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MicroRNA-21: expression in oligodendrocytes and correlation with low myelin mRNAs in depression and alcoholism

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Abstract

MiR-21 is a microRNA implicated in cancer, development, and cardiovascular diseases and expressed in the central nervous system (CNS), especially after injury. However, the cellular expression of miR-21 in the adult CNS has not been clearly established either in mice or human subjects, while its alteration in psychiatric disorders is unknown. MiR-21 expression was characterized in reporter mice expressing β -galactosidase (LacZ) under the endogenous miR-21 promoter (miR-21/LacZ). Brain co-localization of miR-21/LacZ with specific neural markers was examined by double immunofluorescence in reporter mice, while extent of immunostaining for myelin basic protein and PDGFR α was determined in miR-21 knockout and wild-type mice. Levels of miR-21, and mRNAs of selected miR-21 targets, miR-21 regulator STAT3 and myelin-related proteins were measured by qRT-PCR in the white matter (WM) adjacent to the left postmortem orbitofrontal cortex (OFC) of human subjects with major depressive disorder (MDD), alcoholism, comorbid MDD plus alcoholism (MDA) and non-psychiatric control subjects. MiR-21/LacZ was highly expressed in cell bodies of WM and myelinated portions of gray matter (GM). Labeled cell bodies were identified as oligodendrocytes, while miR-21/LacZ was barely detectable in other cell types. MiR-21, as well as the mRNAs of several myelin-related proteins, were reduced in the WM of subjects with MDD and alcoholism. MiR-21 positively correlated with mRNA of myelin-related proteins and astrocytic GFAP. High expression of miR-21 in adult oligodendrocytes and the correlation of miR-21 decrease with mRNA of some myelin proteins,

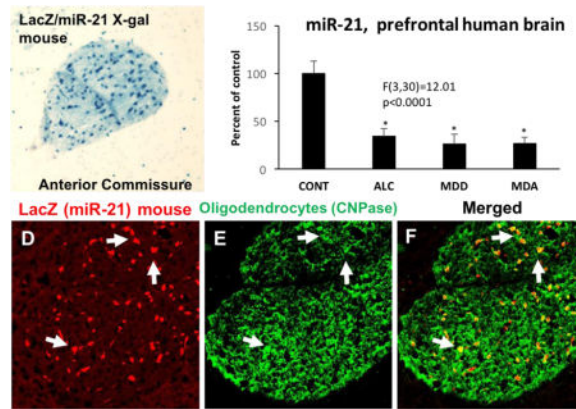
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regulator STAT3, and oligodendrocyte-related transcription factors suggest an involvement of miR-21 in WM alterations in depression and alcoholism.

Graphical Abstract



Keywords

Glia; prefrontal; miR-21; astrocytes; mRNA; knockout; postmortem; human; mouse

1. INTRODUCTION

MicroRNAs (miRNAs) are small non-coding RNAs, 22-nucleotides long, that are mostly generated from longer RNA precursors (Iwakawa & Tomari, 2015). However, miRNAs are capable of post-transcriptionally regulating the translation of target proteins by binding to specific complementary sequences in protein-encoding mRNAs (Bushati & Cohen, 2007; Valencia-Sanchez, Liu, Hannon, & Parker, 2006).

Some miRNAs are constitutively expressed in differentiated cells, including CNS cells (Krichevsky, King, Donahue, Khrapko, & Kosik, 2003; Nelson et al., 2006; Ziats & Rennert, 2014), but most vary their expression following cell injury, significant physiological alterations or during developmental transitions (Krichevsky et al., 2003; Liu & Xu, 2011). In the CNS, specific miRNAs fluctuate in neurons (Jovicic et al., 2013), astrocytes and oligodendrocytes (Lau et al., 2008) following injury or metabolic stress (Feng & Feng, 2011; Liu & Xu, 2011). More recently, miRNA alterations in particular brain regions have been associated with psychiatric disorders such as depression, schizophrenia (Dwivedi, 2014; Kocerha, Dwivedi, & Brennand, 2015; Smalheiser et al., 2014) or alcoholism (Rajesh C. Miranda, 2014; R. C. Miranda et al., 2010), suggesting pathophysiological involvement of miRNAs. MiRNAs in the GM of relevant brain regions such as the prefrontal cortex (PFC) (Serafini et al., 2014; Smalheiser et al., 2012; Smalheiser et al., 2014) and basal ganglia, or in the blood circulation have been linked to psychopathology or neurodegeneration (Dwivedi, 2016). Less is known, however, about the cellular localization of miRNAs in the WM and possible disturbances of WM miRNAs in psychiatric disorders.

Among pathologically relevant miRNAs, miR-21 has received considerable attention because miR-21 dysregulation is linked to inflammatory diseases (Lu & Rothenberg, 2013) and cancer, including gliomas (Krichevsky & Gabriely, 2009; Kumarswamy, Volkman, & Thum, 2011). Its role in cancer appears to derive from its ability to interfere with translation of apoptosis-promoting proteins (Buscaglia & Li, 2011). In addition, miR-21 is differentially expressed at various stages during CNS development, where it regulates the expression of proteins involved in cell growth and survival (Sathyan, Golden, & Miranda, 2007). Altered miR-21 expression also occurs in some neurological disorders and their animal models (J. Peng et al., 2013; Yelamanchili, Chaudhuri, Chen, Xiong, & Fox, 2010). Likewise, prolonged alcohol exposure drastically reduces the levels of some miRNA species, including miR-21, in developing neural cells (Balaraman, Winzer-Serhan, & Miranda, 2012). By contrast, increased miR-21 expression may be neuroprotective in animal models of ischemia and traumatic brain injury (Buller et al., 2010; Ge et al., 2014). However, it remains to be established whether detectably high miR-21 expression is conspicuous in specific cell types of adult animals or is related to particular pathological or developmental stages (Kumarswamy et al., 2011).

Most miR-21 studies in the CNS have targeted GM or entire brain regions. However, despite mounting interest in WM glial pathology in psychiatric disorders (Tham, Woon, Sum, Lee, & Sim, 2011), little is known about the expression of miR-21 in these cells or whether miR-21 is changed in psychiatric disorders such as depression and alcoholism, in which alterations in myelin proteins and in patterns of white matter-related connectivity have been identified (J.M. Lewohl et al., 2000; J. M. Lewohl, Wixey, Harper, & Dodd, 2005; H. J. Peng et al., 2013; Rajkowska et al., 2015; Rosenbloom, Sullivan, & Pfefferbaum, 2003). Here we describe the rich expression of miR-21 in oligodendrocytes of reporter mice that express the reporter gene β -galactosidase (LacZ) under the endogenous miR-21 promoter, and identify changes in oligodendrocyte markers in miR-21 knockout mice. In addition, we report miR-21 expression levels as well as mRNA levels for miR-21 targets, miR-21 regulator STAT3 and oligodendrocyte-related proteins in the white matter of human subjects with depression, alcoholism or comorbid depression plus alcoholism as compared to non-psychiatric controls.

2. METHODS

2.1 Animals

MiR-21 reporter mice (miR-21-lacZ) were generated by crossing *Mir21a^{tm1Mtm}* mice (Jackson Labs # 036060), a knock-in mouse strain that expresses the β -galactosidase (lacZ) reporter gene under the endogenous miR-21 promoter (Park et al., 2012) with CAG-Cre mouse (Sakai & Miyazaki, 1997) to remove the neomycin-miR-21-loop loxP-flanked cassette to avoid interference in the β -galactosidase expression. miR-21-lacZ mice were backcrossed for more than 10 generations into C57BL/6J genetic background. miR-21-lacZ were maintained as a heterozygous strain carrying one copy of WT miR-21 gene in the complementary chromosome. The generation of miR-21 knockout mice (miR21KO) has been reported (Lu et al., 2011). MiR21KO mice were backcrossed for more than 10 generations into C57BL/6N genetic background, and maintained heterozygous.

Homozygous miR21KO and WT littermates were generated by breeding miR21KO heterozygous animals.

2.2 β -galactosidase histochemistry

Twelve-week old male miR-21-lacZ mice were anesthetized and intracardially perfused with saline (0.9% NaCl). Tissues were embedded in OCT and 8 μ m sections processed for β -galactosidase histochemistry using standard techniques (Saunders, 2003).

2.3 Immunohistochemistry

Twelve-week old male mice were anesthetized and intracardially perfused with saline (0.9% NaCl) followed by freshly prepared 4% paraformaldehyde in PBS. Tissues were removed, postfixed with 4% paraformaldehyde, loaded with 30% sucrose in PBS by immersion, embedded in OCT and 10- μ m thick sections were obtained and mounted on slides.

For LacZ immunodetection we used a primary antibody raised in chicken because distribution of its binding closely matched the X-gal signal for miR-21/LacZ, (see Supplemental materials for information on antibody choice). The cellular phenotype of cells positive for LacZ double-labeling experiments was determined by simultaneous incubation with the LacZ antibody and antibodies to either Neuron Nuclear antigen (NeuN), CNPase, Iba1 or GFAP (all antibodies raised in species different from chicken). After washing in Tris buffer saline (pH 7.6), species-specific fluorescent secondary antibodies were used to identify binding to LacZ and either of the other primary antibodies. Dual fluorescence was assessed at a Nikon NC1 laser-scanning confocal microscope.

Effects of miR-21 absence on PDGFR α and MBP immunohistochemistry were measured in four 12 weeks-old miR21KO and four wild type mice according to immunohistochemical and cell counting procedures described in the Supplemental Information.

2.4 Human subjects

Collection of human brain samples was performed according to protocols approved by the Institutional Review Boards of the University Hospitals of Cleveland and the University of Mississippi Medical Center. The protocol included obtaining written consent from next-of-kin for retrospective interviews to compile relevant diagnostic information according to procedures detailed in previous publications (Miguel-Hidalgo et al., 2014; Whittom et al., 2014). Blocks of postmortem tissue were collected during autopsy at the Cuyahoga County Medical Examiner's Office, Cleveland, Ohio. Subjects with evidence of neurological injury or disorder, prolonged agonal states or coma were excluded from the study. Weight of the whole brain and postmortem delay (time from death to collection of brain sample) were determined. Subjects included in this study belonged into 4 diagnostic groups: Non-psychiatric comparison subjects (n=10), subjects with alcohol dependence (n=9), subjects with major depressive disorder (MDD, n=10) and subjects with dual diagnosis of MDD and alcohol dependence (MDA, n=10). Table 1 presents summaries of the age at time death, sex, brain pH, brain weight, postmortem delay and mode of death (suicide vs. non-suicide). Estimates of onset and duration of the psychiatric diagnoses are also shown in Table 1.

2.5 Collection of brain tissue sections for miRNA and RNA determinations

Blocks containing the OFC and the adjacent WM were immediately frozen at autopsy and stored at -80°C . Two pH values were obtained per brain using frozen cerebellar tissue.

Sections containing OFC WM were cut at a thickness of $50\ \mu\text{m}$, and $5\ \text{mm}$ -diameter punches were collected from the sectioned WM adjacent to cortical area 47. This area was identified according with published morphological criteria (Uylings et al., 2010). Levels of miR-21 and mRNAs of interest were measured in brain tissue as detailed in the Supplemental Information.

2.6 *In situ* hybridization

Formalin-fixed paraffin embedded $5\ \mu\text{m}$ thick tissue sections were dewaxed in xylenes, and rehydrated through a standard ethanol dilution series. Tissue sections were digested with $5\ \mu\text{g}/\text{mL}$ proteinase K for 10 minutes at room temperature, and then loaded onto Ventana Discovery Ultra (Ventana Medical Systems) for *in situ* hybridization analysis. The tissue slides were hybridized with 5' - and 3' -double digoxigenin-labeled miRCURY LNA microRNA detection probes (Exiqon; miR-21-5p: cat # 619870-360; U6: cat # 699002-360; scramble control: cat # 699004-360) for 2 hours at 50°C . Hybridized probes were detected with a polyclonal anti-DIG antibody and Alkaline Phosphatase-conjugated secondary antibody (Ventana Medical Systems) using NBT-BCIP as the substrate and counter stained with Nuclear Fast Red.

2.7 Statistical Analysis

The density of immunoreactive cells (cells/mm^3) or area fraction of immunoreactivity was statistically compared between wild type and KO mice by using two-tailed t-tests with significance evaluated at $p < 0.05$.

The effect of psychiatric diagnosis on miR-21 and different mRNAs was evaluated in postmortem samples of human subjects using analysis of covariance (ANCOVA) with age at the time of death, postmortem interval (time between death and freezing of brain samples), tissue pH, and brain weight at autopsy as covariates. Among the covariates examined, the mean age appeared to differ the most between controls and MDD subjects. Hence, ANOVA was used to evaluate age between the 4 groups, and for only controls and MDD subjects. There was no significant difference in age among the four groups ($F(3, 34) = 0.476$, $p = 0.701$) or between controls and MDD subjects alone ($F(1, 18) = 1.123$, $p = 0.285$). The co-variables considered in this study were included because they have been found to variably influence molecular parameters in previous studies of the human brain or WM. When ANCOVA showed significant diagnosis effect ($p < 0.05$) pairwise comparisons to controls were made using Dunnett's tests.

Pearson analysis of correlations between miR-21 levels and the levels of each of the mRNA species included in this study were Bonferroni-corrected by the number of comparisons (16 comparisons).

3. RESULTS

3.1 EXPRESSION of miR-21/LacZ IN REPORTER MICE

In miR-21 reporter mice expressing LacZ under the control of the endogenous miR-21 promoter (miR-21/LacZ mice) there was abundant detection of miR-21/X-gal signal in the WM and in cell bodies of myelinated parts of the GM all across the brain (Fig. 1). Closer inspection revealed that the strongest intensity of miR-21/LacZ expression corresponded to LacZ-positive cell bodies occurring all throughout the WM and myelinated parts of the GM (Fig. 2). To further confirm that the histochemical reaction catalyzed by X-gal activity corresponded to the localization of miR-21/LacZ we subjected coronal histological sections at several rostrocaudal levels to immunohistochemistry of LacZ using three different antibodies (see Methods section). We based our study on the polyclonal antibody raised in chicken against LacZ, which resulted in a distribution of immunostaining virtually identical to X-gal histochemistry staining in both white and gray matter (Figs. 3–5).

3.2 CELL TYPE IDENTIFICATION OF miR-21/LacZ POSITIVE CELL BODIES

The size and distribution of LacZ positive cell bodies in white and GM suggested that most of them corresponded to oligodendrocytes. To determine whether these cell bodies could also be labeled with antibodies to oligodendrocyte markers, alternating coronal sections at various rostrocaudal levels of the reporter mouse brain were labeled with primary antibodies to LacZ in combination with either antibodies to CNPase (marker of oligodendrocytes)(Fig. 3), CSPG4 (marker of NG2 cells, oligodendrocyte precursors) (Fig. 4A,B,C), GFAP (marker of astrocytes) (Fig. 4C,D,E), Iba1 (marker of microglia) (Fig. 4G,H,I) or NeuN (general marker for neurons) (Fig. 5). These double labeling experiments showed that miR-21/LacZ co-localized with CNPase in the great majority of CNPase positive cell bodies (Fig. 3), while most cells bodies identified with the primary antibodies to the other glial or neuronal markers were mostly devoid of miR-21/LacZ immunoreactivity. (Figs. 4 and 5). The presence of conspicuous miR-21/LacZ immunoreactivity in oligodendrocyte cell bodies was further ascertained with double-fluorescent immunolabeling using the antibody to LacZ in combination with an antibody to glutathione S-transaminase Pi, which is also known for labeling the cytoplasm of mature and early oligodendrocytes with a high degree of specificity (Tamura et al., 2007; Tansey & Cammer, 1991). Examination of sections containing the corpus callosum, anterior commissure or myelinated portions of the medial GM showed that many GSTPi cells were immunopositive for miR-21/LacZ (not shown).

3.4 MiR-21 *IN SITU* HYBRIDIZATION IN MOUSE AND HUMAN BRAIN

In the brain of wild-type mice, *in situ* hybridization of miR-21 resulted in the labeling of small size cell bodies in the WM that had morphological features of oligodendrocytes, as exemplified in the corpus callosum and anterior commissure (Fig. 6).

Using the same protocol as in the mouse brain, histological sections containing WM adjacent to the OFC of a 61 years-old male human subject were subjected to miR-21 *in situ* hybridization. As compared to hybridization with scrambled RNA probes, sections hybridized with miR-21-complementary probes demonstrated hybridization in relatively small cells in both white and GM as well as at the WM/GM boundary (Supplemental Figure

1 in Supplemental information file). In the human brain, cell bodies of labeled cells were mostly oval or rounded in shape and smaller than 10 μm in diameter, with morphologies consistent with those of oligodendrocytes or astrocytes, although, given that no cell type-specific markers were used, labeling of other cell types such as small-sized neurons cannot be entirely ruled out.

3.5 IMMUNOHISTOCHEMISTRY OF OLIGODENDROCYTE-RELATED PROTEINS IN miR-21 KO MICE

To determine whether miR-21 absence results in changes of myelinated fiber tracts we compared wild-type mice to mice with a full knock out of miR-21 (KO mice) using antibodies to platelet derived growth factor receptor alpha (PDGFR α , a marker of premyelinating oligodendrocytes) (Supplemental Figure 2), chondroitin sulfate proteoglycan 4 (CSPG4, a marker of oligodendrocyte precursor cells and premyelinating oligodendrocytes), and antibodies to myelin basic protein (MBP, the major myelin component of mature oligodendrocytes) (Supplemental figure 3). In sections of the corpus callosum and anterior cingulate cortex, the packing density of PDGFR α immunoreactive cells was significantly higher in miR-21 KO mice as compared to wild type mice ($t=4.77$, $df=6$, $p=0.009$) (Fig. 7A), but density of CSPG4-positive cells in KO mice was not significantly different from controls ($t=-0.90$, $df=6$, $p=0.42$) (Fig. 7B). In contrast, area fraction of MBP immunoreactive fibers localized to the anterior cingulate gyrus (where, unlike in the corpus callosum, fiber bundles can be resolved against a non-immunoreactive background) was significantly lower in KO as compared to wild type mice ($t=2.45$, $df=6$, $p=0.01$) (Fig. 7C).

3.6 DOUBLE-LABELING FOR miR-21/LacZ and PROTEIN TMEM49 (VMP1)

The 22-nt long miR-21 is generated from a longer pri-miR-21 sequence regulated by its own promoter. Pri-miR-21 DNA sequence and its promoter overlap with the TMEM49 sequence (also known as VMP1) both in the human and mice, with the promoter sequences for pri-miR-21 being located in intron 10 of the same protein (Cai, Hagedorn, & Cullen, 2004; Kumarswamy et al., 2011). We found very scarce to no evidence for co-localization of miR-21/LacZ with TMEM49 (see Supplemental information file).

3.7 miR-21 LEVEL IN THE OFC WM OF HUMAN NON-PSYCHIATRIC CONTROL SUBJECTS AND SUBJECTS WITH MAJOR DEPRESSION OR ALCOHOLISM

ANCOVA of RT-qPCR data revealed a significant effect of psychiatric diagnosis on miR-21 levels (Fig. 8) among human subjects with diagnoses of MDD, alcoholism (ALC), comorbid alcoholism plus MDD (MDA), and non-psychiatric comparison subjects ($F(3,29)=12.01$, $p<0.0001$). Pairwise contrasts revealed that miR-21 was significantly lower in each of the psychiatric groups as compared to controls (ALC vs control: $d=0.723$, $p<0.001$; MDD vs control: $d=-0.816$, $p<0.001$; MDA vs. control: $d=-0.807$, $p<0.001$).

3.8 mRNA LEVELS OF miR-21 TARGETS, STAT3 AND MYELIN-RELATED PROTEINS IN THE OFC WM IN DEPRESSION AND ALCOHOLISM

Among validated miR-21 targets, PTEN, BMFR, and APAF1 did not differ between control and diseased subjects ($F(3,29)=0.168$, $p=0.971$; $F(3,29)=1.3$, $p=0.327$; $F(3,29)=1.292$, $p=0.296$, respectively) (Fig. 9). MiR-21 levels were inversely correlated with levels of either APAF1 mRNA or PTEN mRNA when including all subjects (Table 2). Levels of mRNA of miR-21 targets SOX5 and TGF β R2 were reduced in the overall population of psychiatrically-diagnosed subjects ($F(1,31)=6.02$, $p=0.02$; $F(1,31)=7.221$, $p=0.012$, respectively) as compared to controls (Fig. 10A–D).

We also determined STAT3 mRNA levels in WM because STAT3 is a well-known upstream regulator of miR-21 (Ou, Li, & Kang, 2014). STAT3 was considerably decreased in psychiatric patients when all diseased subjects were combined as compared to non-psychiatric subjects ($F(1,31)=7.02$, $p=0.013$) (Fig. 10E, F). A positive correlation existed between STAT3 mRNA and miR-21 when including either all subjects or only subjects with psychiatric diagnoses (Table 2).

mRNA of MBP, PLP1, MAG, MOG, GJC2 (connexin 47), OLIG1, OLIG2 and CSPG4 (NG2), all proteins of either oligodendrocytes or their precursors, were also quantified in the OFC WM. ANCOVA showed that there was no significant effect of diagnosis on MBP, CSPG4 and OLIG 2 mRNA levels, but significant reduction of PLP1, MAG, MOG, and OLIG1 mRNA (PLP1 $F(3,29)=3.195$, $p=0.038$; MAG $F(3,29)=3.779$, $p=0.021$; MOG $F(3,29)=3.039$, $p=0.045$; OLIG1 $F(3,29)=3.917$, $p=0.018$) in the psychiatric groups (Figs. 10–12). Comparisons to controls (Dunnett's tests) showed significantly lower level of PLP1 mRNA in MDA subjects, and lower MAG mRNA in each of the psychiatrically diagnosed groups. GJC2 mRNA levels were lower in the overall group of psychiatric subjects ($F(1,31)=7.364$, $p=0.011$) (Fig. 13A, B).

Since astrocytes are abundant in the WM and have important roles in myelin maintenance, mRNA levels of astrocyte-related GJA1 (also known as connexin-43, which pairs GJC2 to form heterologous gap junctions with oligodendrocytes) and glial fibrillary acidic protein (GFAP) were also determined. ANCOVA revealed that the combined psychiatric subjects had significantly higher GJA1 mRNA than control subjects ($F(1,31)=4.321$, $p=0.047$) (Fig. 13C, D). GFAP mRNA was significantly different among the 4 diagnostic groups ($F(3,29)=3.557$, $p=0.026$) (Fig. 14), and Dunnett's tests demonstrated lower GFAP mRNA in MDD subjects as compared to controls (Fig. 14).

miR-21 mRNA levels in all subjects were positively and significantly correlated with GFAP mRNA but not with GJA1 mRNA levels (Table 2).

4. DISCUSSION

4.1 miR-21 expression in oligodendrocytes

Several microRNAs play crucial roles during the development of oligodendrocytes as well as in the formation of myelin (Fitzpatrick, Anderson, & McDermott, 2015; Jovicic et al., 2013). Dicer deletion causes oligodendrocyte damage and disturbs myelin formation (Shin, Shin,

McManus, Ptacek, & Fu, 2009). MiRNAs miR-335 and miR-9 are important for the differentiation of glial progenitors into oligodendrocyte precursors, immature and myelinating oligodendrocytes (Lau et al., 2008). However, less is known about miRNAs expressed in differentiated oligodendrocytes. The present data suggest that there are high miR-21 expression levels in the WM and myelinated portions of GM of the mouse CNS. Likewise, double immunofluorescence indicates that miR-21 is expressed in mature oligodendrocytes, but much less abundantly in other cell types. Nevertheless, it cannot be ruled out that smaller amounts of miR-21 are also present in other cell types, because miR-21 has been shown to be significantly induced in astrocytes, microglia or some neurons after specific treatments in cell cultures or in models of brain injury and neurodegeneration (Buller et al., 2010; Ge et al., 2014; Montalban et al., 2014; J. Peng et al., 2013; Sahni et al., 2010; Yelamanchili et al., 2010).

Consistent with the observations of high miR-21 expression in oligodendrocytes is that cell bodies with oligodendrocyte morphology were patent in the WM after miR-21 *in situ* hybridization in mice and, although sparser, in a non-psychiatric human subject. Thus, possible constitutive expression of miR-21 in oligodendrocytes of adult rodents and humans may lead to a degree of enduring regulation of miR-21 targets at the translational level. However, it is difficult to determine the precise impact of such a regulation in the expression of miR-21 target proteins without further investigating the concurrent activity of other factors that control the transcription of specific mRNAs and their translation into target proteins.

MiR-21 expression during development, in cancerous cells, or after brain injury has been linked to increases in cell survival or resilience (Buller et al., 2010; Buscaglia & Li, 2011; Ge et al., 2014; Ou et al., 2014). Since abundant miR-21 expression in adult oligodendrocytes apparently occurs in the absence of developmental or pathogenic challenges, there is the possibility that the normal function of oligodendrocytes or the interactions of these with their environment require continuous activation of survival-related mechanisms involving miR-21. However, as shown in miR21KO mice, mere absence of miR-21 might be insufficient to dramatically affect development, myelin formation and general behavior of mice. The possibility still remains that under specific challenges constitutive miR-21 may buffer the effects of deleterious alterations of impulse conduction caused by oligodendrocyte pathology. In the same vein, it is possible that putative behavioral alterations caused by miR-21 reduction are only detectable after specific physiological challenges or exposure to stressing circumstances. Thus, it remains to be ascertained whether miR-21 depletion alters behavior or whether challenging cognitive or behavioral conditions in the absence of oligodendrocyte miR-21 may lead to significant cognitive or behavioral abnormalities. Studies using such challenges will be necessary to determine the role of elevated miR-21 expression in CNS oligodendrocytes.

4.2 Downregulation of miR-21 and myelin-related proteins in the human OFC WM in depression and alcoholism

Low miR-21 in the human prefrontal WM appears to be associated with the diagnoses of depression and alcoholism. Low miR-21 in these disorders would be consistent with miR-21

involvement in mechanisms of myelin dysregulation, and possibly with connectivity alterations such as those observed in functional and DTI neuroimaging studies of both depression (Fox, Buckner, White, Greicius, & Pascual-Leone, 2012; Korgaonkar, Cooper, Williams, & Grieve, 2012; H. J. Peng et al., 2013; Tham et al., 2011; Zeng et al., 2012; Zhu et al., 2012) and alcoholism (McQueeney et al., 2009; Rosenbloom et al., 2003; Schulte, Muller-Oehring, Pfefferbaum, & Sullivan, 2010). Consistent with this suggestion, several myelin-related mRNAs were also reduced in the WM of psychiatric groups, while significant positive correlations of miR-21 levels and levels of oligodendrocyte proteins such as the major myelin structural protein PLP1, were observed even when the correlation only included psychiatric subjects. Low PLP1 was also found in a different cohort of MDD and control subjects (Rajkowska et al., 2015), indicating that PLP1 mRNA dysregulation in the OFC WM may be a feature of WM pathology in MDD and possibly in alcoholism.

The reduced area fraction of MBP-immunoreactive fibers in the anterior cingulate cortex and the increased density of PDGFR α -positive cells in the corpus callosum of the miR21 KO mice are consistent with an involvement of miR-21 in myelin disturbances. Interestingly, the level of MBP mRNA appears to be unchanged in the OFC WM of human diseased subjects as compared to controls in this study, which agrees with unchanged MBP mRNA found also in the OFC WM of a different cohort MDD subjects (58). Since the levels of PLP1, but not of MBP, were found to be significantly and strongly correlated with miR-21 levels in the human OFC WM, it is possible that miR-21 is associated with downregulation of PLP1 and not MBP. In miR-21 KO mice, we used MBP as a marker for myelin, finding that miR-21 knockout resulted in a reduction in the area fraction of MBP-immunoreactivity. It remains to be determined how the structural alteration of MBP-positive fibers may relate to changes in the levels of MBP mRNA and protein or to PLP1 changes in miR21 KO mice and the human prefrontal WM and how miR-21 could be related to the regulation of those two main myelin proteins.

Low mRNA levels of miR-21 targets SOX-5 and TGFBR2 in the aggregated psychiatric subjects may indicate disrupted regulation of myelin formation (Palazuelos, Klingener, & Aguirre, 2014; Wegner, 2008), particularly on the case of Sox5. Sox5 together with other Sox-family factors regulates oligodendrocyte differentiation (Wegner, 2008). In this process, the expression of PDGFR α in oligodendrocyte precursors and of MBP and PLP1 in differentiated astrocytes is affected by SOX5, SOX 6 and SOX10 (Stolt et al., 2006). SOX5 and SOX6 activity results in delayed MBP and PLP1 expression and the persistence of oligodendrocyte precursors as undifferentiated, while absence of those factors rather favors premature oligodendrocyte differentiation. It remains to be determined whether a putative SOX5 decrease in depression and alcoholism affects oligodendrocyte differentiation, and how the low SOX5 level may interact with low transcription factor OLIG1 (important positive factor in oligodendrocyte differentiation)(Snaidero & Simons, 2017), and with low miR-21 to result in oligodendrocyte pathology.

miRNAs other than miR21 may also regulate oligodendrocyte markers and transcription factors and could additively or synergistically act with miR-21 in the regulation of oligodendrocyte pathology. For example, miR-219 and miR-338 suppress the expression of PDGFR α and SOX5 (Simons & Nave, 2015), which is decreased in the human white matter

(mRNA), and white matter of the miR-21 KO mice (protein), respectively. Since SOX5 is also a target of miR-21 and the knock out of miR-21 alters PDGFR α positive cell numbers, it will important to determine the relative contribution of these other miRNAs and miR-21 in the regulation of myelin-related changes in psychiatric disorders.

Differences between psychiatric and control subjects, although suggestive, do not necessarily demonstrate that miR-21 is a mediator in myelin and oligodendrocyte alterations because both myelin and miR-21 disturbances may depend on other upstream pathogenic changes, as suggested by the lack of differences among groups in some of the major miR-21 targets. However, since several other miR-21 targets were not examined here, further study of these targets may be necessary to establish mechanisms affected by miR-21 reduction and their link to myelin maintenance.

Determining the mechanisms ultimately responsible for low miR-21 in depression will require further research in animal models and postmortem tissue. However, low WM miR-21 in alcoholism may be related to direct actions of prolonged alcohol exposure, as suggested by the effects of ethanol on cultured neocortical neurospheres (Balaraman et al., 2012; Sathyan et al., 2007). Alcoholism is also associated with significant reductions in the expression of myelin-related proteins and their mRNAs in human subjects (J.M. Lewohl et al., 2000; J. M. Lewohl et al., 2005). Hence, given the correlations between mRNAs of myelin-related proteins and miR-21 there may be a mechanistic link between miR-21 deficits and myelin dysregulation in alcoholism. The molecular factors responsible for reduced miR-21 in subjects with MDD (and without alcoholism) remain to be elucidated.

4.3 Correlation of miR-21 and mRNA levels of astrocyte proteins

Overall, there was strong correlation between miR-21 and GFAP mRNA, the major astrocyte intermediate filament protein, while GFAP mRNA was significantly lower in the psychiatric subjects than in controls. In addition, GJA1 mRNA level, which is expressed mainly by astrocytes in neocortical areas (Yamamoto, Ochalski, Hertzberg, & Nagy, 1990), was higher in the combined psychiatric subjects than in controls, but not correlated with miR-21. Differences between control and psychiatric subjects regarding GFAP and GJA1 mRNA levels in WM, although in opposite directions, are consistent with the known roles of astrocytes in support of oligodendrocyte maturation and physiology, and the known myelin-destabilizing effects of GFAP alterations or astrocyte gap junction disturbance (Li, Giaume, & Xiao, 2014; Liedtke et al., 1996; Magnotti, Goodenough, & Paul, 2011; Sargiannidou, Markoullis, & Kleopa, 2010; Stumpf et al., 2003). Whether the correlation of GFAP mRNA and miR-21 levels reflects a causal relationship between these two factors or just a dependence on other depression related alterations remains to be determined. In addition, since other studies have found miR-21 upregulation in astrocytes after brain injury (Bhalala et al., 2012; Ge et al., 2014), the exact contribution of astrocytes to miR-21 changes and its relevance to oligodendrocyte physiology remains to be established. Future studies in animal models and the human brain should determine the exact roles of miR-21 in WM oligodendrocytes, astrocytes or other glial cells and how they may contribute to connectivity alterations in depression and alcoholism.

5. Conclusion

MiR-21 was highly expressed in adult oligodendrocytes in the white matter and myelinated portions of the gray matter. Significantly low levels of miR-21 were found in the white matter adjacent to the orbitofrontal cortex of human subjects with depression and alcoholism as compared to non-psychiatric controls. The correlation of the miR-21 decrease with some mRNAs of myelin proteins, regulator STAT3, and oligodendrocyte-related transcription factors suggest an involvement of miR-21 in WM alterations in depression and alcoholism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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HIGHLIGHTS

- miR-21 is highly expressed in adult oligodendrocytes
- miR-21 level in orbitofrontal white matter is low in depression and alcoholism
- miR-21 correlates with low mRNA of PLP1, transcription factor OLIG1 and STAT3
- miR-21 may be implicated in the white matter pathology of depression and alcoholism

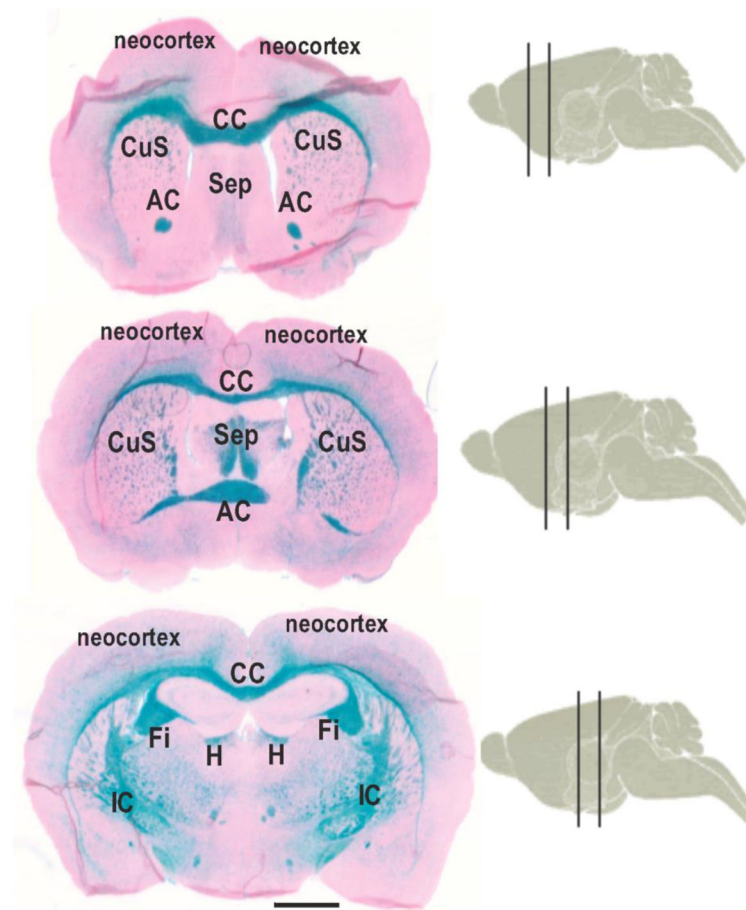


Fig. 1. X-Gal histochemistry in coronal sections of a miR-21/LacZ reporter mice at three different successive rostro caudal levels each level falling between two vertical lines drawn on sagittal cartoons of the mice brain. Note the abundant X-gal labeling (blue) in the major WM bundles of the mouse brain. **CC**: Corpus Callosum, **AC**: Anterior Commissure, **OT**: Olfactory Tract, **CuS**: Corpus Striatum, **Sep**: Septum, **IC**: Internal capsule.

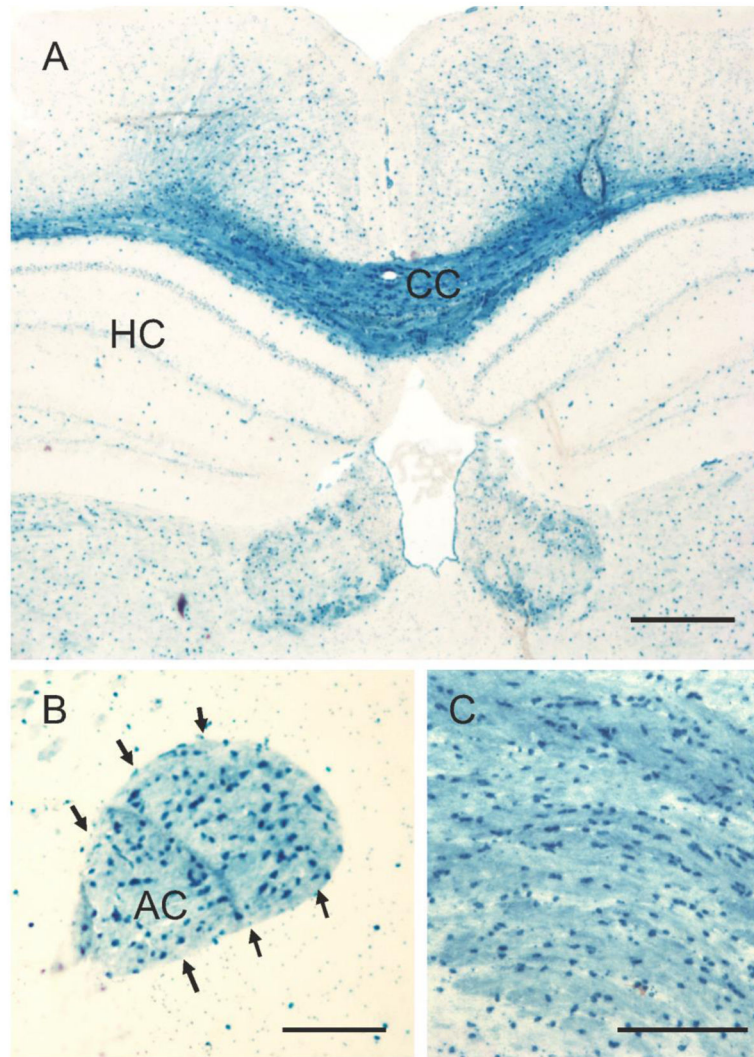


Fig. 2. High power micrographs of white and GM regions in reporter mice with X-Gal labeling. Note that the corpus callosum (CC in panel A and all panel C), the anterior commissure and some GM in deep cortical layers contain cells intensely labeled with X-Gal reaction. Calibration: 300 μ m in A, 100 μ m in B and C.

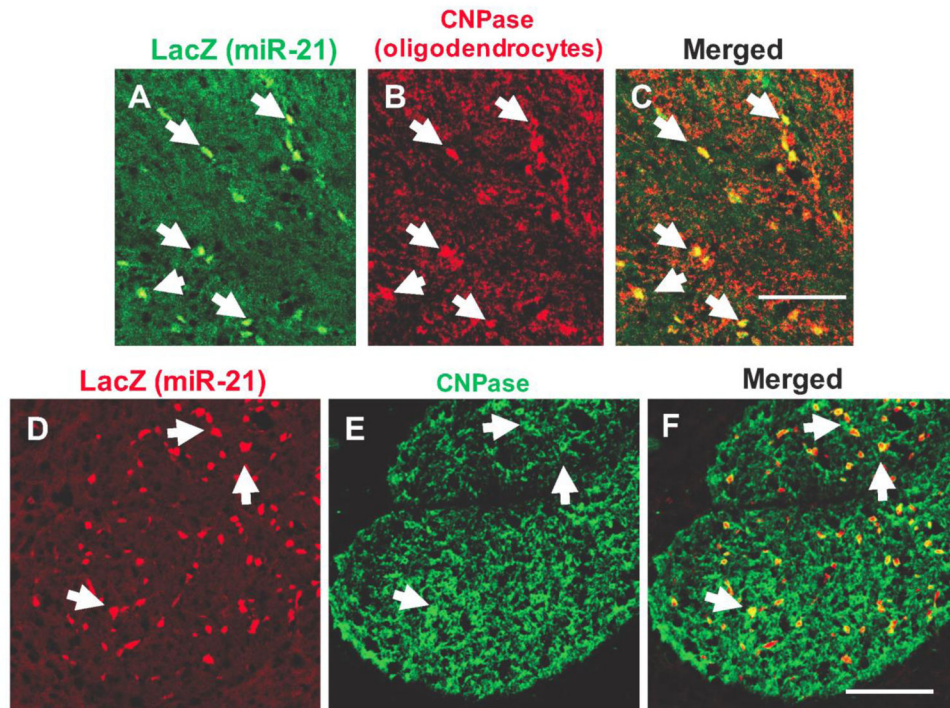


Fig. 3. Immunofluorescence labeling of miR-21/LacZ showing co-localization with CNPase immunofluorescence in individual cells of the corpus callosum (A to C) and anterior commissure (D to F). Dual-labeled cells are denoted with arrows in the three panels and appear as yellow structures in panels C (merged picture of A and B) and F (merged picture of D and E). Calibration bars: 100 μ m.

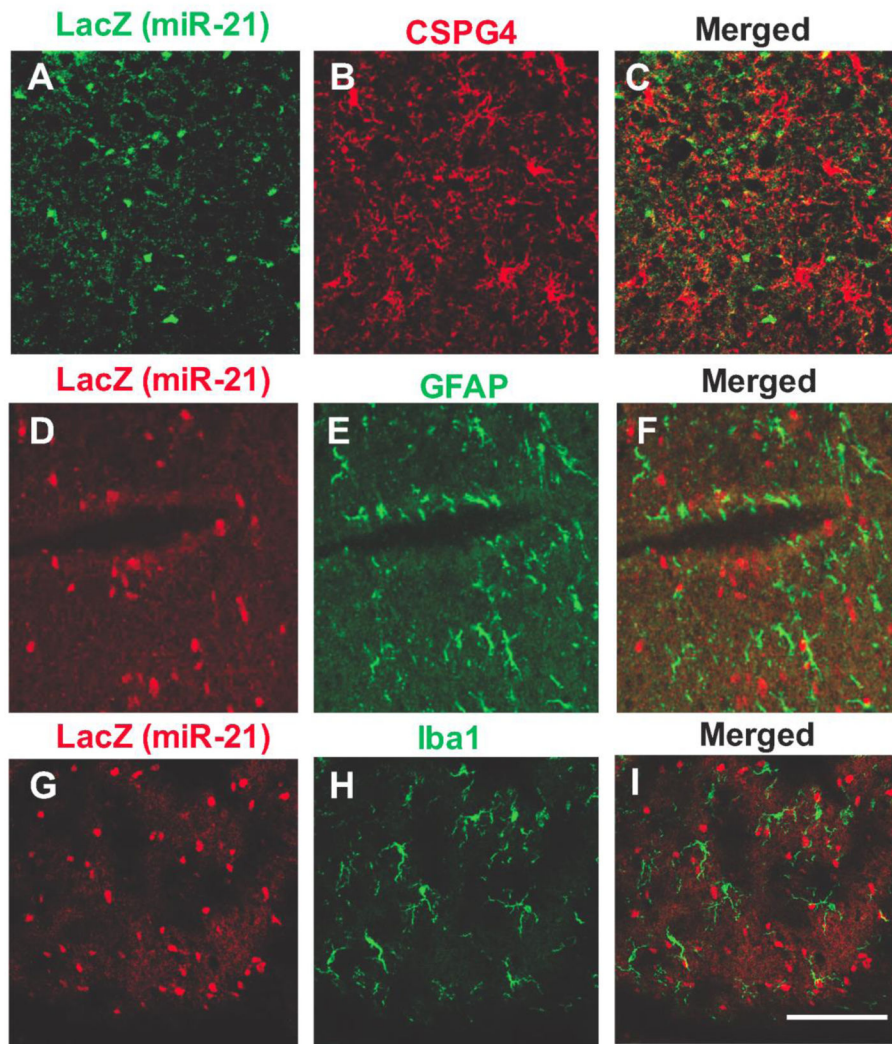


Fig. 4. Micrographs of double-labeling immunofluorescence detection of miR-21/LacZ and either of the three glial markers CSPG4 for NG2 cells (A, B, C), GFAP for astrocytes (D, E, F) and Iba1 for microglia (H, I, J). Calibration bar: 100 μ m.

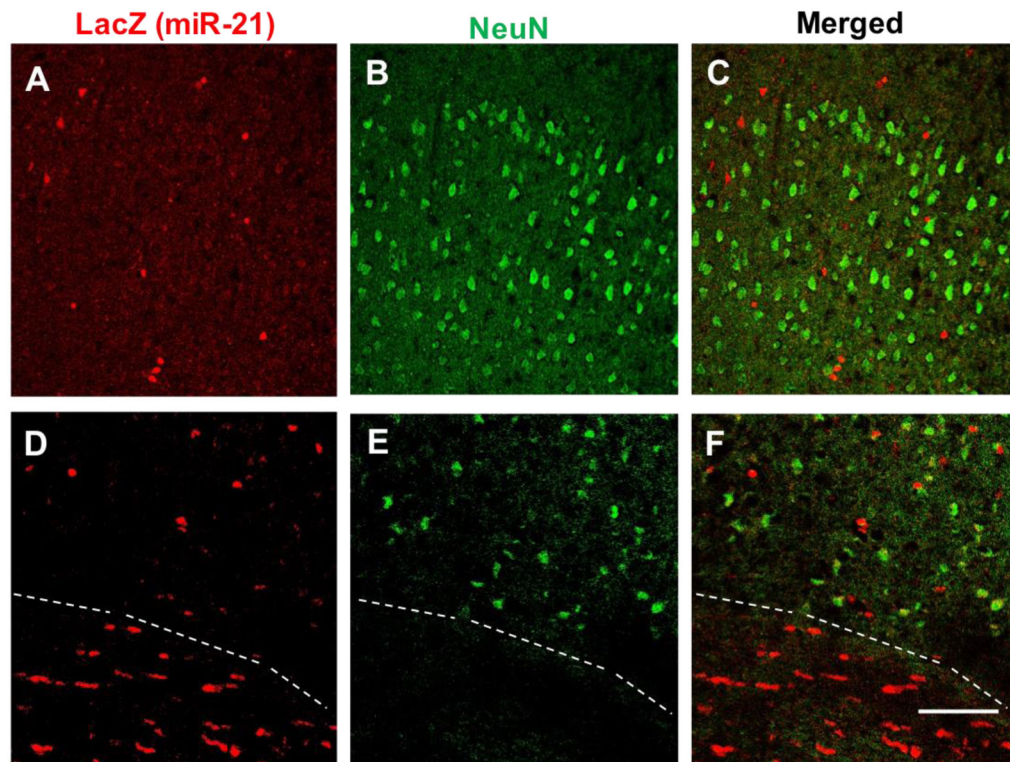


Fig. 5. Micrographs of dual-labeling immunofluorescence detection of miR-21/LacZ (red) and neuronal marker NeuN (green) in the outer half of the cortical GM of the mouse somatosensory neocortex (A to C) and around the boundary of GM-WM (D to F; GM on top of broken line, WM at the bottom). Calibration bar: 100 μ m.

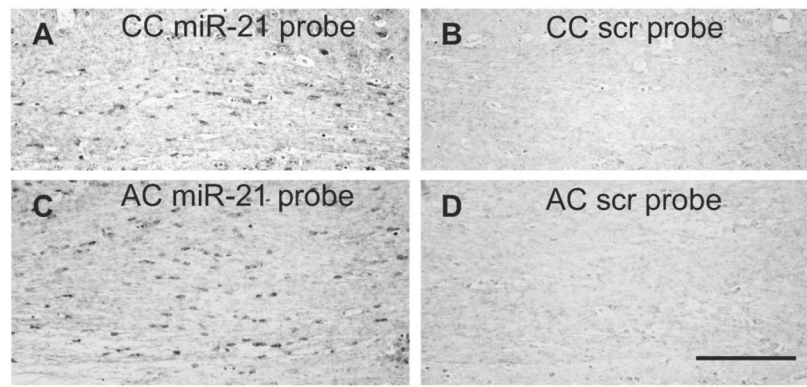


Fig. 6. In situ hybridization signal for the miR-21-specific probe (A, C) or a scrambled probe (B, D) in coronal sections through the corpus callosum (CC) and the anterior commissure (AC). Calibration: 100 μ m.

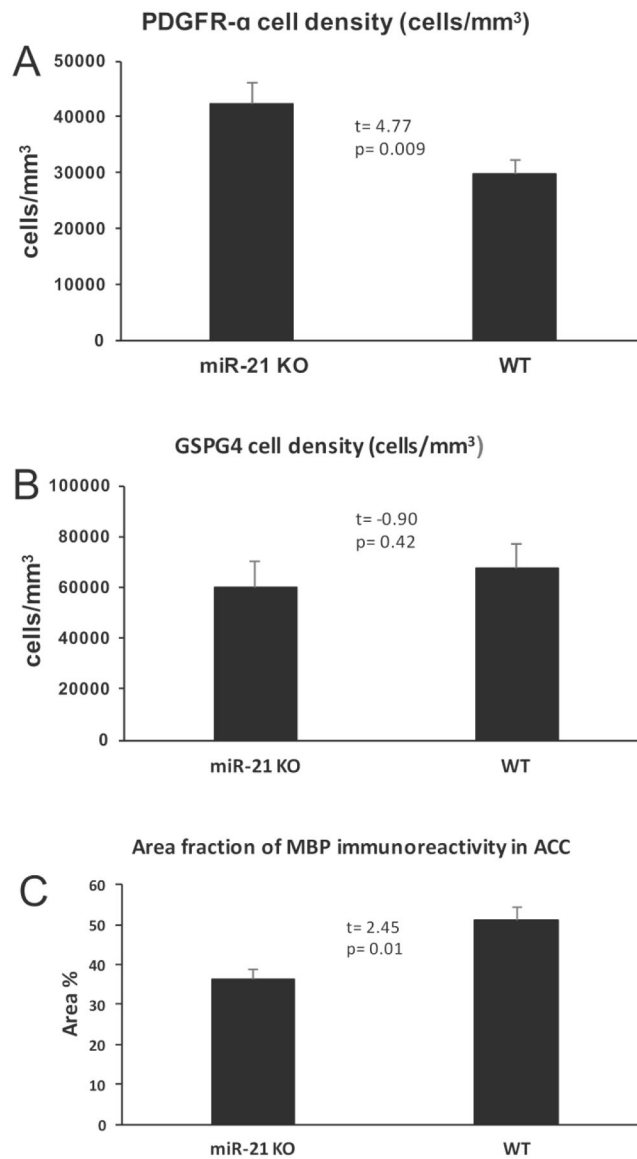


Fig. 7. Quantification of the packing density of oligodendrocyte precursor cells using antibodies to markers PDGFR α (A) and CSPG4 (B) in the corpus callosum as well as of the area fraction of immunoreactivity of myelin marker MBP (C) in the anterior cingulate cortex in miR-21 KO and wild type (WT) mice.

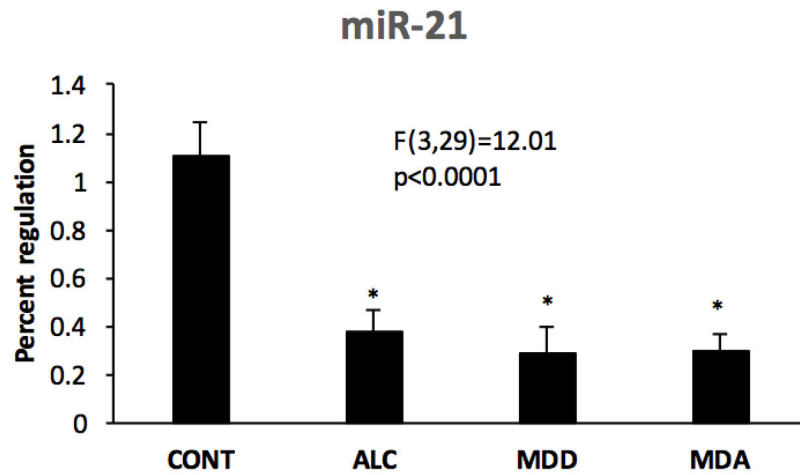


Fig. 8. miR-21 levels in human postmortem WM adjacent to the OFC in non-psychiatric controls (CONT) as compared to subjects diagnosed with alcohol dependence (ALC), major depressive disorder (MDD) or comorbid major depression and alcoholism (MDA). Asterisks indicate significant differences as compared to the control groups (ALC vs control: $d = -0.723$, $p < 0.001$; MDD vs control: $d = -0.816$, $p < 0.001$; MDA vs. control: $d = -0.807$, $p < 0.001$).

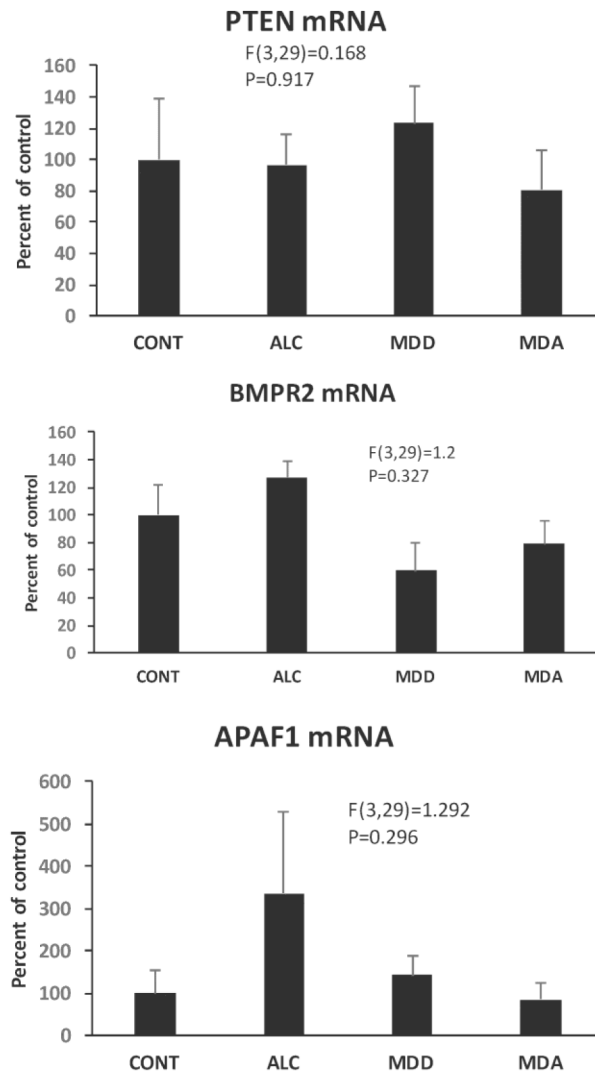
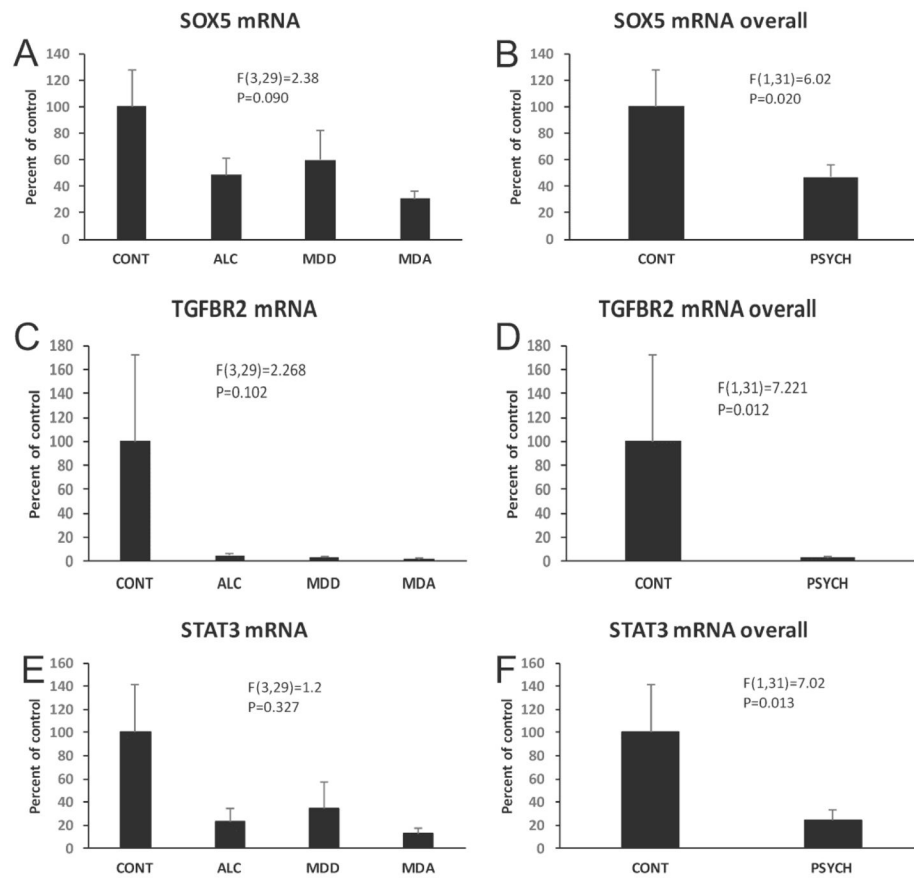


Fig. 9. Relative levels of mRNA for miR-21 targets PTEN (top), BMPR2 (middle), and APAF1 in CONT, ALC, MDD and MDA subjects. Diagnostic group abbreviations as in Fig. 8.

**Fig. 10.**

Relative levels of mRNA for miR-21 targets SOX5 (top) and TGFBR2 (middle), as well as for major regulator of miR-21 expression STAT3 (bottom) in CONT, ALC, MDD and MDA subjects. Graphs on the right compare levels of the three mRNAs in controls subjects to the overall group of psychiatric subjects. Diagnostic group abbreviations as in Fig. 8.

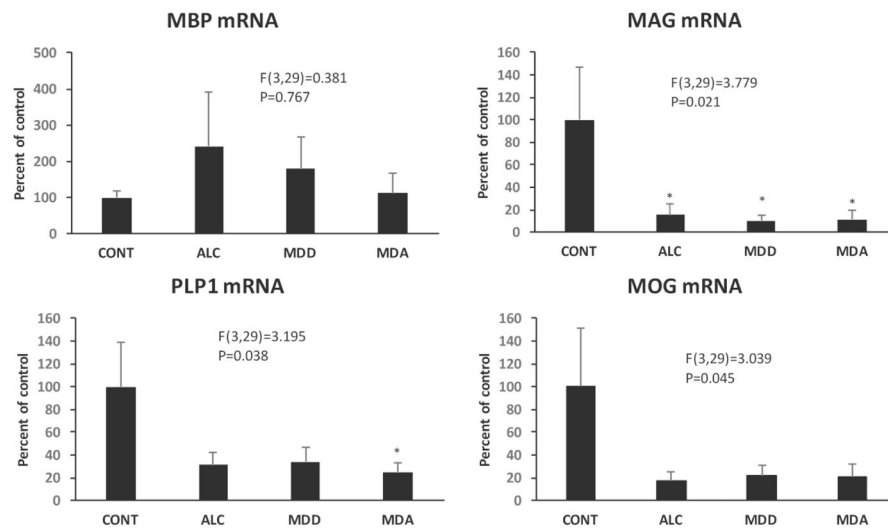


Fig. 11. Levels of mRNA for myelin-related proteins MBP, MAG, PLP1 and MOG in the four diagnostic groups. Diagnostic group abbreviations as in Fig. 8.

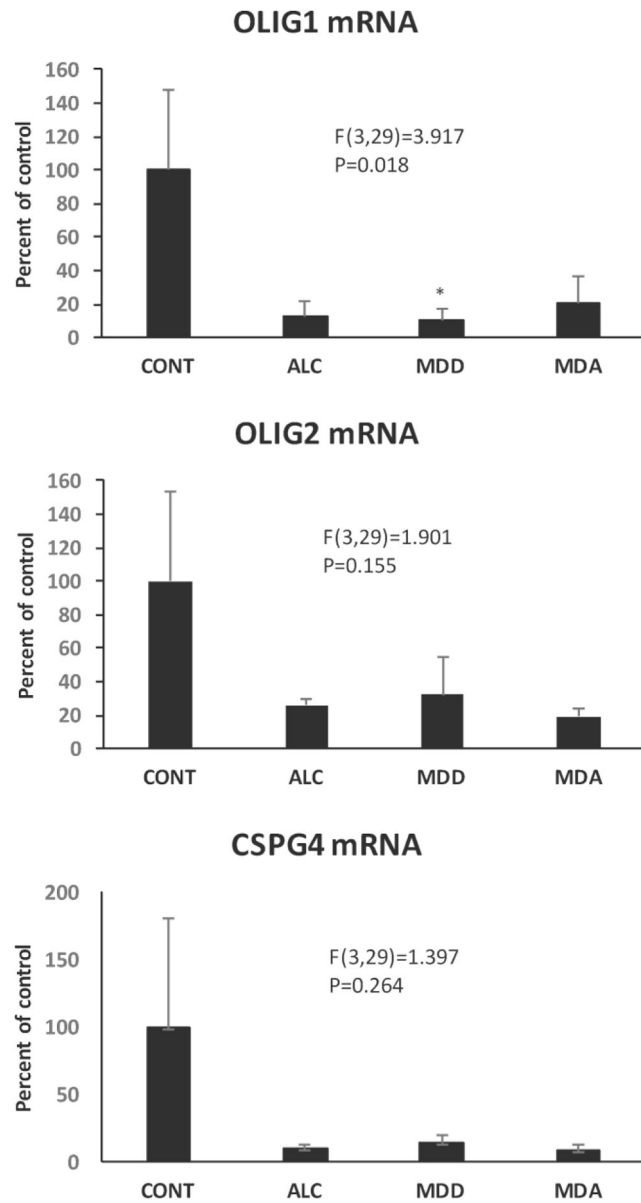


Fig. 12. Levels mRNA for oligodendrocyte-related transcription factors OLIG1 and OLIG2 as well as for CSPG4 in the four diagnostic groups. Diagnostic group abbreviations as in Fig. 8.

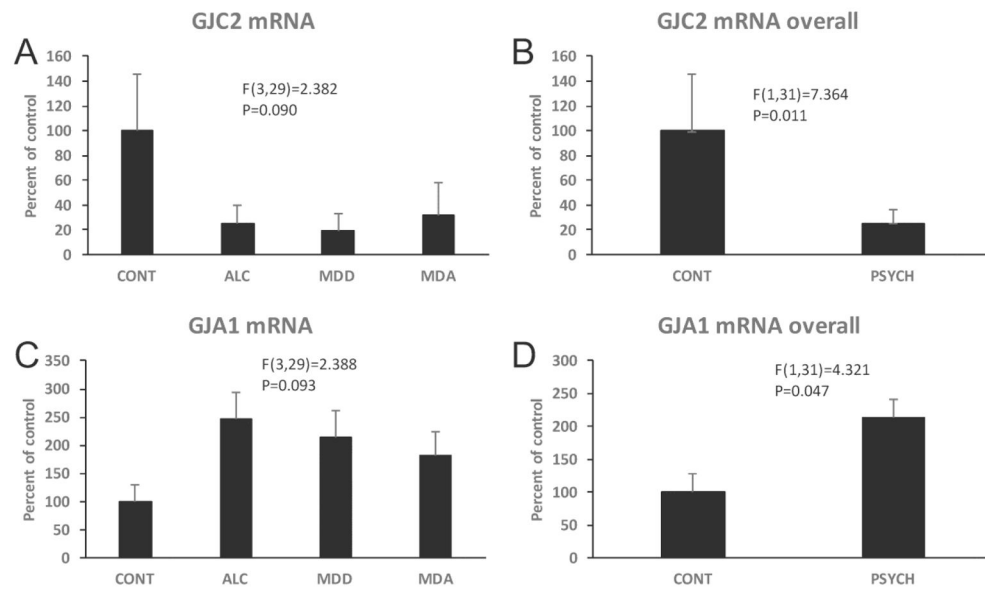


Fig. 13. Levels of mRNAs for gap junction proteins GJC2 (A, B) and GJA1 (C,D) in the WM of that four diagnostic groups. Graphs on the right compare levels of the three mRNAs in controls subjects to the overall group of psychiatric subjects. Diagnostic group abbreviations as in Fig. 8.

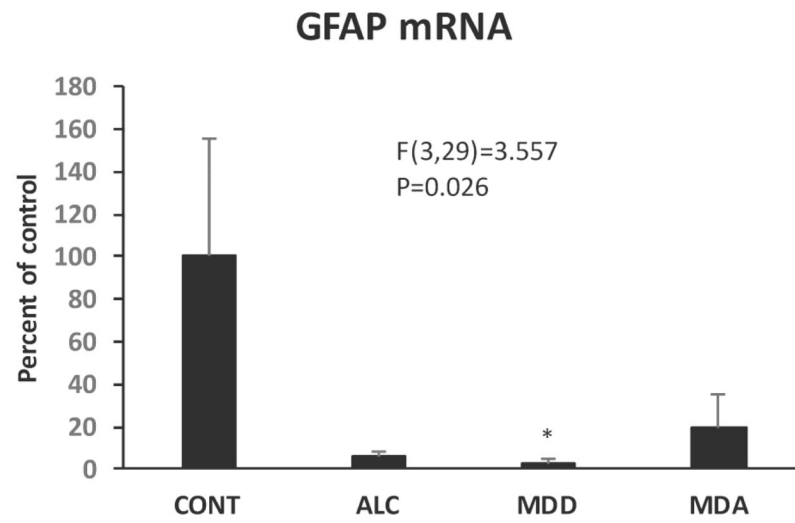


Fig. 14. GFAP mRNA levels in the human postmortem WM adjacent to the OFC in the four diagnostic groups. Diagnostic group abbreviations as in Fig. 8.

Table 1

Characteristics of the human subjects included in this study

	CONT	ALC	MDD	MDA
PMI (hours)	23.30 ±1.72	21.08±4.40	24.02 ±1.87	26.42 ±2.62
GENDER	8M, 2F	7M, 2F	5M, 5F	7M, 3F
brain pH	6.54±0.08	6.63±0.05	6.54 ±0.07	6.62±0.08
Age (years)	51.10±4.03	47.33 ±3.02	45.80 ±2.63	50.40 ±4.14
Brain weight (grams)	1404.10±58.64	1378.44±56.50	1427.30±53.67	1359.50±47.20
Suicide (S)	NA	4S, 5Non-S	8S, 2Non-S	5S, 5Non-S
Depression onset (years)	NA	NA	34.6 ±4.14	31.9 ±3.59
Depression duration (years)	NA	NA	10.76±3.86	12.08±3.56
Alcoholism onset (years)	NA	25.11±1.80	NA	23.7±3.42
Alcoholism duration (years)	NA	21.67±3.33	NA	21.18±3.66

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Table 2

	Correlations of miR-21 with mRNA levels of target and other relevant proteins			
	ALL SUBJECTS IN THE STUDY		SUBJECTS WITH PSYCHIATRIC DIAGNOSES	
	Corr. Coef.	p (Bonf. adj.)	Corr. Coef.	p (Bonf. adj.)
PLP1	0.678	0.0001	0.638	<0.0001
MBP	0.071	0.679	0.329	0.094
MAG	0.563	0.0001	0.382	0.045
MOG	0.516	0.001	0.505	0.006
OLIG1	0.636	0.0001	0.456	0.015
OLIG2	0.315	0.074	0.128	0.532
CSPG4	0.562	0.0001	0.648	0.0001
GJC2	0.535	0.001	0.23	0.238
GFAP	0.586	0.0001	0.163	0.408
GJA1	-0.029	0.871	0.376	0.058
STAT3	0.612	0.0001	0.21	0.292
BMPR2	-0.27	0.106	-0.329	0.087
SOX5	0.689	0.0001	0.704	0.0001
APAF1	-0.07	0.679	0.122	0.535
TGFBR	0.322	0.055	-0.026	0.896
PTEN	-0.236	0.159	-0.523	0.004

Corr. Coef: Correlation coefficient; Bonf. Adj: After Bonferroni adjustment