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MOLECULAR MECHANISMS UNDERLYING INCREASED *PDYN* AND DYNORPHIN EXPRESSION IN THE PREFRONTAL CORTEX OF ALCOHOLIC MEN

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To my wife

ABSTRACT

Alcohol dependence is a chronic relapsing disorder caused by drug x gene x environment interactions for which pharmacotherapy is but moderately effective. The prefrontal cortex is a brain region important for cognitive control / behavioral flexibility which function is impaired in alcoholics. Although the exact role of the dynorphin / κ-opioid receptor system in alcohol dependence is unknown, it has been suggested to contribute to the psychopathology of this disorder. Consistently, *prodynorphin* gene and dynorphin peptide expression is increased in the prefrontal cortex of alcoholic men. The aim of this thesis was to identify molecular mechanisms underlying these alterations. In line with this aim, we have shown that *prodynorphin* is regulated by the transcriptional control protein repressor element 1 silencing transcription factor *in vitro* and that repressor element 1 silencing transcription factor target gene and protein expression is altered in the prefrontal cortex of alcoholic men.

LIST OF PUBLICATIONS

I. **Richard Henriksson, Alexander Kuzmin, Anna Ökvist**, **Clive Harper, Donna Sheedy, Therese Garrick, Tatjana Yakovleva, Georgy Bakalkin.** Elevated synaptophysin I in the prefrontal cortex of human chronic alcoholics. *Synapse. 2008; 62(11):829-33*.

II. **Hiroyuki Watanabe, Richard Henriksson, Yoshinori N. Ohnishi, Yoko H. Ohnishi, Clive Harper, Donna Sheedy, Therese Garrick, Fred Nyberg, Eric J. Nestler, Georgy Bakalkin, Tatjana Yakovleva.** FOSB proteins in the orbitofrontal and dorsolateral prefrontal cortices of human alcoholics. *Addict Biol. 2009; 14(3):294-7*.

III. **Richard Henriksson, Cristina M. Bäckman, Brandon K. Harvey, Ranjan Sen, Toni S. Shippenberg.** REST regulates *PDYN* and REST target gene and protein expression is altered in the prefrontal cortex of alcoholic men. *Manuscript in preparation*.

CONTENTS

LIST OF ABBREVIATIONS

5-CSRTT	5-choice serial reaction time task
αCREM	α cAMP-responsive element modulator
AP-1	activator protein-1
BA9	Brodmann's area 9
cAMP	Cyclic adenosine monophosphate
ChIP	Chromatin immunoprecipitation
CTCF	CCCTC-binding factor
DLPFC	Dorsolateral prefrontal cortex
DSM-IV	Diagnostic and statistical manual of mental disorders, 4th edition
DREAM	Downstream regulatory element-antagonist modulator
ER	Estrogen receptor
FOSB	FBJ murine osteosarcoma viral oncogene homolog B
GATA-1	GATA binding protein 1 (globin transcription factor 1)
GR	Glucocorticoid receptor
HIV	Human immunodeficiency virus
HMBS	Hydroxymethylbilane synthase
JUND	JUN D proto-oncogene
MEIS-1	MEIS homeobox 1
MIR	MicroRNA
NF-κB	Nuclear factor κ-light-chain-enhancer of activated B cells
NPTX1	Neuronal pentraxin 1
OPRK1	Opioid receptor, κ 1
PDYN	Prodynorphin
PFC	Prefrontal cortex
PMI	Postmortem interval
RE1	Repressor element 1
REST	Repressor element 1 silencing transcription factor
RIN	RNA integrity number
RPLP0	Ribosomal protein, large, P0
SFG	Superior frontal gyrus
SNAP-25	Synaptosomal-associated protein 25
SNARE	Soluble *N*-ethylmaleimide-sensitive attachment protein receptor
SNP	Single nucleotide polymorphism
SP1	Specificity protein 1
SYP	Synaptophysin
SYX1A	Syntaxin 1A
U6	Small nuclear RNA U6
Ta-BF	T-allele binding factor
VAMP	Vesicle-associated membrane protein
VTA	Ventral tegmental area
YY1	Yin-yang 1

1 INTRODUCTION

1.1 ALCOHOL DEPENDENCE

Alcohol dependence is diagnosed according to DSM-IV criteria as a chronic relapsing disorder (1). Its lifetime prevalence is ~10 % (2), and it is thought to be caused by drug x gene x environment interactions with heritability estimates of > 50 % (3,4). Although treatment (e.g. psychosocial support) is effective in 50-60 % of patients (1), fewer than 25 % of afflicted individuals ever receive such (2). Moreover, there is no pharmacotherapy which improves treatment outcome by more than 20 % (still the benefit to cost ratio of such is 1.9-39) (1,5). These numbers reflect stigmatization (6), and limited knowledge about disease etiology, respectively. Thus, more research about alcohol dependence is warranted to counter stigma, further our understanding of the etiology of this disorder and identify new pharmacological targets for its treatment.

1.2 PREFRONTAL CORTEX

1.2.1 Function

The PFC is important for cognitive control / behavioral flexibility. This function rests on reinforcement learning theory such that the PFC guides behavior in accordance with internal goals, reward prediction errors coded by mesocortical dopamine neurons and memories; the neural correlate of which may be persistent activity (7,8). Consistently, the PFC is anatomically well positioned to integrate reward prediction errors, memories and motor responses (7). It follows that PFC recruitment is dependent on the need for cognitive control a task imposes such that in the absence of a functional PFC habitual responses would predominate and, where those don’t exist, behavior would be haphazard (7). Such impulsive and disorganized behavior is a hallmark of PFC damage.

1.2.2 Role in alcohol dependence

Alcohol dependence is associated with atrophy, morphological changes and altered glucose metabolism, gene and protein expression in the PFC (9). Moreover, alcoholics perform worse on tasks dependent on PFC function (e.g. reward evaluation) (10).

Although the role of these alterations in the etiology of alcohol dependence remains to be determined, it has therefore been argued that the PFC plays a central role in this disorder (9). A mechanistic basis for such a role was recently provided in a study showing that acute ethanol affects persistent activity *in vivo* in the rat PFC in a manner influenced by mesocortical dopamine neurons *in vitro* in organotypic culture (11).

1.3 DYNORPHIN / K-OPIOID RECEPTOR SYSTEM

1.3.1 Function

The κ-opioid receptor and its endogenous ligands, the dynorphin peptides, are involved in a wide variety of physiological responses including hedonic processing (12). Consistently, the κ-opioid receptor and dynorphin peptides are expressed in many of the brain regions that comprise the reward system (e.g. PFC and VTA) wherein they have been suggested to modulate neurotransmission (13). The expression of κ-opioid receptors in mesocortical, but not mesolimbic, dopamine neurons in the VTA, and the modulation of dopamine dynamics in the PFC by local and VTA κ-opioid receptors, are of particular interest as they make the dynorphin / κ-opioid receptor system anatomically well positioned to influence PFC function (e.g. reward evaluation).

1.3.2 Role in alcohol dependence

The role of the dynorphin / κ-opioid receptor system in the behavioral effects of ethanol appears to vary with dose and mode and length of exposure (13). This system may initially reduce ethanol intake by opposing the positive reinforcing effects of this drug, while it may increase ethanol intake after repeated, heavy consumption by contributing to the negative reinforcing effects of drug withdrawal. Although the role of the PFC in these effects is yet to be determined, the evidence presented above is suggestive of that it may be involved. Consistently, *PDYN* gene and dynorphin peptide expression is increased in the PFC of alcoholic men (14), and genetic variants in *PDYN* and *OPRK1* have been associated with alcohol dependence (15,16).

1.4 REGULATION OF *PDYN* AND DYNORPHIN EXPRESSION

1.4.1 C*is*

Dynorphin peptides are derived from PDYN which gene has four exons and multiple transcription start sites (Fig. 1) (17-19). *PDYN* is regulated in *cis* by sequence variants located in the promoter of this gene (20-23). Among these, rs1997794 (SNP) has been associated with allelic imbalance (differential expression of alleles at one or more loci) in *PDYN* gene expression in the PFC (22,24). Interestingly in this regard, rs1997794 is located in a response element for the transcriptional control protein AP-1 (25), and influences its binding to this site *in vitro* (26). However, the causative variant may be one in linkage disequilibrium (nonrandom association of alleles at two or more loci) with rs1997794. A candidate variant is rs910080 (SNP) as: 1) it was used as a reporter SNP in the two studies demonstrating allelic imbalance in *PDYN* gene expression in the PFC (22,27); 2) allelic imbalance in striatal *PDYN* gene expression was not observed in the one subject analyzed who was homozygous for this locus (25); and 3) rs910080 is located in a RE1 bound by the transcriptional control protein REST *in vivo* (28).

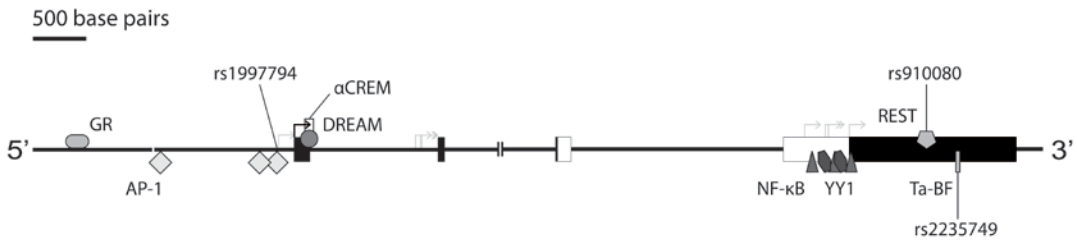

Figure 1. To scale schematic of *PDYN*. Shown are functional sequence variants associated with alcohol dependence (e.g. rs910080) and transcriptional control proteins (e.g. REST) implicated in *PDYN* regulation based on DNA-binding data or protein-protein interactions (i.e. DREAM-αCREM) (13,15,26,29). Transcriptional control proteins for which there are *in vivo* evidence of DNA-binding are depicted above the sequence (e.g. REST), and those for which only *in vitro* evidence are available below the sequence (e.g. AP-1). ‖ = genomic regions omitted for clarity; boxes = exons; white boxes = coding regions; black arrow = transcription start site; grey arrows = alternative transcription start sites.

1.4.2 Epigenetic

Methylated CpG islands (genomic regions containing a high frequency of CpG dinucleotides) and dinucleotides have been identified in *PDYN* (29,30), but the role of this epigenetic mark in *PDYN* regulation is unknown. A gene encoding a long non-coding RNA, *AK090681*, is transcribed from the opposite strand of *PDYN* (31), and such RNAs are involved in epigenetic control of gene expression (32). However, *PDYN* and *AK090681* may be separate but overlapping transcription units as suggested by the exon locations of *AK090681*. CTCF-mediated chromatin looping may provide a mechanistic basis for this separation as: 1) two transcripts lacking exons 3 and 4 have been reported for *AK090681* (19,33); 2) these exons enclose *PDYN* and exon 3 and intron 3 of *AK090681* contain response elements bound CTCF and cohesin *in vivo* (34,35); and 3) CTCF may regulate higher-order chromatin structure (36).

1.4.3 *Trans*

Transcriptional control proteins implicated in *PDYN* regulation are αCREM, AP-1, DREAM, ER, GATA-1, GR, MEIS-1, NF-κB, REST, SP1, Ta-BF and YY1 (Fig. 1) (13,29). (Note that transcriptional control proteins implicated in *PDYN* regulation based on *in silico* data alone (i.e. ER, GATA-1, MEIS-1 and SP1) have been omitted from Fig. 1 as this type of analysis is plagued by a high false positive rate (37)). Of these, αCREM, AP-1, DREAM, REST and YY1 have been shown to influence gene expression via their respective response elements in *PDYN* or protein-protein interactions. αCREM and AP-1 activates *PDYN* (13,20,23), while DREAM, REST and YY1 repress this gene *in vitro* (38-41) (paper III). However, whether αCREM, AP-1, DREAM or YY1 endogenously regulate *PDYN* remains to be determined as the gene expression data on these proteins are from reporter assays. Moreover, *PDYN* has never been identified as a target for any AP-1 (e.g. JUND) family member *in vivo* (42).

1.4.4 Post-transcriptional

Alternatively spliced *PDYN* transcripts have been identified (18,19). The role of this processing step in *PDYN* regulation is unknown, but it may contribute to dynorphin / κ-opioid receptor system diversity (43). Consistently, the translation products of some of

these messages may have novel functions as suggested by their sequence similarity to transcriptional control proteins (44), and localization to the cell nucleus *in vitro* (18).

1.4.5 Post-translational

Although additional processing enzymes have been implicated in dynorphin peptide biosynthesis *in vitro* (45,46), this process appears to be governed primarily by the non-selective proteases cathepsin L, prohormone convertase 1, 2 and 3 and carboxypeptidase E *in vivo* (47-49). Thus, brain regional differences in dynorphin peptide expression may reflect the expression patterns of these processing enzymes.

2 SPECIFIC AIM OF THESIS

To identify molecular mechanisms underlying increased *PDYN* gene and dynorphin peptide expression in the prefrontal cortex of alcoholic men.

3 MATERIALS AND METHODS

3.1 SUBJECTS

All studies were approved by Stockholm's ethic vetting board (permit # 04-849/4). Postmortem samples from 16 control and 16 alcoholic men were obtained from the New South Wales Tissue Resource Center; 13 per group of whom were included in all three studies. Cases were collected by qualified pathologists under full ethical clearance (protocol # X03-0117) and with informed, written consent from the next of kin. All subjects were Caucasian, negative for HIV and hepatitis B and C, had no brain abnormalities on gross examination and no history of cerebral infarction, head trauma, developmental or neurological disorders or illicit drug use, while the majority had a smoking history. Control men consumed ≤ 20 grams of ethanol / day for the majority of their adult lives with one exception who drank 35 grams / day, while the corresponding number for alcoholic men was ≥ 80 grams of ethanol / day with one exception who consumed 60 grams of ethanol / day. Alcoholic men met DSM-IV criteria for alcohol dependence and did not have Wernicke–Korsakoff´s syndrome or liver cirrhosis. Detailed clinical and demographic data tables are provided in papers I-III.

3.2 CELL CULTURE AND TRANSFECTION

Human HeLa carcinoma cells were grown in high glucose Dulbecco's modified eagle medium containing 10 % fetal bovine serum at 37 ^{o}C and 5 % CO_2 and transfected with lipofectamine™ (Invitrogen) 24 hours post plating according to the manufacturer's instructions. Human neuroblastoma SH-SY5Y cells (kind gift from Dr. Freed WJ) were maintained in high glucose Dulbecco's modified eagle medium containing 17 % fetal bovine serum at 37 ^{o}C and 5 % CO_2 and transfected with lipofectamine™ 2000 (Invitrogen) 24 hours post plating according to the manufacturer's instructions. Extractions were performed 24-48 hours post transfection as described below.

3.3 RNA EXTRACTION AND REAL-TIME PCR

3.3.1 CELLS

RNA was extracted using either RNAqueous® (Ambion) (> 200 nucleotides) or TRIzol® (Invitrogen) (total) according to the manufacturer's instructions and, in the latter case, purified using butanol / ether extraction (50). RNA was reverse transcribed using either iScript™ cDNA synthesis kit (Bio-Rad) (total) or TaqMan® microRNA reverse transcription kit (Applied Biosystems) (MIRs). If necessary, cDNA was preamplified using TaqMan® preamp master mix (Applied Biosystems). HotStar Taq DNA polymerase (Qiagen) was used for two-step real-time PCR in Applied Biosystems 7900HT fast real-time PCR system. Universal Probe Library assays (Roche Applied Science) and TaqMan® microRNA assays (Applied Biosystems) are listed in Table S2 in paper III. Assay cDNA specificities were verified on agarose gels. Assay efficacies were determined using standard curves based on RNA from untransfected cells. Relative gene and MIR expression was determined using the Pfaffl method (51). Gene expression was normalized against *HMBS* and MIR expression against U6.

3.3.2 TISSUE

RNA extraction and real-time PCR were performed as above with the exceptions that RNA from human PFC was used for determination of assay efficacies and that RNA integrity was analyzed using RNA 6000 nano kit (Agilent) in an Agilent 2100 bioanalyzer. RIN was determined using B.02.03 (Agilent). Gene expression was normalized against *RPLP0* and MIR expression against U6. *RPLP0* was previously identified using geometric averaging as the most stably expressed housekeeping gene of those tested in the PFC of 26 out of the 32 subjects in our sample population (13 control and 13 alcoholic men) (52), while U6 was selected as it resembles the mean expression value of the three MIRs analyzed (i.e. MIR-9, MIR-191 and U6) (53).

3.4 CHROMATIN EXTRACTION AND IMMUNOPRECIPITATION

Protein and DNA were cross-linked with 1 % formaldehyde, the reaction quenched with 125 millimolar glycine and chromatin extracted using 1 % SDS lysis buffer supplemented with phosphatase and protease inhibitors. Chromatin was shredded using a bioruptor (Diagenode) to ca 500 base pair fragments. Sonciated lysates were pre-cleared using protein A agarose beads blocked with salmon sperm (Millipore) and

incubated with primary antibodies. Immunocomplexes were collected using blocked protein A agarose beads and DNA eluted in TE buffer, pH 10 supplemented with proteinase K. Assays were designed using RealTime PCR (Integrated DNA technologies) and are listed in Table S2 in paper III. iQ™ SYBR® green supermix (Bio-Rad) was used for one-step real-time PCR in Applied Biosystems 7900HT fast real-time PCR system. Assay cDNA specificities were verified on agarose gels. Assay efficacies were determined using standard curves based on human genomic DNA (Roche Applied Science). The ChIP signal was normalized against input chromatin.

3.5 PROTEIN EXTRACTION AND WESTERN BLOTTING

3.5.1 CELLS

Protein was extracted using either RIPA buffer (Sigma-Aldrich) (total) or NE-PER® nuclear and cytoplasmic extraction reagents (Thermo Scientific) supplemented with phosphatase and protease inhibitors according to the manufacturer's instructions. Protein concentration was determined using Bio-Rad protein assay. Protein was loaded onto 5 % tricine SDS gels, separated and transferred onto nitrocellulose membranes (Bio-Rad). Membranes were incubated with primary antibodies, the appropriate horseradish peroxidase-conjugated secondary antibody (Millipore) and ECL solution (GE Healthcare). Chemiluminescence was detected using a LAS-3000 charge-coupled device camera (Fujifilm). Densitometric analysis was performed in Multi gauge V3.0 (Fujifilm). Protein expression was normalized against the total protein load which was determined using MemCode™ reversible protein stain (Thermo Scientific) (54).

3.5.2 TISSUE

Protein was extracted in 4 % SDS extraction buffer supplemented with phosphatase and protease inhibitors (total). Western blotting was performed as above with the exceptions that: 1) 10 % tricine SDS gels were used in papers I and II; 2) chemiluminescence was detected on hyperfilm ECL (GE Healthcare); 3) densitometric analysis was performed in Image gauge V3.12 (Fujifilm); and 4) in papers I and II, the linear range of detection for each protein was determined using blots made from serial dilutions of interblot control samples and analysis of the linear dependence of the protein optical densities on the corresponding MemCode optical densities.

3.6 STATISTICAL ANALYSIS

3.6.1 CELLS

Outliers were identified using Grubb's test (www.graphpad.com). Data normality was assessed using Shapiro-Wilks test. Group means of gene, MIR and protein expression were compared between untransfected and transfected cells using one-way ANOVA followed by Dunnett's post-hoc test. Statistical significance was set at $p < 0.05$. Statistical analysis was performed in Graphpad prism™ V5.0 (Graphpad Software).

3.6.2 TISSUE

Outliers were identified as above. Data normality was assessed using either Kolmogorov-Smirnov or Shapiro-Wilks test. Group means of gene, MIR and protein expression were compared between control and alcoholic men using backwards elimination to identify covariates among age, brain pH, PMI, RIN and storage time. Identified covariates were included in ANCOVAs. If no covariates were identified, group differences were evaluated using either Student's t-test or Mann-Whitney U-test depending on data normality. Likewise, correlations were analyzed using either Pearson's correlation or Spearman's rank correlation. Statistical significance was set to $P < 0.05$. Statistical analysis was performed in Statistica™ V10 (StatSoft).

4 RESULTS

4.1 PAPER I

Elevated synaptophysin I in the prefrontal cortex of human chronic alcoholics

PDYN is responsive to Ca^{2+} and cAMP *in vitro* (55). Repeated ethanol exposure may alter signaling via these second messengers through effects on neurotransmitter release (56). Thus, in addition to the hypothesis put forth in paper I (i.e. that repeated ethanol exposure affects modulators of synaptic strength but not executors of neurotransmitter release) we hypothesized that the increased *PDYN* gene and dynorphin peptide expression observed in the PFC of alcoholic men (14) results from altered neurotransmitter release. To this end, we compared the expression of the presynaptic release machinery (i.e. SNAP-25, SYX1A and VAMP) (57), and the presynaptic marker, SYP (58), in the SFG, BA9 between 14 control and 14 alcoholic men.

We used western blotting. SYP expression was significantly increased in the PFC of alcoholic men (ANCOVA, F (1, 25) = 13, P = 0.01), while the expression of SNAP-25, SYX1A and VAMP in this brain region did not differ significantly between control and alcoholic men. Consistent with the hypothesis put forth in paper I, these results indicate that repeated ethanol exposure affects modulators of synaptic strength but not executors of neurotransmitter release. However, contrary to our predictions they do not support a role of altered neurotransmitter release in the increased *PDYN* gene and dynorphin peptide expression observed in the PFC of alcoholic men (14), which prompted us to publish our findings on the presynaptic release machinery and SYP separately.

4.2 PAPER II

FOSB proteins in the dorsolateral and orbitofrontal cortices of human alcoholics

The transcriptional control protein / AP-1 family member ΔFosB targets the mouse *Pdyn* promoter *in vivo* and represses it *in vitro* (59). The role of this protein in gene regulation appears to be time- and expression level-dependent with persistent, high expression favoring gene activation over gene repression (60). ΔFosB is accumulated in

the PFC of rats repeatedly exposed to ethanol (61). Thus, in addition to the hypothesis put forth in paper II (i.e. that drug-induced accumulation of ΔFOSB in the PFC is directly involved in addiction maintenance) we hypothesized that the increased *PDYN* gene and dynorphin peptide expression observed in the PFC of alcoholic men (14) results from accumulation of ΔFOSB. To this end, we compared the expression of FOSB proteins in the SFG, BA9 between 15 control and 15 alcoholic men.

We used western blotting. Three FOSB proteins were detected in the adult human PFC, one of which was ΔFOSB. ΔFOSB expression was very low and FOSB expression in this brain region did not differ significantly between control and alcoholic men. Contrary to the hypothesis put forth in paper II, these results indicate that ΔFOSB is not accumulated in the PFC of alcoholic men and may not be directly involved in addiction maintenance. Moreover, they do not support a role of ΔFOSB in the increased *PDYN* gene and dynorphin peptide expression observed in the PFC of alcoholic men (14), which again prompted us to publish the findings on FOSB proteins separately.

4.3 PAPER III

REST regulates *PDYN* and REST target gene and protein expression is altered in the prefrontal cortex of alcoholic men

PDYN is targeted by REST *in vivo* (28). REST is negatively regulated by the microRNA MIR-9 *in vitro* (62). Murine Rest and miR-9 are responsive to ethanol *in vitro* (63,64). Thus, we hypothesized that: 1) REST regulates *PDYN*; 2) the increased *PDYN* gene and dynorphin peptide expression observed in the PFC of alcoholic men (14) results from MIR-9- and REST-mediated derepression of REST target genes and proteins. To these ends, we correlated the effects of dominant negative REST and murine miR-9 on endogenous *PDYN* gene expression in human neuroblastoma SH-SY5Y cells with REST binding to the RE1 in *PDYN* and compared the expression of MIR-9, REST protein and the REST target genes, *NPTX1* and *SYP*, and protein, NPTX1, in the SFG, BA9 between 15 control and 15 alcoholic men.

We used real-time PCR, ChIP and western blotting. Dominant negative REST or MIR-9 increased endogenous *PDYN* gene expression in SH-SY5Y cells (ANOVA, F (3, 8) = 38.3, P = < 0.001 and F (3, 8) = 38.3, P = < 0.05, respectively), which correlated with

decreased REST binding to the RE1 in *PDYN* (ANOVA, F (3, 8) = 10.5, P = < 0.05 and F (3, 8) = 10.5, P = < 0.01, respectively). *NPTX1* gene and protein expression was increased in the PFC of alcoholic men (Student's t-test, P < 0.01 and P < 0.05, respectively), while *SYP* gene, MIR-9 and REST protein expression in this brain region did not differ significantly between control and alcoholic men. Consistent with the hypothesis put forth in paper III, these results indicate that REST is involved in transcriptional control of *PDYN* and may underlie the increased *PDYN* gene and dynorphin peptide expression observed in the PFC of alcoholic men (14). However, contrary to our predictions the latter effects appear not to be mediated by MIR-9.

5 GENERAL DISCUSSION

PDYN gene and dynorphin peptide expression is increased in the PFC of alcoholic men (14). The specific aim of this thesis was to identify molecular mechanisms underlying these alterations. In line with this aim, we have shown that *PDYN* is regulated by REST *in vitro* and that REST target gene (i.e. *NPTX1*) and protein (i.e. NPTX1 and SYP) expression is altered in the PFC of alcoholic men. These findings implicate REST in the increased *PDYN* gene and dynorphin peptide expression observed in the PFC of alcoholic men (14), while our results on the SNARE proteins (i.e. SNAP-25, SYX1A and VAMP), FOSB proteins and MIR-9 fail to support such a role for these factors.

We believe it necessary to validate mechanistic data from animal studies of disorders with a cognitive component such as alcohol dependence in humans as: 1) organism complexity may stem from progressively more elaborate patterns of gene regulation (65); and 2) the behavioral flexibility of humans and requirement for cognitive control that it imposes may be unique to our species (7). Thus: 1) while a given gene may be responsive to ethanol across species the underlying mechanisms may differ; and 2) the role of its altered expression in brain regions implicated in cognitive control (e.g. PFC) in alcohol dependence may not be easily inferred from animal models as the severity of loss of cognitive control may decrease with descending organism complexity.

In line with this reasoning, negative findings were considered important and worth publishing. However, we decided not to further pursue their biological causes as we had limited resources (i.e. tissue). Thus, the reach of our conclusions depended on the available evidence in favor of the tested hypotheses, which were arguably better for the role of ΔFosB in addiction maintenance than the effects of repeated ethanol exposure on modulators of synaptic strength. These things said, we acknowledge that our findings are correlative and that result interpretation is made difficult by limitations related to specificity, postmortem tissue, sample heterogeneity (e.g. incomplete abstinence and toxicology data and lack of data on cognitive status) and size.

On the notion that organism complexity stems from progressively more elaborate patterns of gene regulation: 1) *PDYN* appears not to be targeted by AP-1 *in vivo* (42); 2) the RE1 in *PDYN* is not conserved; 3) Rest appears not to regulate mouse *Pdyn* (66,67);

and 4) rs910080 is located in the RE1 in *PDYN* (Fig. 1) (27). Together with these findings, our results on FOSB proteins and REST lend further support to a role of *cis*-regulatory divergence in transcriptional control of *Pdyn* (21), and are suggestive of that the responsiveness of this gene to ethanol may differ between species and even individuals as rs910080 has been associated with alcohol dependence (15).

On the notion that the behavioral flexibility of humans and requirement for cognitive control that it imposes is unique to our species: 1) the existence of a DLPFC (e.g. SFG, BA9) in non-primate animals is a matter of debate (68); 2) the crucial function of the SFG, BA9 is unknown (69); and 3) areas proximal to this area have been implicated in loss of ability to abstain from drug seeking in abstinent alcoholics (70). Together with these findings, the observation that *PDYN* gene expression is increased selectively in the SFG, BA9 of alcoholic men (14) is suggestive of that similar to the underlying molecular mechanisms, the role of this alteration in the psychopathology of alcohol dependence (if any) may not be easily inferred from animal studies.

We believe it reasonable to assume that all but two of the alcoholic men included in papers I-III were abstinent (only two of them had ethanol in their blood at the time of death). Thus, the altered expression of REST target genes (i.e. *PDYN* and *NPTX1*) and proteins (i.e. dynorphin peptides, NPTX1 and SYP) in the PFC of alcoholic men observed in our laboratories may result from abstinence-induced alterations in neurotransmission (e.g. increased glutamate transmission) and be involved in behavioral phenotypes associated with abstinence (e.g. compulsive drug seeking). Testing these hypotheses poses challenges related to species-specificity, but is worth the effort as doing so may identify new therapeutic targets for alcohol dependence.

6 FUTURE PERSPECTIVES

The role of increased PFC *PDYN* gene and dynorphin peptide expression in alcohol dependence could be investigated in a mouse model of this disorder (71). *PDYN* gene and dynorphin peptide expression in the medial PFC of alcohol dependent, abstinent conditional *PDYN* knockout mice (transgenic mice carrying floxed *PDYN*) (need to be generated) could be correlated with the performance of these mice on the 5-CSRTT (a test of cognitive control / behavioral flexibility) (72,73). If increased gene and peptide expression in the medial PFC correlated with task performance, canine adenovirus type 2 carrying Cre recombinase could be injected into the medial PFC to preferentially knockout *PDYN* in neurons this brain region and its afferent projection areas (74). Regional specificity could be investigated by injection of adeno-associated virus type 2 carrying Cre recombinase into the medial PFC (limited retrograde transport). Causality could be established by rescue of dynorphin peptide expression via injection of viruses of either type carrying floxed *PDYN* (need to be generated) into this brain region.

If abstinence-induced *PDYN* gene and dynorphin peptide expression in the medial PFC affected cognitive control / behavioral flexibility, the effects of abstinence on REST protein localization, expression and localization of splice variants of REST (75), and *PDYN* gene expression could be determined in transduced cells from the medial PFC of conditional *PDYN* knockout mice. If increased *PDYN* gene expression correlated with altered REST protein localization and / or expression and / or localization of splice variants of REST, the effects of viruses carrying floxed *REST* (need to be generated) could be investigated. If knockout of *PDYN* and overexpression of REST protein in the medial PFC similarly affected the performance of abstinent conditional *PDYN* knockout mice on the 5-CSRTT, the expression of other REST target genes and proteins could be determined in postmortem tissue from the SFG, BA9 of alcoholics to further our understanding of the role of this transcriptional control protein in alcohol dependence and aiding evaluation of its therapeutic potential for this disorder.

The contribution of κ-opioid receptors to the effects of increased PFC *PDYN* gene and dynorphin peptide expression on cognitive control / behavioral flexibility could be investigated using the 5-CSRTT. The effects of κ-opioid receptor agonists and antagonists injected into the medial PFC on the performance of naïve mice on this task

could be investigated. If the effects of either type of κ-opioid receptor ligand on task performance mimicked those observed after abstinence, the therapeutic potential of the dynorphin / κ-opioid receptor system for alcohol dependence could be investigated as above. κ-opioid receptor occupancy could also be correlated with the performance of abstinent mice and alcoholics on the 5-CSRTT and a recently developed incentive conflict task (test of reward evaluation) (70), respectively, pending the approval of κ-opioid radiotracers for use in humans (76-78). This could provide: 1) an *in vivo* measure of dynorphin peptide expression in the PFC by competition with cold ligand; and 2) a means to tap into the function of the SFG, BA9 and investigate its role in alcohol dependence provided that increasing cognitive load (i.e. task difficulty), which results in progressively stronger PFC recruitment (7), would eventually engage it.

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