Downregulation of the neuronal opioid gene expression concomitantly with neuronal decline in dorsolateral prefrontal cortex of human alcoholics

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Abstract
Molecular changes in cortical areas of addicted brain may underlie cognitive impairment and loss of control over intake of addictive substances and alcohol. Prodynorphin (PDYN) gives rise to dynorphin (DYNs) opioid peptides which target kappa-opioid receptor (KOR). DYNs mediate alcohol-induced impairment of learning and memory, while KOR antagonists block excessive, compulsive-like drug and alcohol self-administration in animal models. In human brain, the DYN/KOR system may undergo adaptive changes, which along with neuronal loss, may contribute to alcohol-associated cognitive deficit. We addressed this hypothesis by comparing the expression levels and co-expression (transcriptionally coordinated) patterns of PDYN and KOR (OPRK1) genes in dorsolateral prefrontal cortex (dlPFC) between human alcoholics and controls. Postmortem brain specimens of 53 alcoholics and 55 controls were analyzed. PDYN was found to be downregulated in dlPFC of alcoholics, while OPRK1 transcription was not altered. PDYN downregulation was confined to subgroup of subjects carrying C, a high-risk allele of PDYN promoter SNP rs1997794 associated with alcoholism. Changes in PDYN expression did not depend on the decline in neuronal proportion in alcoholics, and thereby may be attributed to transcriptional adaptations in alcoholic brain. Absolute expression levels of PDYN were lower compared to those of OPRK1, suggesting that PDYN expression is a limiting factor in the DYN/KOR signaling, and that the PDYN downregulation diminishes efficacy of DYN/KOR signaling in dlPFC of human alcoholics. The overall outcome of the DYN/KOR downregulation may be disinhibition of neurotransmission, which when overactivated could contribute to formation of alcohol-related behavior.

Introduction
Alcohol consumed in moderate and large amounts causes acute and delayed impairments in cognitive and executive functions that guide complex behavior through planning, decision-making, and response control1–6. Total alcohol consumption and binge drinking patterns are risk factors for dementia7–9. Alcohol-induced cognitive deficit is a factor underlying the habitual drug seeking and taking that characterize addiction and dependence10,11.

The mechanism of alcohol-induced cognitive impairments remains unknown but may involve neurodegeneration and aberrant neurotransmission. Several studies indicate that cognitive effects of alcohol may be mediated through dysregulation of the dynorphin/k-opioid receptor (DYN/KOR) system in the prefrontal cortex (PFC) and hippocampus12–16. DYN opioid peptides have been implicated in cognitive decline17–22. Administration of synthetic DYN into dorsal hippocampus impairs spatial
learning in rats. In elderly humans, prodynorphin (PDYN) gene polymorphisms play a role in memory function. In individuals with Alzheimer’s disease, PDYN is elevated in PFC and this increase correlates with neuropathological lesions.

A role of the DYN/KOR system in impairment of spatial learning and memory was identified in a rat model of cognitive deficit induced by alcohol binge drinking. Selective KOR antagonist nor-binaltorphimine normalized animal performance in spatial learning and memory tasks possibly by reversion of ethanol-induced elevation in glutamate overflow.

Molecular and cellular allostatic changes in addicted brain may underlie addictive behavior and associated phenomena including impairment of cognitive functions. Changes in the DYN/KOR system may be involved in allostatic processes in the addicted brain as demonstrated in animal studies. The aim of the present study was to examine whether the DYN/KOR system undergoes adaptive changes at the transcriptional level in dorsolateral prefrontal cortex (dlPFC) in human alcoholics. We previously conducted a pilot analysis of a limited number of human alcoholics and controls that revealed no differences in the expression of the PDYN gene in the orbitofrontal cortex (OFC) and OPRK1 gene (opioid receptor kappa 1 gene encoding KOR protein) in dlPFC, and elevation with a borderline significance of PDYN and KOR mRNA levels in dlPFC and OFC, respectively, of alcoholics. The number of subjects is a critical factor in molecular analysis of human brain. Reasoning that our previous study was possibly underpowered, in the present work the number of subjects was increased nearly 4-fold and more stringent statistical analysis was applied.

Analysis of absolute expression levels revealed a co-regulated (transcriptionally coordinated) pattern of PDYN (prodynorphin gene encoding precursor of DYN peptides) and OPRK1 expression in human nucleus accumbens (NAc). This pattern was significantly different between alcoholics and controls. To assess whether the PDYN and OPRK1 genes are co-regulated in dlPFC, and whether this co-regulation is altered in addicted dlPFC, we compared PDYN and OPRK1 co-expression along with average expression levels of these genes in dlPFC between alcoholics and controls.

A number of neurons is markedly reduced in alcoholics in dlPFC as demonstrated in early postmortem morphological studies and confirmed by analyses of neuronal proportion quantified from epigenome-wide DNA methylation profiles and expression of neuronal marker (RBFOX3 encoding NeuN protein). To attribute potential expression changes to transcriptional events or changes in cell composition in alcoholics, we examined whether PDYN and KOR (OPRK1) mRNAs are expressed strictly in neurons in human brain and then analyzed effects of the decline in neuronal proportion and RBFOX3 expression on PDYN and OPRK1 mRNA alterations in alcoholic brain. Effects of PDYN and OPRK1 single-nucleotide polymorphisms (SNPs) strongly associated with alcoholism on expression of both genes were also studied.

Materials and methods

Human samples

Human frozen brain tissues were collected at the New South Wales Brain Tissue Resource Centre (NSW BTRC), University of Sydney, Australia (https://sydney.edu.au/medicine/pathology/btrc/; see Table 1 for short summary and Supplementary Table 1 for detailed information). Tissue samples from 55 control and 53 alcoholic subjects, all males of European descent, were analyzed. Alcoholics were the subjects that met Diagnostic and Statistical Manual for Mental Disorders, 4th edition (DSM-IV) criteria for Alcohol Abuse or Alcohol Dependence and consumed 206 ± 20 g (mean ± S.E.M.) of ethanol per day in average for the majority of their adult lives. Controls had either abstained from alcohol completely or were social drinkers who consumed 17 ± 3 g of ethanol per day on average. Methods used to classify alcoholics were described previously. Cases with a history of polydrug abuse (with evidence that the individual abused other drugs such as cocaine or heroin) or with medical complications such as Wernicke–Korsakoff syndrome or alcoholic cases with concomitant diseases were excluded. Cases with a history of cerebral infarction, head injury, or neurodegenerative disease (e.g., Alzheimer’s disease) were also excluded. dlPFC samples were dissected from superior frontal gyrus/Brodmann area 9. Informed written consent for autopsy was obtained from the next-of-kin and collection was approved by the Human Research Ethics Committees of the Sydney Local Health District (X15-0199) and the University of Sydney. The study was approved by the Swedish Central Ethical Review Board. Smoking status information was available for 94% of subjects (Supplementary Table 1; “ex-smokers” were grouped with “non-smokers”).

RNA purification

Total RNA was purified using RNeasy Lipid Tissue Mini Kit (Qiagen) and treated with RNase-free DNase I (Qiagen) on column, according to the manufacturer’s recommendations. RNA concentrations and 260/280 and 260/230 ratios were measured with a Nanodrop. RNA quality indicator (RQI) was measured using Bio-Rad Experion (Bio-Rad) with Eukaryote Total RNA StdSens assay, according to the manufacturer’s protocol. 500 ng RNA were reverse-transcribed to cDNA in duplicates with the High-Capacity RNA-to-cDNA kit (Applied Biosystems), according to the manufacturer’s recommendations.
Gene expression analysis

TaqMan assays (Applied Biosystems) for GFAP (Hs00909233_m1), OPRK1 (Hs00175127_m1), PDYN (Hs00225770_m1), POLR2A (Hs00172187_m1), RBFOX3 (Hs01370653_m1), and RPLP0 (Hs99999902_m1) were used. cDNAs were mixed with TaqMan assay and iTaq Universal Probes supermix (Applied Biosystems) for qPCR with a CFX96 Real-Time Detection System (Bio-Rad), according to the manufacturer’s instructions. Levels of each gene of interest mRNA were normalized to geometric mean of expression levels of two control genes POLR2A and RPLP0 selected by geNORM program (https://genorm.cmgg.be/)42 (see also our studies35,43,44). In each experiment, internal control gene-stability measure M42 was controlled for and did not exceed the limit of 0.5.

Radioimmunoassay (RIA)

The procedure has been described elsewhere45,46. Briefly, 1 M hot acetic acid was added to finely powdered frozen brain tissues, and samples were boiled for 5 min, ultrasonicated and centrifuged. Tissue extracts were run through SP-Sephadex ion exchange C-25 column, and peptides were eluted and analyzed by RIA. Anti-Dyn B antiserum showed 100% molar cross-reactivity with Big DYN, which consists of Dyn A and Dyn B sequences, 0.8% molar cross-reactivity with Leu-morphine (29 amino acid C-terminally extended Dyn B), and <0.1% molar cross-reactivity with Dyn A (1–17), Dyn A (1–8), α-neoendorphin, and Leu-enkephalin47.

Isolation of cell nuclei

Tissue samples were Dounce homogenized in the lysis buffer (0.32 M sucrose; 5 mM CaCl2; 3 mM magnesium acetate; 0.1 mM EDTA; 10 mM Tris-HCl, pH 8.0; 0.1% Triton X-100; 1 mM DTT). Homogenized samples were gently suspended in sucrose solution (1.7 M sucrose; 3 mM magnesium acetate; 1 mM DTT; 10 mM Tris-HCl, pH 8.0), and layered onto a sucrose cushion. Ultracentrifugation was carried out at 30,000 × g for 2.5 h at 4 °C (Beckman; L8-70 M; SW28 swing bucket rotor). After centrifugation, the supernatant was removed by aspiration. Nuclei pellets resuspended in PBS were filtered through a 40 μm Nitex mesh to remove remaining clumps.

Flow cytometry

Neuronal nuclei were isolated by fluorescence-activated nuclei sorting (FANS) after labeling with neuron-specific monoclonal antibody against NeuN (MAB377, Millipore). NeuN antibodies conjugated with mouse IgG labeling reagent (Alexa 488, Molecular Probes) were incubated with nuclear suspension for 30 min in the dark and directly sorted in the RLT lysis buffer (Qiagen). FANS was performed using a FACSARia III cell sorter (BD BioSciences), nuclei were pelleted by centrifugation at 3000 x g for 5 min at 4 °C, and stored at −80 °C. To ensure sorting of single but not aggregated nuclei preparations were stained with Hoesch dye, and a gate was set to isolate singlets only that were readily discerned from doublets, triplets, and higher-order aggregates based on their fluorescence intensity. The purity of neuronal nuclei was confirmed by FANS analysis of the sorted preparations.

Droplet digital PCR

The assay was described elsewhere48. Total RNA from FANS-sorted nuclei was purified using RNeasy Plus Mini kit (Qiagen) and 15–60 ng RNA were reverse-transcribed with the High-Capacity RNA-to-cDNA kit (Applied Biosystems), according to the manufacturer’s recommendations. cDNAs

Table 1 Summary of demographic data and tissue characteristics of human subjects (for details, see Supplementary Table 1)

| N   | Age            | PMI     | pH       | RQI         | Neuronal proportiona |
|-----|----------------|---------|----------|-------------|-----------------------|
|     |                | n       |          |             | n.s.                  |
| 55  | 54.7 ± 9.1     | 30.0 ± 12.5 | 6.6 ± 0.2 | 8.1 ± 1.0   |                       |
| 53  | 56.2 ± 8.9     | 35.8 ± 15.3 | 6.5 ± 0.3 | 7.4 ± 1.3   |                       |
|    | P-value        |          |          |             | n.s.                  |
| 55  | 54.7 ± 9.1     | 28.5 ± 13.6 | 6.6 ± 0.2 | 8.1 ± 1.1   | 0.24 ± 0.04           |
| 53  | 58.6 ± 9.5     | 31.9 ± 15.3 | 6.5 ± 0.3 | 7.4 ± 1.3   | 0.21 ± 0.07           |
|    | P-value        |          |          |             | 0.017 ± 0.03          |

Values are means ± SD for each cohort; SD standard deviation, N a number of subjects, Age age in years, PMI post-mortal interval in hours, pH brain pH, RQI RNA quality indicator

Unpaired t-test was used to calculate P-values. n.s. not significant

aData are taken from our previous paper39. Neuronal proportion was quantified from epigenome-wide DNA methylation profiles
were mixed with TaqMan assay, ddPCR Supermix for Probes (Bio-Rad) and Droplet Generation Oil (Bio-Rad), partitioned into 14,000–17,000 droplets in QX200 Droplet Generator and used for PCR with T100 Thermal Cycler (Bio-Rad), according to the manufacturer’s instructions. The fluorescence intensity of the droplets was measured using the QX200 Droplet Reader (Bio-Rad). The data analysis was performed with QuantaSoft droplet reader software (Bio-Rad). mRNA amount was calculated using the Poisson statistics. The absolute transcript levels were expressed in mRNA copies per ng of total RNA. Correlation between PDYN expression levels obtained using ddPCR and qRT-PCR was positive and significant (Pearson \( R = 0.9, P = 0.0004 \)) (Supplementary Fig. 1).

Genotyping

SNPs were determined by SNP&SEQ Technology Platform at Uppsala University using Illumina HumanOmni5Exome-4v1 beadchip.

Computation of neuronal proportions

Genome-wide DNA methylation data for 482,421 CpGs in DNA from total tissue was profiled by SNP&SEQ Technology Platform at Uppsala University using Illumina Infinium HumanMethylation450 beadchip and processed using R package Cell Epigenotype-Specific CETSe mapper. CETSe predicts neuronal proportions from methylation levels of the top 10,000 marker CpGs, which demonstrated the most significant methylation differences between neuronal and non-neuronal DNA.

Statistical analysis

Statistical analysis was performed using R version 3.3.2 (https://www.R-project.org/). Statistica 13 (StatSoft Scandinavia, Sweden) was used for analysis of absolute expression levels by unpaired Student’s t-test and for sample size calculation of one-way ANOVA by statistical power analysis with target Type I error \( a = 0.05 \) and statistical power \( \beta = 0.8 \). For analysis of gene expression linear regression model adjusting for age, PMI, brain pH, RQI, and alcoholism followed by post hoc Tukey HSD test on least squares means was performed using car and lsmeans packages. Gene expression was adjusted for RBFOX3 mRNA levels as a surrogate measure of neuronal proportion and/or activity; it was further adjusted for neuronal proportion instead of RBFOX3 mRNA levels. An interaction effect between alcoholism and KOR (OPRK1) mRNA levels was also considered. Smoking, mean and total alcohol consumption, DSM-V severity of alcohol use disorder and four alcoholism-associated SNPs were added to the model one at a time to test if they can account for gene expression variance. Overly influential points with Cook’s distance ≥1.0 were removed from analysis of the above models. One subject was excluded as a biological outlier; and two subjects were excluded as statistical outliers. R package effects was used to construct effect displays (component and residual plots). Bootstrapped P-values, and bias-corrected and accelerated bootstrap percentile 95% confidence intervals (CI) for regression coefficients, both of which do not require the assumption of normality, were estimated using car package with \( R = 5 \times 10^3 \) resampled cases. Script to analyze data and generate figures is written using publicly available software R and is available from authors upon request. A significance level of \( P < 0.05 \) was accepted as statistically significant and all tests were two-tailed.

Results

Fifty-three DSM-IV alcoholic and 55 control subjects were analyzed in the study. Effects of alcoholism on the whole tissue levels of PDYN and OPRK1 mRNAs in dlPFC were examined after adjusting for demographical data and tissue characteristics including age, PMI, brain pH, and RQI (Table 1). We and others demonstrated that the number of neurons in dlPFC is markedly reduced in alcoholics. Tissue expression levels may differ between the subject groups due to changes in cell composition if genes of interest are transcribed in specific cell types. Therefore, neuronal proportion computed by using genome-wide DNA methylation data, and mRNA levels of neuronal marker RBFOX3, which correlates with neuronal proportion, were included as confounding factors in the analysis (for details, see ref. 39 and Table 1).

PDYN and OPRK1 gene expression in human dlPFC: absolute levels and cell-type specificity

Receptor activation depends on concentration of its ligands, which may be produced in shortage or in excess relative to the receptor. We examined whether a limiting factor in the DYN/KOR signaling in dlPFC may be defined at the level of PDYN or KOR (OPRK1) gene transcription by comparing absolute levels of PDYN and OPRK1 mRNAs by ddPCR using cDNA prepared from total dlPFC tissue samples from control subjects (Fig. 1a). The PDYN mRNA levels were significantly 2-fold lower compared to those of OPRK1 levels \( (t_{(18)} = -5.3864, P = 4.1 \times 10^{-5}; \) Fig. 1a). Considering rapid DYN degradation and relatively large extracellular volume in which the peptides are diluted, the concentration of DYNs in the vicinity of the receptor molecules should be low resulting in the receptors’ underactivation. Thus, transcriptional processes may already establish proportion in the expression of PDYN and KOR (OPRK1) genes rendering the DYN production to be a limiting factor in the DYN/KOR signaling in dlPFC.

Expression levels of PDYN and KOR (OPRK1) in dlPFC may depend on proportion of cell type(s), specifically neurons, transcribing these genes in the tissue. To
confirm neuronal expression of PDYN and OPRK1 in human dIPFC, we analyzed levels of PDYN and KOR (OPRK1) mRNAs by ddPCR in neuronal and non-neuronal cell nuclei isolated from this tissue samples by FANS with antibodies against NeuN, the neuronal marker transcribed from the RBFOX3 gene (Fig. 1b). mRNAs of RBFOX3 and glial fibrillary acidic protein (GFAP) genes, the neuronal and astrocyte markers analyzed as positive and negative controls were localized in neuronal and non-neuronal nuclei, respectively. Both PDYN and OPRK1 mRNAs were highly enriched, approx. 10-fold in neuronal compared to non-neuronal nuclei (Fig. 1b). Neuronal proportion and mRNA levels of neuronal marker NeuN (RBFOX3) were then included as covariates in statistical models for comparison of PDYN and OPRK1 tissue expression levels between pathological and control brain.

Effects of alcoholism on PDYN expression; influence of cell composition

We next examined whether the tissue PDYN expression levels calculated as the ratio to reference genes were affected by alcoholism. PDYN mRNA levels in alcoholics compared to controls were found to be significantly lower, 1.24-fold (main effect of alcoholism, mean and 95% CI estimated by bootstrap resampling, −0.133 [−0.220, −0.041], \(P = 0.004\); Fig. 2a). When adjusted for the levels of RBFOX3 mRNA, a neuronal marker, or for cell composition, PDYN mRNA levels in alcoholics compared with controls were lower, respectively, with a trend (−0.088 [−0.183, 0.019], \(P = 0.086\); 1.15-fold; Fig. 2b), or significantly (−0.166 [−0.285, −0.051], \(P = 0.006\); 1.29-fold; Fig. 2c). Inclusion of smoking status as covariate in statistical models did not affect significance of differences identified in dIPFC (for tissue levels: −0.122 [−0.217, −0.012], \(P = 0.019\) and when corrected for cell composition (−0.146 [−0.265, −0.025], \(P = 0.019\)). No effects of severity of alcohol use disorder (\(P = 0.834\)), mean lifetime alcohol consumption (\(P = 0.127\)), or lifetime alcohol consumption (\(P = 0.581\)) on PDYN expression were found. PDYN mRNA significantly correlated with RBFOX3 mRNA levels (main effect of RBFOX3 mRNA, mean and 95% CI, 0.197 [0.068, 0.340], \(P = 0.007\); Fig. 2d), but not with neuronal proportion (Fig. 2e). These results suggest that PDYN transcription is downregulated in dIPFC in alcoholics. This decrease does not depend on changes in cell composition, and consistently PDYN does not correlate with neuronal proportion. However, PDYN expression may be associated with that of neuronal marker NeuN (RBFOX3).

Effects of alcoholism on KOR expression; influence of cell composition

No significant effects of alcoholism on KOR (OPRK1) mRNA were evident for the tissue levels (Fig. 3a) and also the levels adjusted for RBFOX3 expression levels (Fig. 3b) and for cell composition (Fig. 3c). Inclusion of smoking status as covariate revealed no differences between the subject groups. No effects of severity of alcohol use disorder (\(P = 0.768\)), mean lifetime alcohol consumption (\(P = 0.415\)), or lifetime alcohol consumption (\(P = 0.479\)) on OPRK1 expression were found. No significant correlation was found between OPRK1 and RBFOX3 mRNA levels.

**Fig. 1 Expression of the PDYN and KOR (OPRK1) genes in human dIPFC.** a Absolute levels of PDYN and OPRK1 mRNAs measured by ddPCR using total tissue RNA (n = 10 control subjects), presented as a number of mRNA copies/ng of total RNA. Student’s t-test, ***\(P < 0.001\). In box plots, middle line is the median, box spans the interquartile range (IQR), and whiskers extend 1.5 × IQR from box limits. b Neuronal expression of PDYN and OPRK1 genes in dIPFC. Levels of RBFOX3 (neuronal marker), PDYN, and OPRK1 mRNAs are high in neuronal nuclei (NeuN+), while those of GFAP (astrocyte marker) in non-neuronal (NeuN−) nuclei. Bar graphs show average mRNA amount ± S.E.M. in NeuN+ and NeuN− fractions as % of total amount of these mRNAs. Nuclei were isolated by FANS individually from dIPFC tissue of n = 3 (used for analysis of PDYN, RBFOX3, and GFAP mRNAs) or n = 2 (used for analysis of OPRK1 mRNA) subjects. mRNA levels were analyzed by ddPCR and normalized to total RNA content.
Fig. 2 Effects of alcoholism on the tissue PDYN expression levels, and the expression levels controlled for cell composition in human dlPFC. 

**a** PDYN mRNA levels were significantly lower in alcoholics ($P = 0.004$). **b** Expression of PDYN controlled for RBFOX3 mRNA levels differed between the two groups with a trend ($P = 0.086$). **c** Expression of PDYN controlled for neuronal proportion was significantly lower in alcoholics ($P = 0.006$). **d** Relationship between PDYN and RBFOX3 mRNA levels; correlation was positive and significant ($P = 0.007$). **e** PDYN and neuronal proportion did not significantly correlate ($P = 0.453$). Data from the cohort of 55 controls and 53 alcoholics (**a, b, d**), or the cohort of 35 controls and 30 alcoholics (**c, e**) were analyzed via linear regression. mRNA levels are shown in arbitrary units. In box plots middle line is the median, box spans the interquartile range (IQR), and whiskers extend 1.5 × IQR from box limits. Lines and shading represent the estimated slopes and 95% confidence intervals, respectively. **$P < 0.01$ by ordinary bootstrap with $5 \times 10^5$ nonparametric resampling of cases**.
(Fig. 3d), and OPRK1 mRNA levels and neuronal proportion (Fig. 3e). These data imply that the number of neurons producing PDYN or KOR is not proportional to the total number of neurons in dlPFC, and that the PDYN and KOR expressing neuronal subtypes and total neuronal population may be differentially affected by alcoholism.

**Correlation between PDYN and KOR**

The PDYN and OPRK1 genes may be co-expressed (i.e., transcriptionally co-regulated) and their co-expression pattern may be affected upon transition from normal to alcoholic state. To address this hypothesis we compared the slopes of regression lines for PDYN and OPRK1 mRNA levels between controls and alcoholics. PDYN and OPRK1 mRNAs correlated with a trend (main effect of OPRK1 mRNA, 0.309 [−0.003, 0.639], P = 0.061; Fig. 4a), while interaction effect of alcoholism on PDYN—KOR correlation was not significant. Thus, correlation between PDYN and OPRK1 expression did not differ between alcohols and controls; the slope of regression line was similar in controls and alcoholics. The effect of alcoholism was not sensitive to (i) changes in cell composition assessed using neuronal marker NeuN (RBFOX3) or neuronal proportion, and to (ii) smoking status. This suggests a low level of coordination in regulation of these two genes both in controls and alcoholics.

**Correlation between PDYN mRNA and dynorphin B, a mature peptide PDYN product**

PDYN mRNA correlated with dynorphin B, the peptide product of this gene (main effect of PDYN mRNA, 0.960 [0.550, 1.400], P = 0.0001; Fig. 4b), while no differences in the level of this endogenous kappa-agonist were found between alcoholics and controls (main effect of alcoholism, 0.052 [−0.128, 0.280], P = 0.605).

**Effects of alcoholism-associated SNPs on PDYN and OPRK1 expression in controls and alcoholics**

The level of alcohol-dependent activation of endogenous opioid transmission might be genetically determined. We therefore examined whether adaptive PDYN responses to alcohol may be modulated by PDYN SNPs associated with alcoholism including promoter SNP rs1997794, and SNPs rs6045819 and rs2235749 located in coding exon 4 and 3′ untranslated region (3′-UTR) of the gene. PDYN promoter SNP (rs1997794) was also found to be associated with better episodic memory scores in elderly humans, and may form non-canonical AP-1 binding site and influence gene expression in human brain.

The Fisher’s exact test revealed no significant difference in distribution of PDYN promoter SNP rs1997794 genotypes (P = 0.329) between alcoholics and control subjects (CC and CT genotypes vs. TT genotype; subjects with the C, high-risk genotype were pooled); or other SNPs (rs6045819, P = 0.528; rs2235749, P = 0.679; rs6985606, P = 0.326).

Two-way ANCOVA with group (controls vs. alcoholics) and PDYN promoter SNP rs1997794 genotype as between factors revealed a significant effect of group × genotype interaction (−0.179 [−0.320, −0.030], P = 0.016). Main effect of genotype was significant (0.186 [0.089, 0.287], P = 0.0003), while main effect of alcoholism was not significant (−0.022 [−0.119, 0.077], P = 0.659). For the combined CC and CT genotypes post hoc t-test showed downregulation of PDYN (1.37-fold; P = 0.0002) in alcoholics (Fig. 4c). The effect of alcoholism × genotype interaction was not sensitive to changes in cell composition assessed using neuronal marker NeuN (RBFOX3) (−0.148 [−0.289, −0.0002], P = 0.044). No other SNPs tested significantly affected PDYN or OPRK1 expression between controls and alcoholics.

**Discussion**

The main finding of our study is downregulation of PDYN expression in dlPFC of alcoholics. DYNs have a role in learning and memory acquisition, while their elevated levels are deteriorating for cognitive processes. Impairments of spatial learning and memory by ethanol exposure were found to be mediated through activation of the KOR. KOR antagonists abrogated alcohol-induced pathological processes. In this framework, the decrease in PDYN expression in dlPFC may be interpreted as adaptation that may counteract cognitive decline developed over the years of heavy alcohol drinking and withdrawal.

Postmortem studies of PDYN and KOR (OPRK1) regulation in addicted human brain are limited in a number and mostly focused on the striatal subregions. PDYN was found to be downregulated in NAc core in heroin addicts, while no changes in PDYN or DYN and KOR protein expression in NAc of cocaine addicts were revealed. The PDYN and KOR (OPRK1) gene expression and DYN levels were reported to be elevated in dorsal striatum of cocaine addicts, while the PDYN mRNA levels were decreased in dorsal striatum in alcoholics and in caudate nucleus of cocaine addicts and in caudate nucleus of cocaine addicts carrying PDYN SNP variant associated with cocaine/alcohol codependence. The absence of consistency in these data is likely due to underpowered postmortem human brain analysis in some of these works. Sample size calculation for PDYN expression levels in alcoholics and controls demonstrated that analyses with n ≤ 30, including our pilot study with 14 alcoholics and 14 controls, which were not overlapping with the current sample, and which suggested modest elevation in PDYN expression in dlPFC of alcoholics, are likely underpowered. “Medium” size effects may be detected with n = 90 or larger sample size. In this study,
Fig. 3 Effects of alcoholism on the tissue KOR (OPRK1) expression levels, and the expression levels controlled for cell composition in human dlPFC. a OPRK1 mRNA levels were not altered in alcoholics ($P = 0.802$). b Expression of OPRK1 controlled for RBFOX3 mRNA levels was not different between the two groups ($P = 0.403$). c Expression of PDYN controlled for neuronal proportion was not significantly different in alcoholics ($P = 0.863$). d Relationship between OPRK1 and RBFOX3 mRNA levels; no significant correlation was found ($P = 0.454$). e Relationship between OPRK1 and neuronal proportion; no significant correlation was found ($P = 0.792$). Data from the cohort of 55 controls and 53 alcoholics (a, b, d) or the cohort of 35 controls and 30 alcoholics (c, e) were analyzed via linear regression. mRNA levels are shown in arbitrary units. In box plots middle line is the median, box spans the interquartile range (IQR), and whiskers extend 1.5 × IQR from box limits. Lines and shading represent the estimated slopes and 95% confidence intervals, respectively. $P$-values were estimated by ordinary bootstrap with $5 \times 10^5$ nonparametric resampling of cases.
we aimed to detect “large” effects by ensuring final sample size $n \geq 90$. Totally 108 subjects were analyzed that is the largest cohort of alcoholics and controls investigated to date. In addition, we applied a more stringent statistical analysis that included available confounding factors (age, PMI, brain pH, RQI, and smoking status). Alcoholism is associated with the decline in neuronal adaptations in dlPFC and dorsal striatum in alcoholics is a limiting factor in the DYN/KOR signaling, while its expression may be a causal characteristic of the analyzed population of alcoholics, who were males of European descent. Importantly, the in vitro study supports this notion by demonstrating a significant decrease in the expression of $PDYN$ in human neuroblastoma SH-SY5Y cells following exposure to ethanol.

This study focuses on changes in expression and co-expression levels of the $PDYN$ and KOR genes in alcoholics. Statistical analysis did not reveal effects of cell composition, suggesting that the observed expression differences may be caused primarily by adaptations in transcriptional mechanisms. These transcriptional changes may have functional consequences contributing to the downregulation of the DYN/KOR circuits in dlPFC of addicted individuals. In dlPFC the absolute expression levels of $PDYN$ were significantly lower compared to those of KOR ($OPRK1$). Thus, $PDYN$ expression may be a limiting factor in the DYN/KOR signaling, while its downregulation suggests the diminished efficacy of the DYN/KOR signaling in dlPFC of human alcoholics.

Development of drug/alcohol dependence may be viewed as a maladaptive habit formation during transition from recreational to compulsive use that is associated with a diminishing cognitive control over drug seeking and taking behavior $^{27,29,64-66}$. These processes are characterized by a shift from prefrontal cortical to striatal control over drug/alcohol use, and a progression from the ventral to dorsal striatum in the addicted brain $^{64,67}$. This shift is associated with the downregulation of the DYN/KOR system in both dlPFC (present study), and caudate and putamen $^{44}$. Whether the paralleled DYN/KOR adaptations in dlPFC and dorsal striatum in alcoholics is a part of molecular processes underlying transition to the addictive state is a subject for future studies.
Associations between alcohol dependence and nine SNPs in the PDYN promoter and 3′-UTR and five SNPs in intron 2 of OPRK1 were previously reported56. We selected three PDYN SNPs (rs1997794 located in the promoter, rs6045819 in coding exon 4, and rs2235749 in 3′-UTR) and one OPRK1 SNP (rs6985606 located in intron 2) with highest significance of association (with P < 0.01) for analysis. PDYN promoter SNP rs1997794 and also 3′-UTR SNP rs910080 (located in the haplotype block with rs2235749) were also found to be associated with better episodic memory scores in elderly humans22. PDYN promoter SNP rs1997794 associated with alcoholism56,57 may form non-canonical AP-1 binding site and influence gene expression in human brain58,68.

For PDYN promoter SNP rs1997794, a significant effect of group × genotype interaction and significant main effect of genotype were found. PDYN downregulation was significant in the subgroup of subjects carrying C, high-risk allele of PDYN SNP rs1997794. This finding corroborates previous findings for the putamen, where PDYN mRNA and Leu-enkephalin-Arg, a peptide derived from PDYN, were significantly downregulated in the same subgroup of subjects44. No other SNPs tested significantly affected PDYN or OPRK1 expression between controls and alcoholics.

As limitations, the identified associations may be only applicable to males of European descent because no female subjects and no other ethnic groups were analyzed. Human subjects were paired between two groups according to age, PMI, and brain pH and all confounding factors (age, PMI, brain pH, RQI, smoking status) were included in the analysis (Supplementary Table 1). The postmortem human findings may be interpreted in two ways, either as molecular adaptations in DYN/KOR expression patterns caused by heavy alcohol consumption and withdrawal or as manifestation of inherited molecular differences between controls and alcoholics.

The important limitation of the study is the absence of experimental background for functional implementation of the molecular findings at both molecular/cellular and behavioral levels. While PDYN mRNA correlated well with dynorphin B, the peptide product of this gene, no differences in the level of this endogenous kappa-agonist were found between alcoholics and controls. The DYN levels are regulated though synthesis and trafficking of their protein precursor molecule, processing of this molecule to mature opioid peptides, and release and degradation of the peptides. Furthermore there is a mismatch in anatomical localization of mRNA and mature peptide products. This mismatch occurs due to trafficking of the protein precursor molecules along neuronal projections to axon terminals in other brain areas, where these molecules are processed to mature DYNs47. These processes may be affected in alcoholic brain and, while not destroying the correlation, may shadow less pronounced differences between alcoholics and controls. We did not analyze relationship between the adaptations in the DYN/KOR system and cognate status of the individuals under study due to the absence of available pre-mortual data. The functional consequence of PDYN downregulation in human alcoholics could be explored by analyzing the binding potential of selective kappa-opioid ligands, the development of which is rapidly progressing69.

In summary, our findings support the notion that the DYN/KOR signaling is dysregulated in human alcoholics. Chronic effects of various addictive substances may cause similar area-nonspecific transcriptional adaptations—the common downstream molecular syndrome mediating the lasting nature of the addictive state66. Downregulation of DYNs in dIPFC (present study), and dorsal striatum of alcoholics44 and cocaine addicts57 may be a part of this general adaptive mechanism.

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Conflict of interest
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