

Published in final edited form as:

Addict Biol. 2009 July ; 14(3): 294–297. doi:10.1111/j.1369-1600.2009.00155.x.

## FOSB proteins in the orbitofrontal and dorsolateral prefrontal cortices of human alcoholics

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### Abstract

The transcription factor  $\Delta$ FosB is accumulated in the addiction circuitry, including the orbitofrontal and medial prefrontal cortices of rodents chronically exposed to ethanol or other drugs of abuse, and has been suggested to play a direct role in addiction maintenance. To address this hypothesis in the context of substance dependence in humans, we compared the immunoreactivities of FOSB proteins in the orbitofrontal and dorsolateral prefrontal cortices (OFC and DLPFC respectively) between controls and alcoholics using semiquantitative immunoblotting. In both structures, we detected three forms of FOSB, one of which was  $\Delta$ FOSB, but in neither case did their immunoreactivities differ between the groups. Our results indicate that the  $\Delta$ FOSB immunoreactivity in the human brain is very low, and that it is not accumulated in the OFC and DLPFC of human alcoholics, suggesting that it may not be directly involved in addiction maintenance, at least not in ethanol dependence.

### Keywords

Alcoholics; dorsolateral prefrontal cortex; ethanol dependence;  $\Delta$ FOSB; FOSB; orbitofrontal cortex

### Introduction

The transcription factor  $\Delta$ FosB, an unusually stable, C-terminally truncated variant of the immediate early gene product FosB is thought to function as a sustained molecular switch for addiction (Nestler, Barrot & Self 2001). Chronic exposure of rodents to most drugs of abuse, including cocaine, morphine,  $\Delta^9$ -tetrahydrocannabinol and ethanol, causes  $\Delta$ FosB to accumulate in the addiction circuitry (Perrotti *et al.* 2008), wherein it has been suggested to regulate the expression of several genes commonly associated with this disease (McClung & Nestler 2003).  $\Delta$ FosB is thought to be directly involved in addiction maintenance by: (1) enhancing the rewarding and incentive motivational properties of drugs of abuse via its actions

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 Individual demographic and clinical data.

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in the nucleus accumbens (Nestler 2008); and (2) producing tolerance to the cognitive-disrupting effects of such drugs via its actions in the prefrontal cortex (PFC) (Nestler 2008). However, to date there has been no attempt to address this hypothesis in the context of substance dependence in humans. To do so, we compared the immunoreactivities of FOSB proteins in the orbitofrontal and dorsolateral prefrontal cortices (OFC and DLPFC, respectively) between human controls and alcoholics using semiquantitative immunoblotting. These two prefrontal cortical subregions are both key parts of the addiction circuitry (Volkow & Fowler 2000; Goldstein & Volkow 2002), and chronic exposure of rats to ethanol or other drugs of abuse has been shown to result in accumulation of  $\Delta$ FosB in their rodent equivalents, i.e. the OFC and medial prefrontal cortex (Perrotti *et al.* 2008; Winstanley *et al.* 2007).

## Materials and Methods

### Subjects

This study was approved by Stockholm's ethic vetting board. Postmortem samples from the orbitofrontal and dorsolateral prefrontal cortices, Brodmann's areas 47 and 9, respectively, of 15 human controls and 15 alcoholics were obtained from the New South Wales Tissue Resource Center. Cases were collected by qualified pathologists under full ethical clearance and with informed, written consent from the next of kin. All subjects were male and Caucasian. Alcoholics drank  $\geq 80$  g of ethanol per day in average, met DSM-IV criteria, did not have Wernicke-Korsakoff's syndrome or liver cirrhosis and had no history of poly drug abuse. Controls had no history of drug abuse. Individual demographic and clinical data for all subjects are given in supporting information Table S1.

### Cell culturing and transfection

Human HeLa carcinoma cells were grown in Iscove's Modified Dulbecco's Media, supplemented with 10% Fetal Bovine Serum in a 37°C incubator with 5% CO<sub>2</sub>. Mouse FosB and  $\Delta$ FosB cDNAs were subcloned into the mammalian expression vectors pcDNA<sup>TM</sup>3.1 / Hygro (+) and pcDNA<sup>TM</sup>3.1 (Invitrogen, Carlsbad, CA), respectively, to generate the plasmids YOF235 and YOF254. Transfections were carried out using Lipofectamine reagent (Invitrogen) according to the manufacturer's instructions. Mock-transfected cells were used as negative controls.

### Immunoblotting

Cell and tissue extracts were prepared by solubilization of pelleted cells and powdered tissue, respectively, in SDS buffer, and sonication. DC assay (Bio-Rad, Hercules, CA) was used for determination of protein concentration. Cell and tissue homogenates were resolved by SDS-PAGE on 10% Tricine gels. Proteins were transferred onto nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) and stained with MemCode (Pierce, Rockford, IL). All blots were made in duplicates, probed with the following antibodies: rabbit polyclonal anti-FosB (cat. # sc-48, Santa Cruz, CA) at 1:200; mouse monoclonal anti-FOSB (cat. # ab11959, Abcam, Cambridge, UK) at 1:500; and mouse monoclonal anti-FOSB (cat. # LS-C528, Lifespan, Seattle, WA) at 1:333; and incubated with the appropriate peroxidase-conjugated secondary antibody (cat. # 170-6515 and 170-6516, Bio-Rad) at 1:25000. sc-48 was blocked by preincubation with 10<sup>-7</sup> M of blocking peptide (cat. # sc-48 P, Santa Cruz). Blots were developed in Amersham's Enhanced Chemiluminescence System (Amersham, Little Chalfont, UK). Films were digitized using a scanner. Densitometric analysis was performed in Image Gauge (V3.12) (Fujifilm, Tokyo, Japan). To enable data normalization, reference samples consisting of pooled OFC tissue extracts from all subjects were loaded onto three wells: the second from the left and right edge, respectively, and the middle one. Protein optical densities (ODs) were expressed as ratio to MemCode<sup>TM</sup> OD.

## Statistical analysis

Data normality was examined with Shapiro-Wilk's test. Depending thereon, group differences were investigated either with Student's *t*-test or Mann-Whitney U test. Likewise, correlations of protein immunoreactivities with age, postmortem interval (PMI), brain pH and storage time were examined either with Pearson's correlation or Spearman's rank correlation. Covariate influence was assessed with analysis of covariance (ANCOVA). Significance was set to  $P < 0.05$ . Statistical analysis was performed in Statistica (V8.0) (StatSoft, Tulsa, OK).

## Results

We tested three commercially available antibodies out of which only sc-48 (Santa Cruz) could be reliably used to detect FOSB proteins in the human OFC (Fig. 1a–c). In HeLa cells ectopically expressing either FosB or  $\Delta$ FosB, this polyclonal antibody detected three FosB proteins of molecular weights, 46, 41 and 39 kDa, and three  $\Delta$ FosB proteins of molecular weights, 37, 30 and 27 kDa. Out of these six, three could be detected in the human brain, namely the 46 and 39 kDa forms of FOSB and the 37 kDa form of  $\Delta$ FOSB. The immunoreactivities of the latter two proteins were low and very low, respectively (Fig. 1e), but all three could be quantified. The mode of quantification has been described elsewhere (but see also Fig. 1d–f) (Henriksson *et al.* 2008). OD values outside of the linear ranges of detection were excluded, i.e. those of the 37 kDa form of  $\Delta$ FOSB in the DLPFC of seven controls and eight alcoholics.

In neither case did the protein immunoreactivities in the OFC and DLPFC differ significantly between controls and alcoholics (Student's *t*-test or Mann-Whitney U test,  $P > 0.05$  in all cases) (Fig. 1f). The 39 kDa form of FOSB and the 37 kDa form of  $\Delta$ FOSB in the OFC correlated significantly with PMI (Spearman's rank correlation, Spearman's  $R = -0.5$ ,  $P < 0.01$  in both cases). No other significant correlations were found (Pearson's or Spearman's rank correlation,  $P > 0.05$  in all cases). In the cases of the 39 kDa form of FOSB and the 37 kDa form of  $\Delta$ FOSB in the OFC, there were no significant differences between controls and alcoholics when the variance introduced by PMI was accounted for (ANCOVA,  $F_{1,27} = 2$ ,  $P > 0.05$  in both cases). Moreover, nicotine abuse and apparent benzodiazepine use had no influence on the protein immunoreactivities as was evident from subgroup analysis of smokers and exclusion analysis, respectively (Student's *t*-test or Mann-Whitney U test,  $P > 0.05$  in all cases).

## Discussion

The absence of significant differences in the immunoreactivities of the 46 and 39 kDa forms of FOSB in the OFC and DLPFC between human controls and alcoholics are consistent with animal data showing that these forms of FosB are not accumulated in the addiction circuitry following repeated drug treatment (Nestler *et al.* 2001). Partially unexpected, though, were the very low immunoreactivities of the 37 kDa form of  $\Delta$ FOSB detected therein; very low immunoreactivities of this form of  $\Delta$ FosB are detected in the PFC of control rats, however, we would have expected much higher immunoreactivities thereof after chronic ethanol exposure (Perrotti *et al.* 2008). Attention was given to potential confounders, i.e. age, PMI, brain pH, storage time, smoking history and apparent benzodiazepine use, neither of which were found to interfere with the interpretation of our results. However, precaution is warranted in this regard, e.g. due to sample size and heterogeneity, and the inherent uncertainty of single observations. Nevertheless, our results indicate that the 37 kDa form of  $\Delta$ FOSB is not accumulated in the PFC of human alcoholics, suggesting that it may not be directly involved in addiction maintenance, at least not in ethanol dependence.

## Supplementary Material

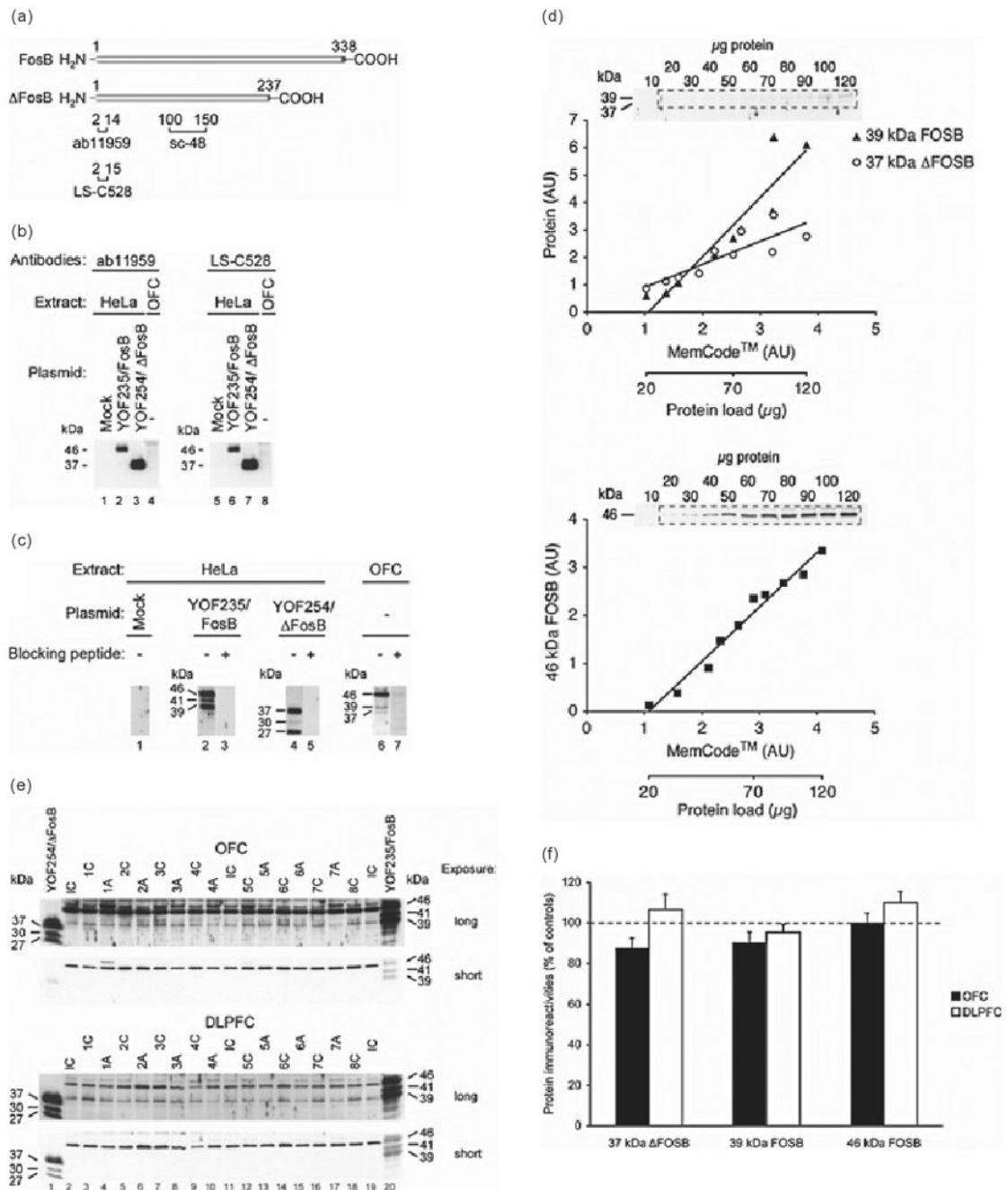
Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

This work was funded by grants from AFA Försäkring, Karolinska Institutet, the Swedish Alcohol Retail Monopoly, the Swedish Research Council, the National Drug Policy Coordinator, the Torsten and Ragnar Söderberg Foundations and Uppsala University. The Australian Brain Donor Programs New South Wales Tissue Resource Centre was supported by the University of Sydney, the National Health and Medical Research Council, the National Institute of Alcohol Abuse and Alcoholism and the New South Wales Department of Health.

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**Figure 1.**

FOSB proteins in the human orbitofrontal and dorsolateral prefrontal cortices (OFC and DLPFC, respectively). (a) Schematic picture of FosB and ΔFosB. The epitopes recognized by the three different antibodies tested are depicted. (b) Immunoblots made with HeLa cell and human OFC tissue extracts, and probed with ab11959 and LS-C528. Lanes 1 and 5: 5 μg of protein from total extracts of Mock-transfected HeLa cells; lanes 2 and 6: 5 μg of protein from total extracts of HeLa cells transfected with YOF235 (plasmid which contains a FosB insert); lanes 3 and 7: 1 μg of protein from total extracts of HeLa cells transfected with YOF254 (plasmid which contains a ΔFosB insert); lanes 4 and 8: 80 μg of protein from total extracts of OFC tissue. (c) Immunoblots made with HeLa cell and human OFC tissue extracts, and probed

with sc-48 preincubated with or without blocking peptide. Lane 1: see (b), lanes 1 and 5; lanes 2 and 3: see (b), lanes 2 and 6; lanes 4 and 5: see (b), lanes 3 and 7; lanes 6 and 7: see (b), lanes 4 and 8. (d) Upper panels: images of blots made with serial dilutions of a reference sample (10–120 µg of protein per well). Dashed areas: bands which optical densities (ODs) were linearly dependent on MemCode™ OD as is shown in the lower panels. Lower panels: linear ranges of protein ODs ( $R^2 > 0.8$  in all cases). AU, arbitrary units. (e) Images of immunoblots made with 100 µg of protein from total OFC and DLPFC tissue extracts. Lane 1: see (b), lanes 3 and 7); lane 20: see (b), lanes 2 and 6. IC, internal control; 1C, control number 1; 1A, alcoholic number 1; etc. (f) Comparison of the immunoreactivities of the 39 and 46 kDa forms of FOSB and the 37 kDa form of  $\Delta$ FOSB in the OFC and DLPFC between human controls and alcoholics. Horizontal bars represent group means and standard error of the mean