 and p50 NF- $\kappa$ B subunits are abundantly expressed in neurons. A substantial fraction of NF- $\kappa$ B (existing as p50/p65 heterodimers) is located in the cell nucleus and is constitutively active (Kaltschmidt et al., 2005; Kaltschmidt et al., 1993; Meffert and Baltimore, 2005). Constitutive NF- $\kappa$ B activity in glutamatergic neurons of the hippocampus and cerebral cortex (Table 1) (Kaltschmidt et al., 1997; Kaltschmidt et al., 2005; Kaltschmidt et al., 1993) can be suppressed by *N*-methyl-D-aspartate, and to a lesser extent AMPA, glutamate receptor antagonists, as well as L-type Ca<sup>2+</sup> channel blockers (Lilienbaum and Israel, 2003; Meffert et al., 2003). Inducible NF- $\kappa$ B is detected in synapses, while glutamatergic stimulation activates retrograde transport of p65 protein from synapses to the cell nucleus (Kaltschmidt et al., 1993; Meberg et al., 1996; Meffert et al., 2003). Thus, NF- $\kappa$ B is involved in translation of short-lasting synaptic signals to persistent changes in gene expression (Meffert et al., 2003; Wellmann et al., 2001). The phosphorylated forms of I $\kappa$ B $\alpha$  and activated IKK were found within the axon initial segment, the site where action potentials are generated (Schultz et al., 2006), suggesting that NF- $\kappa$ B activation is associated with processing of neuronal information at this site.

The dominant view that NF- $\kappa$ B is constitutively active in neurons has been challenged by Barger and collaborators (Table 1) (for review, see (Mao et al., 2009)). Analysis of NF- $\kappa$ B and NF- $\kappa$ B-driven transcription in highly purified murine neuronal cultures prompted these investigators to conclude that neurons are incapable to activating the  $\kappa$ B element with the traditional Rel-family subunits that comprise NF- $\kappa$ B. They alternatively propose that the activation of  $\kappa$ B-mediated transcription in neurons involves SP TFs (SP3 and SP4), while the NF- $\kappa$ B-dependent  $\kappa$ B activation that is detected within the brain occurs within glia. *In*

*in vivo* analysis of interactions of the NF- $\kappa$ B TFs with their target neuronal and glial genes using chromatin immunoprecipitation assay might help to resolve the contradiction between the two sets of experimental observations (Kaltschmidt et al., 2005; Kaltschmidt et al., 1993; Mao et al., 2009; Meffert and Baltimore, 2005).

NF- $\kappa$ B mediated alterations in gene transcription may be essential for synaptic plasticity underlying learning and memory formation (Grilli and Memo, 1999; Guerrini et al., 1995; Kaltschmidt et al., 2006; Kaltschmidt et al., 2005; Mattson, 2005; Meberg et al., 1996; Meffert and Baltimore, 2005; Meffert et al., 2003; O'Neill and Kaltschmidt, 1997; O'Riordan et al., 2006). Activation of this TF is apparently required for development of long-term potentiation (LTP) and long-term depression (LTD) (O'Riordan et al., 2006), fear conditioning (Yeh et al., 2004), and spatial memory (Meffert et al., 2003; O'Mahony et al., 2006). In a hierarchical transcriptional network, NF- $\kappa$ B regulates, through its effects on expression of PKA, phosphorylation of CREB that is essential for transduction of synaptic signals into the cell nucleus and eventually for learning and memory (Kaltschmidt et al., 2006). NF- $\kappa$ B has been also identified as regulator of neuronal morphology, including increases in dendritic arborization and axonal outgrowth that are thought to be important in learning and memory acquisition (Gutierrez et al., 2005).

A role in synaptic plasticity was demonstrated in experiments with several lines of genetically modified mice (reviewed in (Kaltschmidt and Kaltschmidt, 2009)). Mice deficient in tumor necrosis factor receptor superfamily member 1A (TNFR1;  $-/-$ ) or p65 ( $-/-$ ); and conditional knockouts with neuronal promoter-specific ablation of NF- $\kappa$ B predominantly in glutamatergic neurons in the basal forebrain (CamKII-tTA/tetO $\kappa$ B- $\alpha$ ) by expressing the super-repressor I $\kappa$ B- $\alpha$ , all demonstrated impairment in cognitive tests including the radial arm maze or water maze, as well as functional deficits in LTP or LTD (Fridmacher et al., 2003; Kaltschmidt et al., 2006). On the other hand, preferential expression of super-repressor I $\kappa$ B- $\alpha$  (Prion promoter-driven tTA/tetO super-repressor I $\kappa$ B- $\alpha$ ) in inhibitory GABAergic interneurons resulted in enhanced spatial learning and memory (Kaltschmidt et al., 2006; O'Mahony et al., 2006). Thus, the change in balance in the excitatory versus inhibitory activity, respectively, of glutamatergic and GABAergic neurons, caused by the overexpression of the NF- $\kappa$ B super-repressor I $\kappa$ B- $\alpha$  in GABA neurons, improves spatial learning and memory.

A number of studies provide evidence that NF- $\kappa$ B activation protects neurons against amyloid  $\beta$  peptide toxicity (Barger et al., 1995) and excitotoxic or oxidative stress (Mattson, 2005; Sarnico et al., 2009). Overexpression of p65 protected cortical neurons against apoptotic cell death, while I $\kappa$ B super repressor or dominant inhibitory NF- $\kappa$ B - inducing kinase (NIK) reduced the survival of cortical neurons (Bhakar et al., 2002).

The role of NF- $\kappa$ B in glia (we use the term "glia" to collectively refer to astroglia and microglia in this review) has been mainly studied in relation to brain injury (for details, see reviews by (Block et al., 2007; Kaltschmidt and Kaltschmidt, 2009), which is not in the focus of this paper. Briefly, in glia of naïve animals NF- $\kappa$ B is present in a latent form (Table 1) (Bhakar et al., 2002; Schmidt-Ullrich et al., 1996) but may be activated under pathological conditions such as exposure to amyloid  $\beta$  peptide (A $\beta$ ) leading to the production of nitric oxide (Akama et al., 1998) or exposure to HIV-1 Tat (El-Hage et al., 2008). Responses to injury are triggered by endogenous ligands for Toll-like receptors (TLR) and TLR signaling is mediated through the NF- $\kappa$ B (Akira and Takeda, 2004). Inhibition of astroglial NF- $\kappa$ B signaling leads to reduced chemokine expression and leukocyte entry into the injured CNS (Brambilla et al., 2005; Khoroshii et al., 2008) indicating the regulatory effect of astrocytes on immune response (Farina et al., 2007). Microgliosis is a common phenomenon in neurodegenerative disorders and NF- $\kappa$ B plays a

central role in microglia activation. Microglial activation is underscored by the release reactive oxygen species and the production of proinflammatory cytokines that cause secondary neurotoxicity. This activation involves NF- $\kappa$ B, which is a key transcriptional regulator of proinflammatory cytokines such as TNF- $\alpha$ , IL-1 $\beta$ , and interferon- $\gamma$  (Block et al., 2007).

### 3. Alcohol addiction and neurotoxicity

#### 3.1. Animal studies

Acute or short-term ethanol administration has been demonstrated to activate the NF- $\kappa$ B system in the brain, and this in turn triggers the expression of TNF- $\alpha$ , as well as other proinflammatory cytokines and NF- $\kappa$ B-regulated genes (Crews et al., 2006b; Rulten et al., 2006; Ward et al., 1996; Zou and Crews, 2006, 2010). In these experiments, rats received a single intraperitoneal (i.p.) 2 g/kg ethanol dose (Ward et al., 1996), or intragastric administration of ethanol, 8–12 g/kg/day, 3 times per day for 4 days (Crews et al., 2006b; Zou and Crews, 2006, 2010); or mice were given a single i.p. 2.5 g/kg ethanol dose (Rulten et al., 2006). Analysis of whole mouse brain transcriptomes by DNA microarrays consistently identify the NF- $\kappa$ B pathway as involved in the acute response to ethanol (Rulten et al., 2006). While both NF- $\kappa$ B function and RelA expression were upregulated shortly after ethanol administration (Rulten et al., 2006), the increase appears to be transient since it is not sustained following sub-chronic ethanol treatment (a 9% ethanol-containing liquid diet for 15 days) (Mittal et al., 1999).

Although cellular and molecular mechanisms by which alcohol causes brain damage are not fully understood, numerous studies suggest a role of inflammatory processes (Altura et al., 2002; Blanco et al., 2005; Davis and Syapin, 2004; Lee et al., 2004; Martinez et al., 1992; Minambres et al., 2006; Nelson et al., 1989; Valles et al., 2004). At the transcription level, inflammation is primarily regulated by NF- $\kappa$ B and related factors in a variety of cells and tissues. The hallmark of neuroinflammation is the activation of glia and the production of cytokines and inflammatory mediators that can trigger neuronal damage (Minghetti, 2005; Ubogu et al., 2006). Sub-chronic alcohol administration upregulates inflammatory mediators in the rat brain, which includes key neuroinflammatory signaling processes in astrocytes (Altura et al., 2002; Blanco et al., 2005). These processes are initiated by signaling through TLR4/type I IL-1R, which plays roles in the innate host defense against microbial infections, inflammation and injury. This is followed by activation of NF- $\kappa$ B and the upregulation of inducible NO synthase and cyclooxygenase-2 expression. In glial cells, TLR4 is critical for an ethanol-induced inflammatory response (Alfonso-Loeches et al., 2010). Inactivation of TLR4 by siRNA or gene deletion abolished ethanol-induced activation of NF- $\kappa$ B and microtubule-associated protein kinase pathways, and the production of inflammatory mediators in astrocytes. Whereas ethanol exposure upregulates the levels of microglial (CD11b) and astrocyte (glial fibrillary acidic protein) markers, inducible COX-2, interleukin (IL)-1 $\beta$ , IL-6 and TNF- $\alpha$  in the cerebral cortex of wild-type mice, TLR4 ablation protects against ethanol-induced glial activation, induction of inflammatory mediators, and apoptosis. These findings support of the concept that a TLR4-driven NF- $\kappa$ B-mediated response underlies the neuroinflammation, brain injury, and neurodegeneration caused by ethanol exposure.

Increases in NF- $\kappa$ B DNA binding activity induced by ethanol treatment correlate with the increased expression of proinflammatory genes in hippocampal-entorhinal cortex slice cultures (Zou and Crews, 2010). Blockade of NF- $\kappa$ B activation by p65 siRNA or the antioxidant butylated hydroxytoluene, reduces the induction of proinflammatory TNF- $\alpha$ , MCP-1, IL-1 $\beta$ , protease TACE, tissue plasminogen activator (tPA) and inducible nitric oxide synthase by ethanol. In an *in vivo* pharmacological study, butylated hydroxytoluene

treatment prevented both damage to corticolimbic brain areas and reductions in neurogenesis induced by binge-like ethanol administration (Crews et al., 2006a; Crews et al., 2006b). Ethanol treatment also increased the DNA binding activity of NF- $\kappa$ B, COX2 expression, and microglial activation, while antioxidant administration caused the opposite effects in animals. This correlative evidence supports the hypothesis that binge ethanol-induced brain damage involves a neuroinflammatory mechanism that is under control of NF- $\kappa$ B regulated transcription.

Besides brain-derived proinflammatory factors, neuroinflammation can be promoted by serum-derived factors, such as TNF- $\alpha$  and other cytokines, originating from increases in systemic and hepatic inflammation caused by heavy alcohol drinking (Crews et al., 2006b; Wang et al., 2010). Serum cytokines may enter the brain where they activate microglia and astroglia. TNF- $\alpha$  acting through the NF- $\kappa$ B system can inhibit glutamate transporters. Consequently, the excess glutamate, which may attain excitotoxic levels, reportedly contributes to increased drinking and neurodegeneration (Crews et al., 2006b). Altogether, these observations suggest that alcohol effects on immune and inflammatory responses in the brain are mediated through NF- $\kappa$ B, and NF- $\kappa$ B-controlled genes.

In contrast to adult animals, NF- $\kappa$ B may be neuroprotective in the developing brain (Bonthius et al., 2009). Alcohol-induced damage to the developing brain leads to fetal alcohol syndrome; however, mature neurons while maturation of neurons results in acquisition of resistance to ethanol. This process apparently involves the protective NO-cGMP-cyclic GMP-dependent protein kinase pathway G (NO-cGMP-PKG). NF- $\kappa$ B was found as a downstream effector through which the NO-cGMP-PKG pathway signals its neuroprotective effects against alcohol in immature cerebellar granule neurons in culture. Thus, NF- $\kappa$ B – regulated gene transcription may be required for the acquisition of alcohol resistance by maturing neurons.

A role for the NF- $\kappa$ B system in the regulation of ethanol consumption is underscored by meta-analysis of the transcriptome of experimental animals (Mulligan et al., 2006). A comparison of alcohol-preferring and non-preferring mice showed a marked overrepresentation of gene networks associated with NF- $\kappa$ B activation in alcohol-preferring mice suggesting that NF- $\kappa$ B regulates a variety of transcripts involved in establishing a high level of voluntary alcohol drinking.

### 3.2. Human studies

**3.2.1. Association of NFKB1 / p50 with alcoholism**—Human genetic and postmortem molecular studies highlight the importance of NF- $\kappa$ B in alcohol dependence and toxicity (Edenberg et al., 2008; Flatscher-Bader et al., 2005; Okvist et al., 2007). A seminal finding is that polymorphisms in the nuclear factor of  $\kappa$ -light polypeptide gene enhancer in B-cells 1/the p50 protein precursor gene (*NFKB1*) are highly correlated with an increased risk for alcoholism in a family-based association study (Edenberg et al., 2008). *NFKB1* is located on chromosome 4q, which is linked to alcohol dependence, and the association is much stronger in individuals with an early onset of alcoholism. The findings prompt speculation that there may be other important genetic variants that affect expression of *NFKB1* and NF- $\kappa$ B/p50-regulated genes and influence alcohol dependence and toxicity in humans.

**3.2.2. NF- $\kappa$ B in the brain of human alcoholics**—Alcohol abuse and dependence are associated with widespread changes in gene expression in several brain areas including the dl-PFC and striatum in humans (Alexander-Kaufman et al., 2006; Flatscher-Bader et al., 2005; Lewohl et al., 2000; Liu et al., 2004). The differentially expressed genes are implicated in immune responsiveness, cell survival, inflammation, and signal transduction.

Pronounced differences have been found in genes involved in myelination, apoptosis and neurogenesis.

Re-programming gene expression in alcoholics is hypothesized to involve TFs of the NF- $\kappa$ B family. This hypothesis was addressed by analyzing postmortem specimens from the dl-PFC, a region that is involved in alcohol dependence and cognitive processes, and displays functional and histopathological deficits in human alcoholics. The motor cortex (MC), which is not engaged in these processes and less affected by alcohol (Kril et al., 1997; Kril and Harper, 1989), was analyzed as a control area (Okvist et al., 2007). The principal findings are that the DNA-binding of NF- $\kappa$ B and p50 homodimer, as well as *RELA* (p65) expression, are downregulated in the dl-PFC of alcoholics (Fig. 3) (Okvist et al., 2007). Importantly, the p50 homodimer is the dominant  $\kappa$ B-binding factor in the human brain while NF- $\kappa$ B predominates over other members of this family in the adult rodent brain (Fig. 2). The stoichiometry between *RELA*, *NFKB1*, and *IKK $\beta$*  mRNA and between respective p65, p50, and *IKK $\beta$*  proteins is also altered. No significant differences between controls and alcoholics were evident in the MC suggesting that NF- $\kappa$ B-mediated adaptive mechanisms differ between brain areas. Downregulation of NF- $\kappa$ B DNA-binding in alcoholics may result from the decrease in *RELA* expression or from alterations in stoichiometry in p65, p50 and *IKK $\beta$*  proteins (Fig. 3). In addition, NF- $\kappa$ B DNA-binding and transactivation functions in alcoholics may be attenuated through alterations in posttranslational modifications of NF- $\kappa$ B proteins. These alterations may include phosphorylation at several sites by the IKK complex, or reversible lysine methylation (37/218/221) (Ea and Baltimore, 2009; Ghosh and Karin, 2002; Lu et al., 2010; Yamamoto and Gaynor, 2004; Zhong et al., 2002). While p50 homodimer-DNA binding was downregulated in alcoholics, no changes in p50 protein levels were evident suggesting the effects of ethanol may be mediated by post-translational modifications (Fig. 3). p50 phosphorylation by protein kinase A and other kinases regulates binding of the p50 homodimer to DNA, which results in transcriptional repression of NF- $\kappa$ B dependent genes (Guan et al., 2005; Li et al., 1994).

Alcohol withdrawal results in neuronal excitation (Fadda and Rossetti, 1998; Grant and Lovinger, 1995; Grant et al., 1990; Mittal et al., 1999) through activation of glutamate receptors and calcium influx, which in turn activate NF- $\kappa$ B (Grilli and Memo, 1999; Guerrini et al., 1995; Kaltschmidt et al., 2005; Mattson, 2005; Meberg et al., 1996; Meffert and Baltimore, 2005; Meffert et al., 2003; O'Neill and Kaltschmidt, 1997). Cycles of alcohol intoxication and withdrawal initially activate NF- $\kappa$ B; however, when repeated chronically for years, may lead to adaptive changes in the NF- $\kappa$ B system such that the system better tolerates excessive stimulation. One of the adaptive processes (Fig. 3) is the downregulation of the DNA-binding activity of the p50 homodimer, a dominant  $\kappa$ B-binding factor (Fig. 2). This may result in a derepression of genes that possess  $\kappa$ B-elements in their promoters. These types of adaptive changes, which limit the amount of responsiveness to chronic stimulation (due to low NF- $\kappa$ B activity) while stabilizing basal  $\kappa$ B-mediated transcription (due to low p50 homodimer activity), could potentially be exploited as compensatory response that protects brain cells against alcohol neurotoxicity.

In the brain, this adaptation may differ mechanistically from that observed in the immune system (Grundstrom et al., 2004; Hajishengallis and Genco, 2004), which is based on the over-expression of the inhibitory p50 subunit (Biswas and Lopez-Collazo, 2009). The immune system has ability to i) downregulate excessive inflammatory reactions that can damage tissues, and to ii) inhibit excessive immune responses involving T cells (Biswas and Lopez-Collazo, 2009; Grundstrom et al., 2004; Hajishengallis and Genco, 2004; Irla et al., 2010). Prior exposure of monocytic cells to LPS results in a transient repression proinflammatory cytokine responsiveness to a subsequent LPS challenge that is termed "endotoxin tolerance". T cell tolerance is characterized by a cessation in cell proliferation

and diminished expression of Th1 cytokines. The NF- $\kappa$ B system is involved in these processes. In particular, the upregulation of the p50 homodimer, which represses NF- $\kappa$ B-dependent transcription, appears to regulate both endotoxin tolerance and T cell tolerance.

Animal studies demonstrate that acute ethanol administration increases NF- $\kappa$ B DNA-binding activity, while three weeks of ethanol treatment downregulates NF- $\kappa$ B (Rulten et al., 2006; Ward et al., 1996). These data and human postmortem observations are in an apparent contradiction. To resolve it, we may propose that the phase of initial activation of the NF- $\kappa$ B system after acute ethanol administration is followed by the chronic phase when the system is downregulated. Data on acute effects of alcohol on NF- $\kappa$ B in human brain are not available. Even if several first episodes of ethanol intake could activate the NF- $\kappa$ B system, mechanisms of this activation and following repression in humans may be different from those in rodents due to differences in the constellation of NF- $\kappa$ B / c-Rel subunits in the brain.

The hypothesis on activation of the protective mechanisms and inhibition of cell death pathways is supported by human molecular findings. Thus, several components of intrinsic apoptotic pathway including activated caspase-3, the key pro-apoptotic protein, along with both pro-apoptotic PDCD8 (apoptosis-inducing factor, AIF) and BID, were found to be decreased in the dl-PFC of alcoholics (Johansson et al., 2009). No differences were found in the motor cortex. Consistently, the up-regulation of several neuroprotective genes was found in the dl-PFC in human alcoholics (Flatscher-Bader et al., 2005; Okvist et al., 2007). It has been demonstrated that activated caspase-3 and AIF play a critical role in the execution of both neuronal apoptotic and necrotic pathways (Niquet et al., 2003; Niquet et al., 2006; Young et al., 2005). Thus downregulation of these two proteins in the PFC of alcoholics may prevent or delay cell death that may occur by apoptosis or necrosis. Adaptations in regulated cell death pathways could limit the extent of alcohol-induced brain damage, thus protecting cognitive and other dl-PFC functions in alcoholics. Such adaptations may be developed after initial activation of cell death pathways resulting in gross loss of gray and white matter during the first years of heavy alcohol drinking. Albeit this hypothesis should be considered with a caution as based on correlative molecular data. Thus, the possibility that molecular changes identified may contribute to the shift from the apoptotic to necrotic pathway of cell death in the dl-PFC cannot be ruled out.

**3.2.3. NF- $\kappa$ B / p50 homodimer – mediated regulation of genes differentially expressed in alcoholics**—The ability to negatively regulate NF- $\kappa$ B target genes is essential for maintaining cellular homeostasis. Not surprisingly, the sustained activation of NF- $\kappa$ B is associated with several inflammatory diseases (Guan et al., 2005; Rao et al., 2010; Smale, 2010; Yamamoto and Gaynor, 2001). The p50 homodimer is important in maintaining homeostasis through its ability to suppress the transactivation activities of NF- $\kappa$ B by occupying the same  $\kappa$ B sites and recruiting corepressor complexes containing histone deacetylases (HDAC) (Hoberg et al., 2006). Following stimulation, NF- $\kappa$ B displaces DNA-bound p50 / HDAC.

Downregulation of DNA-binding of the p50 homodimer and NF- $\kappa$ B in alcoholics may result in changes in expression of genes regulated through  $\kappa$ B elements. This hypothesis was tested using a computational approach that compared the number of genes containing  $\kappa$ B elements between sets of genes downregulated and upregulated in the dl-PFC of alcoholics (Liu et al., 2006). p50 binds to  $\kappa$ B sites as a homodimer and therefore has a slightly higher affinity for symmetric  $\kappa$ B elements compared to NF- $\kappa$ B (Chen-Park et al., 2002; Chen and Greene, 2004), which is reflected in the composition of the  $\kappa$ B matrix of the “p50 homodimer”-subtype present in the JASPAR database (Fig. 4). Analysis of genes differentially expressed in the dl-PFC of alcoholics demonstrated that 58 genes contain one



or more putative  $\kappa$ B binding sites for the p50 homodimer and NF- $\kappa$ B (the “NF- $\kappa$ B”- subtype), and 19 genes contain at least one putative “p50 homodimer”-subtype  $\kappa$ B binding site (Okvist et al., 2007). Computational analysis showed significant differences in the frequency of genes with each “NF- $\kappa$ B”- or “p50 homodimer”- subtype of  $\kappa$ B elements between the up- and down regulated genes and a control set of genes. In particular, the frequency of each binding site subtype in the downregulated genes was lower compared to the upregulated genes and control genes (Fig. 4) (Okvist et al., 2007). Thus, genes with  $\kappa$ B elements were generally upregulated in the dl-PFC in alcoholics. The upregulated genes may be derepressed due to decreased activity of the p50 homodimer, which inhibits transcription and is the dominant  $\kappa$ B binding factor in human brain.

Expression of several NF- $\kappa$ B-driven genes, for example the murine chemokine keratinocyte chemoattractant (KC) may require stabilization of the constitutively unstable mRNA (Hartupee et al., 2008). To date, dependence of the levels of short-lived mRNAs in brain on postmortem interval, RNA integrity number, and brain pH has not been systematically addressed. Because these mRNAs may rapidly degrade during postmortem period, NF- $\kappa$ B dependent genes identified by the genome-wide scan may be limited to abundant and/or “stable” mRNAs that still produce a reliable microarray signal, and, consequently, a number of NF- $\kappa$ B dependent genes may be underestimated in the computational analysis (Okvist et al., 2007).

Downregulation of p50 homodimers may allow the recruitment of NF- $\kappa$ B if this TF is present within the cell in a transcription competent form and in amounts sufficient to activate transcription from  $\kappa$ B elements. Alternatively, the removal of co-repressor complexes targeted to  $\kappa$ B sites by the p50 homodimer could result in derepression of basal gene transcription that involves other transcription factors besides NF- $\kappa$ B. Indeed, NF- $\kappa$ B cooperates with SP1, nuclear receptors, or AP-1 for binding to both overlapping or non-overlapping target DNA elements (Hirano et al., 1998). It was demonstrated that the interaction of SP1 with  $\kappa$ B elements keeps basal expression of NF- $\kappa$ B-dependent genes at the elevated levels; whereas, the p50 homodimer displaces SP1 from these sites and thereby inhibits basal SP1-driven expression (Hirano et al., 1998). In contrast, the downregulation of NF- $\kappa$ B in alcoholics may lead to decreased transcription of genes that are normally activated by NF- $\kappa$ B.

In the human brain,  $\kappa$ B-driven transcription may be regulated not only by competitive binding between transcriptionally active NF- $\kappa$ B and repressive p50 homodimers, but by the modulation of inhibitory and transactivation potential of the p50 homodimer, a dominant  $\kappa$ B-binding complex (Fig. 2). This modulation may be performed through recruitment of several I $\kappa$ B proteins including BCL-3, I $\kappa$ B $\zeta$  and I $\kappa$ BNS, which have been identified as pleiotropic p50 cofactors (reviewed in (Ghosh and Hayden, 2008)). In contrast to other I $\kappa$ B proteins, BCL-3 has a transactivation domain, and in complex with a p50 homodimer may facilitate or inhibit  $\kappa$ B-mediated transcription depending on its posttranscriptional modifications. The formation of transcriptionally active BCL-3 – p50-p50 complexes requires phosphorylation or ubiquitylation of BCL-3 protein. BCL-3 may also stabilize repressive p50 homodimers thereby preventing the binding of transcriptionally active NF- $\kappa$ B. Similarly to BCL-3, I $\kappa$ B $\zeta$  associates with the p50 homodimer, and selectively inhibits or activates  $\kappa$ B-driven transcription. A role of these cofactors in the human brain has not yet been studied.

Bioinformatics searches for NF- $\kappa$ B/p50 homodimer target genes identified nine potential NF- $\kappa$ B/p50 targets, and seven genes associated with the NF- $\kappa$ B/p50 pathway within a set of genes that were differentially expressed in alcoholics (Okvist et al., 2007). The upregulated genes included the death-associated protein 6 (*DAXX*), which is involved in life-or-death

processes and in spatial learning. DAXX acts as an anti-apoptotic regulator through its ability to repress gene transcription activated by NF- $\kappa$ B / RelB (Michaelson and Leder, 2003; Puto and Reed, 2008). Daxx may control epigenetic silencing of RelB by DNA methylation through recruitment of DNA methyl transferase 1 to RelB DNA-target sites resulting in synergistic repression (Puto and Reed, 2008). Several other genes relevant for alcohol dependence and neurotoxicity have also been identified as NF- $\kappa$ B targets. They include K-ras, a regulator of alcohol intake discovered in genome-wide gene expression analysis (Repunte-Canonigo et al., 2010), and the opioid receptor and opioid peptide precursor genes including proenkephalin and prodynorphin, which play a role in addictive and cognitive processes (Bakalkin et al., 1994; Chen et al., 2006; Rattner et al., 1991).

**3.2.4. NF- $\kappa$ B -  $\Delta$ FosB interplay in addicted brain**—It has been proposed that NF- $\kappa$ B may crosstalk with other TFs that regulate addictive behavior. In animal studies, upregulation of  $\Delta$ FosB, a product of the *Fosb* gene, is induced by chronic administration of virtually all drugs of abuse and alcohol. The elevated levels persist for several weeks and  $\Delta$ FosB reportedly can trigger the sustained expression of a variety of genes associated with substance addiction. Accordingly,  $\Delta$ FosB has been hypothesized to have a direct role of in the maintenance of addiction (Ehrlich et al., 2002; Hiroi et al., 1997; Hope et al., 1994; Nye and Nestler, 1996; Pich et al., 1997). NF- $\kappa$ B was identified by microarray screening as a  $\Delta$ FosB target in the NAc after chronic cocaine treatment using  $\Delta$ FosB-overexpressing mice (Ang et al., 2001). By analogy,  $\Delta$ FosB may potentially regulate NF- $\kappa$ B in human alcoholics. However, emerging evidence suggests that this is unlikely:  $\Delta$ FosB is produced at very low levels in human brain and, more importantly,  $\Delta$ FosB does not show any changes in the levels of expression in the brains of human alcoholics (Watanabe et al., 2009). Thus,  $\Delta$ FosB may not be involved in the maintenance of the addictive state, at least not in alcohol dependence in humans.

## 4. Conclusions

A remarkable number of the short-term responses and long-term adaptive changes to alcohol are regulated by the NF- $\kappa$ B family of TFs suggesting that they are pivotal in mediating the response of the brain to ethanol. Not only do NF- $\kappa$ B and p50 homodimers respond to acute and chronic ethanol exposure including activation by glutamate, cytokines and oxidative stress, but these TFs regulate synaptic plasticity, neuronal survival and neuroinflammation - the neurobehavioral substrate(s) underlying alcohol addiction (Grilli and Memo, 1999; Guerrini et al., 1995; Kaltschmidt and Kaltschmidt, 2009; Kaltschmidt et al., 2006; Kaltschmidt et al., 2005; Li and Verma, 2002; Meffert et al., 2003; Moynagh, 2005; O'Riordan et al., 2006). Induction of oxidative stress by alcohol, and alcoholism-associated alterations in the expression of inflammatory, cell survival and myelin genes (Alexander-Kaufman et al., 2006; Farris et al., 2010; Flatscher-Bader et al., 2005; Lewohl et al., 2000; Liu et al., 2004) regulated by NF- $\kappa$ B (Huang et al., 2002; Kaltschmidt et al., 2005; Li and Verma, 2002; Mattson, 2005; Nickols et al., 2003) corroborate this notion.

The importance of NF- $\kappa$ B in regulating the response of the brain to alcohol is supported by animal studies demonstrating that acute ethanol administration activates NF- $\kappa$ B and NF- $\kappa$ B-regulated transcription of TNF- $\alpha$ , proinflammatory cytokines, and other genes in the brain (Crews et al., 2006a; Crews et al., 2006b; Rulten et al., 2006; Ward et al., 1996; Zou and Crews, 2006, 2010). The NF- $\kappa$ B - mediated response may be critical for neuroinflammation and neurodegeneration induced by ethanol exposure in rodents. Cellular and molecular mechanisms involved in these ethanol-dependent NF- $\kappa$ B-driven processes are now under investigation in several laboratories. In contrast, a role of the NF- $\kappa$ B system in the regulation of alcohol consumption and in the cognitive control of alcohol seeking and drinking has attracted minimal attention. The mouse genetic models utilized to study the role

of NF- $\kappa$ B in learning and memory (reviewed in (Kaltschmidt and Kaltschmidt, 2009; Meffert and Baltimore, 2005), have not yet been used in the study of alcoholism.

NF- $\kappa$ B is a prevailing form of  $\kappa$ B binding protein in the adult rodent brain, while in adult human brains the inhibitory p50 homodimer strongly predominates over NF- $\kappa$ B (Fig. 2). The distinction suggests that the regulation of  $\kappa$ B-mediated transcription and neurodegeneration may differ between humans and rodents. This assumption imposes limitations on the extent to which information gained on the role of the NF- $\kappa$ B / p50-mediated transcription in rodent models can be generalized to human neuropsychiatric disorders such as alcohol dependence and associated cognitive impairment. Specifically, these constraints may limit the applicability of animal gene expression data for construction of human molecular models of both alcohol dependence, and transcriptional tolerance to repeated effects of ethanol. It would be essential to compare the role of the  $\kappa$ B-mediated transcription in alcohol-induced neurodegeneration and neuroinflammation in human and rodent brain cells.

Human genetic analysis demonstrated an association of polymorphisms in the *NFKB1* (p50) gene with alcohol dependence (Edenberg et al., 2008), while molecular analysis of human brain identified a downregulation of the NF- $\kappa$ B/p50 homodimer system in the dl-PFC, an area affected by alcohol exposure in human brain (Okvist et al., 2007). These adaptations in NF- $\kappa$ B/p50 may underlie a shift to a new “steady-state” or “allostatic level” (Koob, 2003), caused by persistent alterations in expression of target genes relevant to alcohol-dependent state. Multiple mechanisms may assure the stability of these alterations. Hypothetically, these could involve (i) the feedback loop between NF- $\kappa$ B and I $\kappa$ B (Kearns et al., 2006); (ii) feed-back and feed-forward NF- $\kappa$ B/p50 interactions with other transcription factors such as DAXX (Michaelson and Leder, 2003; Puto and Reed, 2008), glucocorticoid receptors (Hirano et al., 1998; Rosenfeld et al., 2006), SP1 (Hirano et al., 1998; Teferedegne et al., 2006), or CREB (Kaltschmidt et al., 2006); and (iii) epigenetic regulations (Natoli, 2009; Rao et al., 2010). Allostasis in the NF- $\kappa$ B system may ensure tolerance to excessive ethanol stimulation and that protects brain cells against chronic, repeated exposure to ethanol. Downregulation of the inhibitory p50 homodimer is likely to contribute to the enhanced expression of genes regulated through  $\kappa$ B elements in alcoholics. NF- $\kappa$ B and p50 homodimers are apparently principal but not sole mediators of cellular and behavioral effects of ethanol. They may act in cooperation with other transcription factors including CREB (Kaltschmidt et al., 2006) thus constituting gene regulatory networks that determine the course of both dependence to alcohol and associated impairment of cognitive functions (Farris et al., 2010) through controlling neuroplastic changes, neuroinflammation and neurodegeneration.

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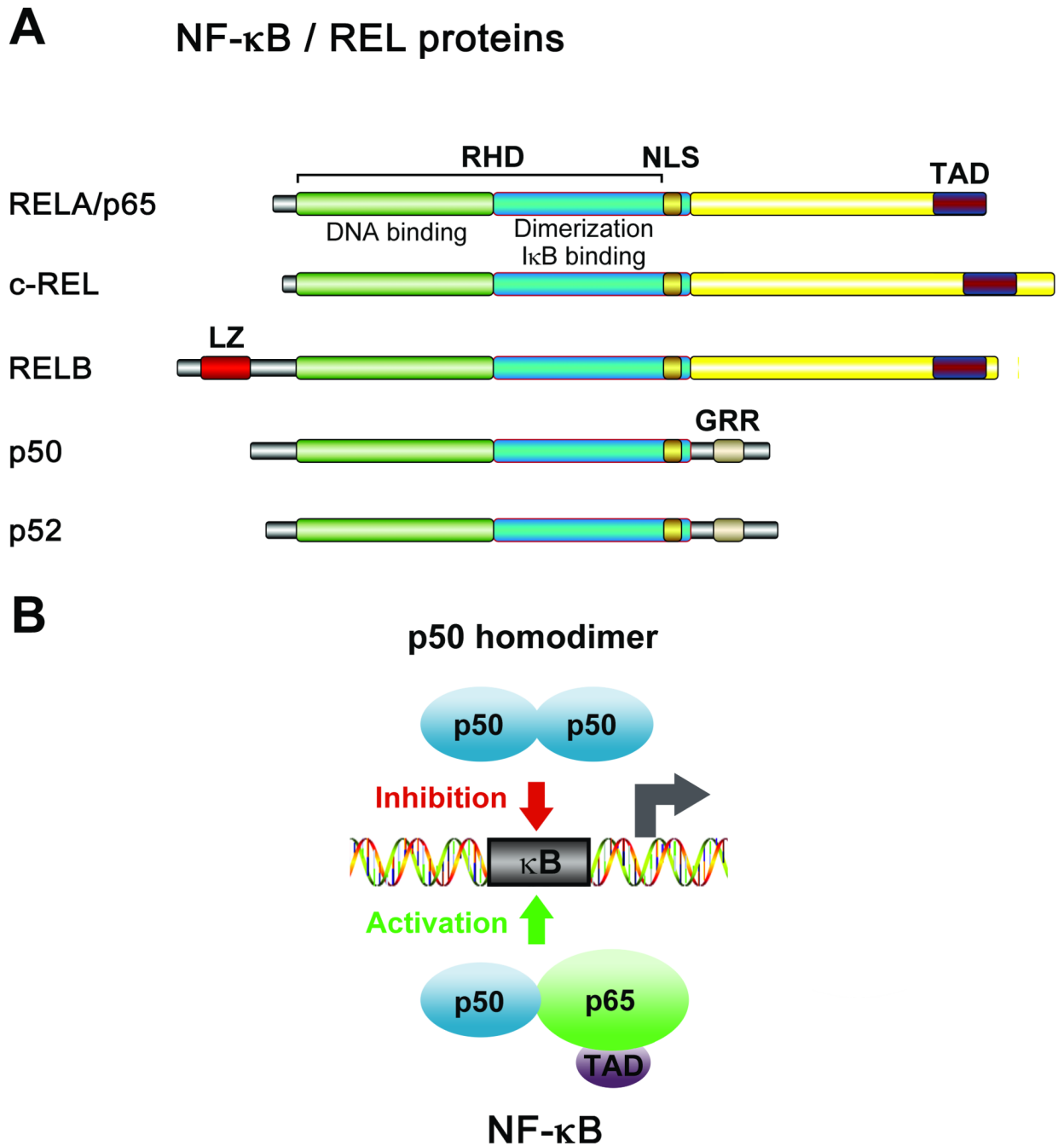
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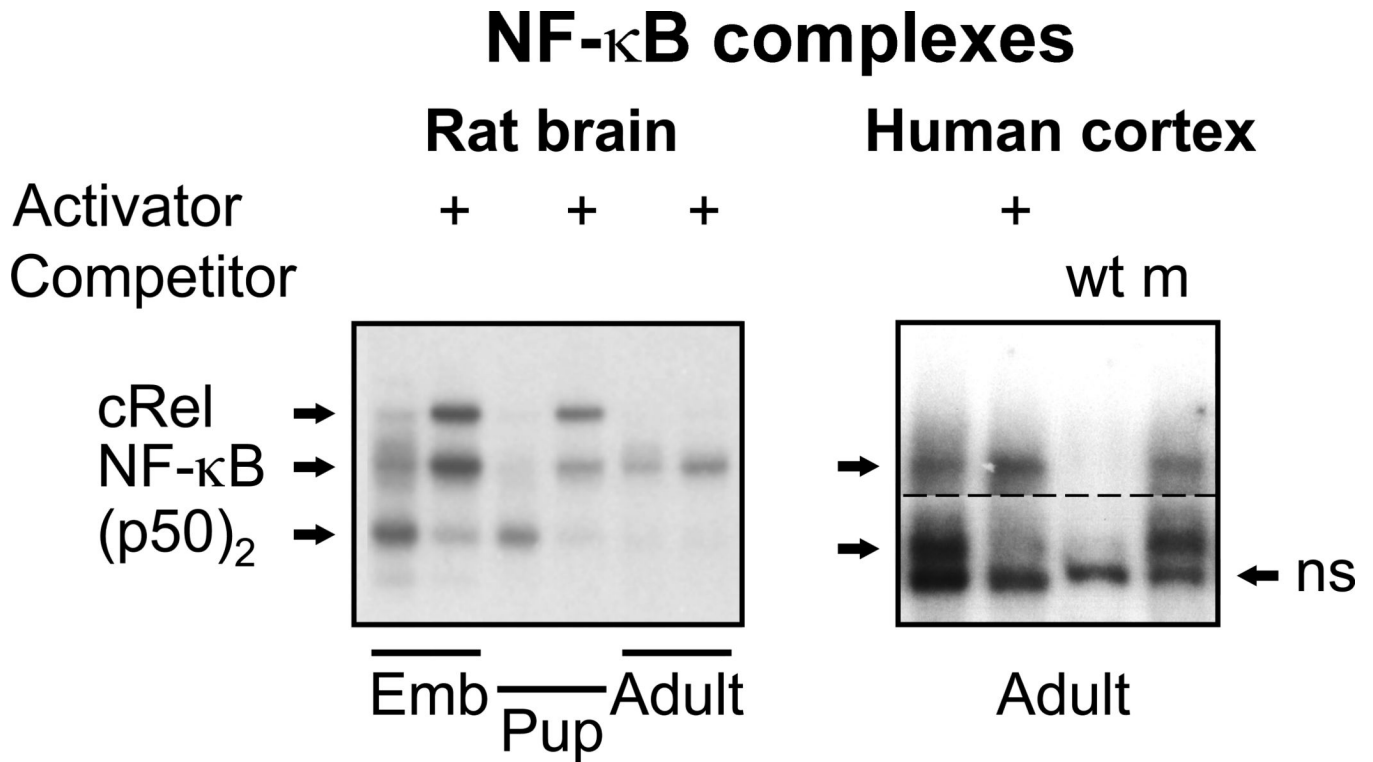
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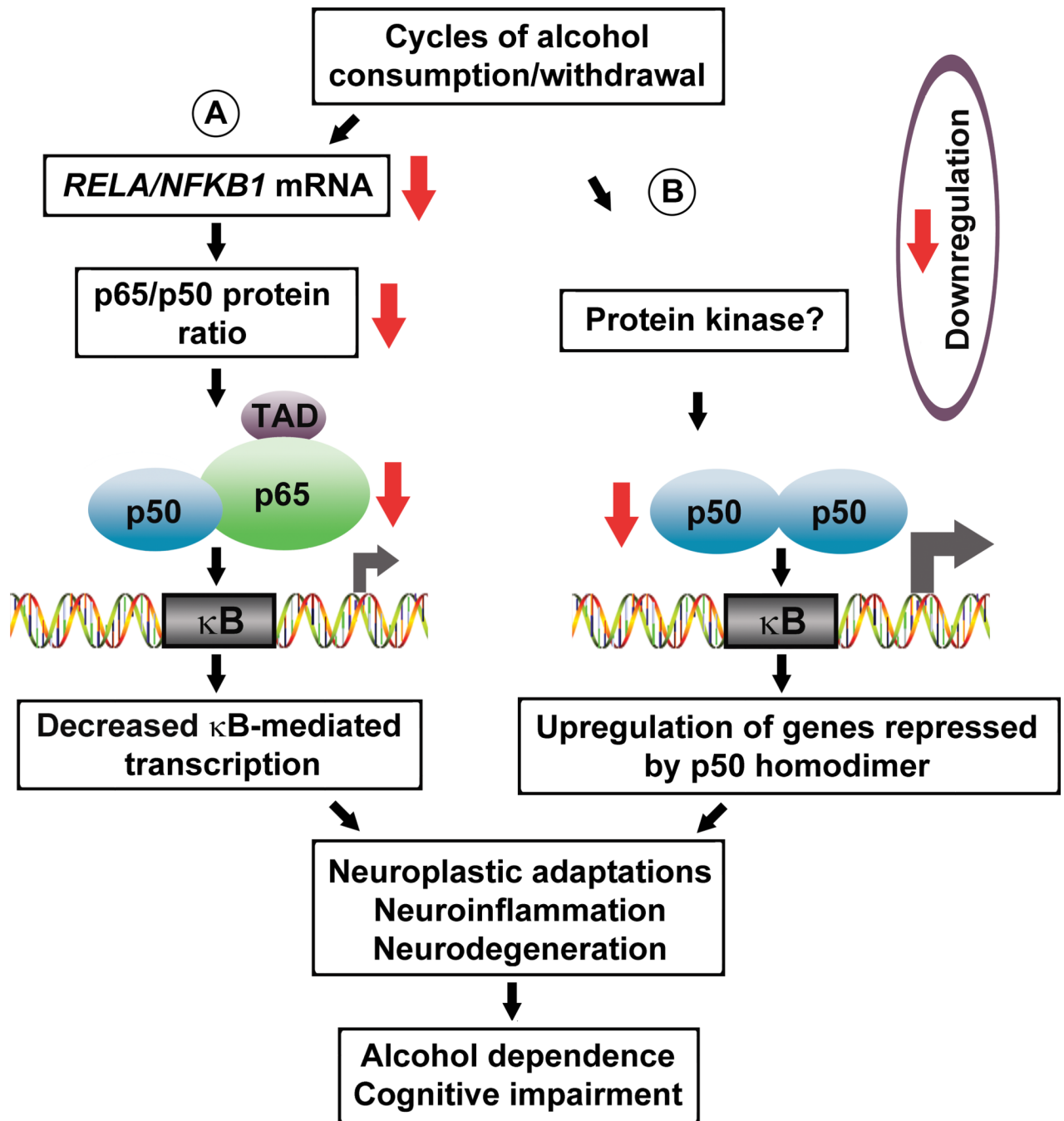


**Fig. 1.** The NF- $\kappa$ B family is composed of five transcription factors, RelA (p65), c-Rel, RelB, p50 and p52 proteins (Chen and Greene, 2004). They are related through an N-terminal, 300 amino acid, DNA binding/dimerization domain, or the Rel homology domain (RHD). Through RHD these proteins form homo- and heterodimers that bind to DNA target elements known as  $\kappa$ B sites in the promoter and enhancer gene regions. RelA (p65), c-Rel, RelB proteins are active in the form of heterodimers, while p50 and p52 may also bind to DNA as homodimers. RelA, c-Rel and RelB contain C-terminal transcription activation domain (TAD), which enable them to activate target gene expression. p50 and p52 do not have TAD; therefore, p50 and p52 homodimers repress transcription unless they are bound

to a protein containing a TAD, such as RelA, c-Rel or RelB. NLS, a nuclear localization signal. GRR, a glycine rich region is involved in posttranslational processing of the respective precursor proteins. LZ, the leucine zipper is required for transactivation by RelB.

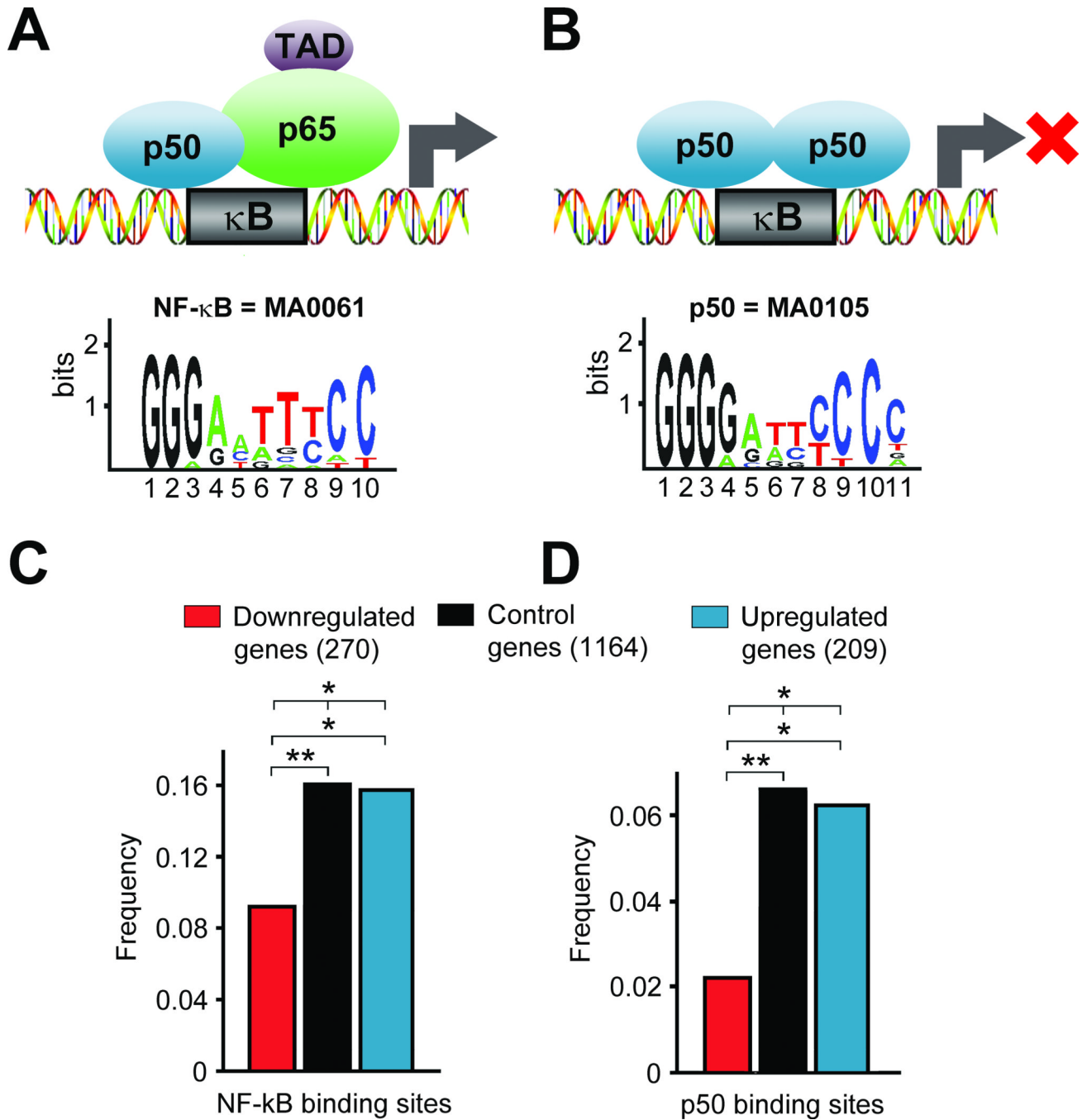


**Fig. 2.**  $\kappa$ B-binding factors identified by an electromobility shift assay (EMSA) in the brain of 17-day-old rat embryos (Emb), 5-day-old rat pups and adult rats (the left panel), and in the dl-PFC of human male subject (the right panel). The constitutively active  $\kappa$ B-DNA binding activity (c) was determined by incubation of tissue extracts with labeled  $\kappa$ B – HIV enhancer oligonucleotide. To measure total (t; constitutive plus latent) DNA-binding activity of the NF- $\kappa$ B factors, latent factors were activated by treatment with 0.6% deoxycholate that dissociated I $\kappa$ B inhibitory protein from complexes with NF- $\kappa$ B. The specificity of DNA-protein complexes was assessed by competition with wild type- $\kappa$ B (wt) or mutant- $\kappa$ B (m) oligonucleotides. Three complexes in the rat brain consisted of cRel/p65, NF- $\kappa$ B (p65/p50) and p50 homodimer, respectively. Two upper sequence-specific complexes in the human dl-PFC consisted of NF- $\kappa$ B (p65/p50) and p50 homodimer, respectively. The lower complex (ns) showed no DNA-binding sequence specificity and was likely formed by Ku protein. For the right upper image, film was exposed with a gel for longer time than for the lower one. Figure was reproduced from (Bakalkin et al., 1993) and (Okvist et al., 2007) with modifications and permission from the publishers.



**Fig. 3.** Model for molecular adaptations in NF- $\kappa$ B and p50 homodimer induced by cycles of alcohol consumption and withdrawal in the dl-PFC of human alcoholics (Okvist et al., 2007). (A) Chronic alcohol intake inhibits *RELA* expression and induces alterations in stoichiometry of *RELA/NFKB1* mRNAs and p65/p50 proteins, resulting in downregulation of NF- $\kappa$ B DNA-binding. In cells with dominant NF- $\kappa$ B this adaptation results in inhibition of NF- $\kappa$ B-dependent transcription. (B) Alcohol may act upstream of an unidentified protein kinase that modulates DNA-binding of the p50 homodimer leading to hypophosphorylation of p50 and downregulation of p50 homodimer DNA-binding. The p50 homodimer is

dominant  $\kappa$ B – binding factor in human brain. In control individuals, the p50 homodimer inhibits gene transcription acting through  $\kappa$ B - binding sites because it prevails over NF- $\kappa$ B. In alcoholics, downregulation of the p50 homodimer eliminates transcriptional repression leading to upregulation of basal or  $\kappa$ B-mediated transcription. Dysregulation of the NF- $\kappa$ B/ p50 homodimer-dependent gene transcription contributes to neuroplastic adaptations, neuroinflammation and neurodegeneration. Figure was reproduced from (Okvist et al., 2007) with modifications and permission from the publisher.



**Fig. 4.**  $\kappa$ B elements in genes differentially expressed in alcoholics (Okvist et al., 2007). Matrices from the JASPAR database were used to identify “NF- $\kappa$ B” (A) and “p50 homodimer” (B) subtypes of  $\kappa$ B binding sites. Both NF- $\kappa$ B (p65/p50 heterodimer) and p50 homodimer bind to the “NF- $\kappa$ B” subtype of  $\kappa$ B elements with high affinity and specificity, whereas p50 homodimer has higher affinity for more symmetric “p50 homodimer”-subtype of  $\kappa$ B binding sites (Chen-Park et al., 2002). The matrix logos provide visual representation profiles of nucleotide conservation in  $\kappa$ B elements. The maximal conservation amounts are 2 bits for each position. Higher conservation indicates increased biological importance for a



base. **C, D.** Frequencies of occurrence of the “NF- $\kappa$ B”- (**C**) and “p50 homodimer”- (**D**) subtypes of  $\kappa$ B binding sites in 270 and 209 genes downregulated and upregulated in alcoholics, respectively, and in 1164 control genes studied for comparison (Okvist et al., 2007). Both the group of differentially expressed genes (270 and 209 genes), and the group of control 1164 genes randomly selected from the set, which did not include genes differentially expressed in alcoholics, were taken from (Liu et al., 2006). Significance of differences between upregulated, downregulated and control genes in the frequency of occurrence of genes with  $\kappa$ B elements was evaluated with Chi-square test for pair-wise comparison; \*\*  $P < 0.01$ , \*  $P < 0.05$ . Figure was reproduced from (Okvist et al., 2007) with modifications and permission from the publisher.

**Table 1**

NF- $\kappa$ B (p65/p50 heterodimer) and p50 homodimer in the murine and human brain. Activation and localization in neurons and glia.

	<b>Murine brain</b>	<b>Human brain</b>	<b>References</b>
Dominant form	NF- $\kappa$ B	p50 homodimer >> NF- $\kappa$ B	(Bakalkin et al., 1993) (Kaltschmidt and Kaltschmidt, 2009) (Okvist et al., 2007)
	Neurons	Glia	
Cytosol		Latent NF- $\kappa$ B	(Kaltschmidt and Kaltschmidt, 2009)
Nucleus	NF- $\kappa$ B: a) constitutively active b) transcriptionally silent		a) (Kaltschmidt and Kaltschmidt, 2009) b) (Mao et al., 2009)
Synapses	Latent NF- $\kappa$ B		(Kaltschmidt and Kaltschmidt, 2009)