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p38α Mitogen-Activated Protein Kinase Depletion and Repression of Signal Transduction to Translation Machinery by miR-124 and -128 in Neurons

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The p38α to p38δ mitogen-activated protein kinases (MAPKs) are central regulatory nodes coordinating acute stress and inflammatory responses. Their activation leads to rapid adjustment of protein synthesis, for instance translational induction of proinflammatory cytokines. The only known direct link of p38 to translation machinery is the MAPK signal-integrating kinase Mnk. Only p38α and p38β transcripts are ubiquitously expressed. These mRNAs encode highly conserved proteins that equally phosphorylate recombinant Mnk1 *in vitro*. We discovered that expression of the p38α protein, but not the p38β isoform, is suppressed in the brain. This is due to p38α depletion by two neuron-selective microRNAs (miRNAs), miR-124 and -128. Suppression of p38α protein was reversed by miR-124/-128 antisense oligonucleotides in primary explant neuronal cultures. Targeted p38α depletion reduced Mnk1 activation, which cannot be compensated by p38β. Our research shows that p38α alone controls acute stress and cytokine signaling from p38 MAPK to translation machinery. This regulatory axis is greatly diminished in neurons, which may insulate brain physiology and function from p38α-Mnk1-mediated signaling.

The p38 mitogen-activated protein kinases (MAPKs) orchestrate cellular responses to stress and inflammation. Of the four p38 isoforms, only p38α and p38β are universally expressed (p38γ and p38δ have restricted tissue type specificity). p38α and p38β are ~70% identical at the amino acid level and are the only p38 isoforms sensitive to pyridinyl imidazole compounds, inhibitors which have been used extensively to characterize p38 functions. Since they have overlapping sets of substrates and upstream activators, it is difficult to distinguish the physiological roles of p38α and p38β using available inhibitors. Evidence from knockout mice suggests distinct roles for p38α and p38β in development. $p38\alpha^{-/-}$ mice are embryonic lethal, partly because p38α is specifically required for placental development (1–4). On the other hand, $p38\beta^{-/-}$ mice are viable and healthy (1–5). The isoforms may have some overlapping functions, because p38α/β double-knockout mouse embryos display spina bifida, exencephaly, and liver anomalies, which are not seen with either single knockout (6). However, expression of p38β under the *p38α* promoter does not compensate for most of the p38α knockout developmental defects (6).

In line with their role in stress and inflammation, the p38 MAPKs constitute a central regulatory node in the proinflammatory response. Most directly, they coordinate acute induction of proinflammatory cytokines, involving transcriptional and post-transcriptional mechanisms (7). Rapid induction of proinflammatory cytokines upon exposure to inflammatory stimuli occurs prior to an increase of their mRNAs and is highly sensitive to p38 inhibitors (8). Kinases downstream of the p38 MAPKs, Mnk1 (9) and MAPK-activated protein kinase 2 (Mk2) (10) have been implicated in posttranscriptional control upon p38 MAPK activation. Both Mnk1 (11) and Mk2 (12) may stabilize proinflammatory cytokine transcripts via phosphorylation of AU-rich element (ARE)-binding proteins that interact with AREs in cytokine mRNA 3′ untranslated regions (3′UTRs). In addition, upon activation by p38 MAPK, Mnk1 binds to eukaryotic initiation factor 4G (eIF4G) (13, 14) and catalyzes phosphorylation of eIF4E on Ser209 [eIF4E(Ser209)] (15, 16). How MAPK signaling to Mnk1 and eIF4F components affects translation mechanistically remains unclear.

Due to their central switchboard functions as biological response modifiers, the p38 MAPKs likely play important physiological roles in many organs. Their activities, however, may be particularly critical in neuronal systems. This is because (i) the p38 MAPKs are implicated in cognitive function and memory (17); (ii) cytokine-mediated signaling to p38 MAPK alters regulatory circuitry that controls behavior, mood, motivation, and anxiety (18); and (iii) postmitotic neurons are particularly vulnerable to biological stressors associated with p38 MAPK activation (19). Accordingly, the p38 MAPKs are implicated in chronic degenerative disorders with cognitive, behavioral, and neuroinflammatory components, e.g., Alzheimer's and Parkinson's diseases (20).

We report here that p38α protein levels are potently and specifically downregulated in neuronal cells due to targeting of the p38α message by two neuron-specific microRNAs (miRNAs), miR-124 and -128. This effect was partially relieved upon expression of miR-124 or -128 antisense oligonucleotides in explant mouse cerebellar granule cells. Selective depletion of p38α to achieve "neuronal" p38α/p38β expression ratios prevented Mnk1 activation, induction of Mnk1-eIF4G binding and eIF4E(Ser209) phosphorylation. p38β did not compensate for p38α loss, and depletion of p38β itself had no effect on downstream p38 MAPK signaling to Mnk1. Our results show that the p38α isoform is the predominant source of p38 MAPK signals to the translation ap-

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paratus. Controlling p38α levels may be important for proper neuronal function and protection by limiting p38 MAPK activities that are implicated as factors in chronic neuronal inflammation and degeneration.

MATERIALS AND METHODS

Cell lines and transfections. Hek293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Tetracycline (Tet)-inducible Hek293 cells expressing N-terminal myc-tagged and C-terminal Flag-tagged eIF4G1 ($Hek293^{eIF4G}$) or N-terminally hemagglutinin (HA)-tagged Mnk1 ($Hek293^{Mnk1}$) (14) were maintained in DMEM supplemented with 10% FBS, nonessential amino acids, hygromycin B (100 μg/ml; Mediatech), and blasticidin S HCl (15 μg/ml; Invitrogen). Cells were transfected with 0.1 μM pre-miR RNA hairpins (Ambion) or 0.1 μM small interfering RNA (siRNA) (Qiagen) and 15 μl Lipofectamine RNAiMax (Invitrogen) per well in 6-well plates for 18 h, then fresh medium was added, and the cells were allowed to recover for an additional 48 h. For immunoprecipitation (IP) assays, 0.1 μM siRNA was transfected into 15-cm dishes with 50 μl Lipofectamine RNAiMax for 18 h, and then fresh medium was added for an additional 48 h. Transfected Tet-inducible cells were serum starved in serum-free medium with doxycycline (1 μg/ml) for 18 h prior to treatment with inhibitors and harvesting.

Tissue samples. Mouse tissues were dissected from euthanized 6-month-old healthy animals and snap-frozen on dry ice. Healthy human brain samples were obtained from NY Brain Bank (Columbia University). These samples were from unidentified donors with causes of death not related to neurological conditions and without clinical or histopathological evidence for neurological disease. The postmortem time for the samples ranged from ~6 h to ~28 h. Patient glioblastoma or medulloblastoma samples were obtained from the Preston Robert Tisch Brain Tumor Center Tissue Repository (Duke University). The glioblastoma samples were from unidentified donors and were obtained at the time of craniotomy. The samples were snap-frozen in liquid nitrogen immediately after dissection. The medulloblastoma samples are derived from (unidentified) patient tumor material that is maintained through continuous passage as xenografts in athymic animals. These tissues were snap-frozen on dry ice upon dissection. All tissue samples used in this study were homogenized in brain lysis buffer (BLB) (50 mM HEPES [pH 7.3], 10% glycerol, 1 mM EDTA, 5 mM EGTA, 150 mM NaCl, 0.5% NP-40, 1× protease inhibitor [Roche]) following procedures reported elsewhere (21).

Quantitative reverse transcription-PCR (qRT-PCR). RT reactions were performed using TaqMan reverse transcription kit or miRNA reverse transcription kit (Invitrogen) followed by TaqMan qPCR (Invitrogen) on a LightCycler 480 (Roche). Mouse tissue samples were normalized to 18S rRNA, and miRNAs were normalized to U6 snRNA. All assays were performed in triplicate. The ΔΔCt method was used to calculate fold changes.

Cerebellar granule cell cultures and lentiviral vector transduction. We followed established protocols to subcultivate and maintain mouse cerebellar granule cells (22). Briefly, cerebella from postnatal day 5 BALB/c mouse pups were dissected and dissociated in trypsin-DNase and triturated in DNase (both trypsin and DNase from Worthington Biochemical) with fire-polished Pasteur pipettes. The cells were then passed through 40-μm nylon mesh filters, resuspended in granule cell medium, and plated on plates treated with poly-D-lysine (22). The following day, cells were transduced with lentiviral vectors. miR-zip-124 and miR-zip-128 vectors express short hairpin RNA (shRNA) complementary to the corresponding miRNAs under the control of an H1 promoter and, as transduction efficiency readout, green fluorescent protein (GFP) under the control of a cytomegalovirus (CMV) promoter (Systems Bioscience). Lentiviral vectors were produced by transfection of 293FT cells with Virapower packaging mix (Invitrogen) and miR-zip-124, miR-zip-128, pGreenPuro control vector (Systems Bioscience) or pLenti-Flag-p38α transduction vector. Vectors were harvested after 48 h and concentrated using Peg-It (Systems Bioscience).

Cloning of the p38 construct. Mouse p38α was cloned with a Flag tag from the ATCC IMAGE clone using the primers 5′-TGGGATCCATGGATTACAAGGATGACGATGACAAGTCGCAGGAGAGGCCCACGTTC-3′ and 5′-TAGCGGCGGCTCAGGACTCCATTTCTTCTTGGT-3′ and inserted into pEntr3C and then gateway cloned into pLenti6.2 (both Invitrogen).

Reporter assays. For p38α luciferase (Luc) reporters, the full-length p38α 3′UTR was amplified from a cDNA expression clone (ATCC) using primers 5′-GATCTAGATGAGCACCTGGTTTCTGTTC-3′ and 5′-TAGCGGCCGCAACAAGTGGTATTGTCTGAC-3′, digested with XbaI-NotI restriction enzymes, and inserted into pCI-FLuc (F stands for firefly) downstream of the firefly *Luc* open reading frame (ORF). Construction of reporter cDNAs expressing *Renilla* Luc was described earlier (23). Mutations in the miR-124 and -128 seed sequences were made using the QuikChange II mutagenesis kit (Stratagene) and primer pair 5′-CTGTTCTGTTGATCCCACTTCCTCGTGAGGGGAAGGCCTTTTCAT-3′/5′-ATGAAAAGGCCTTCCCCTCACGAGGAAGTGGGATCAACAGAACAG-3′ (miR-128) or primer pair 5′-CAGTATATTTGAAACTGTAAATATGTTTGCCGCTTAAAAGGAGAGAAGAAAGTGTAGATAG-3′/5′-CTATCTACACTTTCTTCTCTCCTTTTAAGCGGCAAACATATTTACAGTTTCAAATATACTG-3′ (miR-124). pcDNA5 miR-124 and miR-128 expression clones were generated using genomic sequences from mir-124/pAD track-CMV and miR-128/pAD track-CMV, generous gifts from Soo-Kyung Lee (Baylor University), that were excised using EcoRV-KpnI and XhoI-KpnI, respectively. Fragments were then inserted into the corresponding sites of pcDNA5 FRT/TO (Invitrogen). Hek293 cells in 24-well plates were transfected with 10 ng of p38α 3′UTR firefly *Luc* reporter DNA, 1 ng *Renilla Luc* reporter (as a control), and 400 ng pcDNA miR-124, pcDNA miR-128, or pcDNA5 (as a negative control) using Lipofectamine 2000. The cell media were changed after 24 h, and the cells were grown another 24 h prior to harvesting and analyzing with the Dual-Luciferase reporter assay kit (Promega) and a Turner Biosystems luminometer. Statistical analysis was performed using JMP10 software. An unpaired Student's *t* test was used to determine significance. P values of <0.05 were taken as statistically significant.

Immunoprecipitation and immunoblotting. Cell lysates were prepared using polysome lysis buffer (20 mM Tris [pH 7.4], 100 mM NaCl, 5 mM $MgCl_2$, 0.5% NP-40, 2 mM dithiothreitol [DTT], 1× protease inhibitor [Roche], Halt phosphatase inhibitor [Thermo Scientific]). Flag-IPs were performed as previously described (14). Briefly, anti-Flag M2-agarose beads (Sigma) were blocked with 1% bovine serum albumin (BSA) in NT2 buffer (50 mM Tris [pH 7.4], 100 mM NaCl, 1 mM $MgCl_2$, and 0.05% NP-40) for 30 min prior to IP, then 25 μl of Sepharose slurry and 1.2 mg lysate were added, and the resulting mixture was incubated for 3 to 4 h at 4°C with gentle rotation. Beads were then rinsed 4 times with NT2 buffer. Precipitated proteins were eluted from beads by resuspending in wash buffer with 0.1 mg/ml Flag peptide (Sigma). Immunoblotting was performed as described elsewhere (24) using antibodies against p38α, p38β (for human samples), Mnk1, phosphorylated Mnk1 (phospho-Mnk1) (Thr197/202), eIF4E, phospho-eIF4E, phospho-Erk1/2, Erk1/2, phospho-p38, Bmi1, Itgb1, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (all from Cell Signaling), tubulin, c-myc (Sigma), or p38β (for mouse samples; Invitrogen). Densitometry analysis was performed using the FluorChem FC2 imaging system and AlphaEase FC program (Cell Biosciences) and with ImageJ software (NIH).

Kinase activators and inhibitors. Anisomycin, SB203580, 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (all from Sigma) were dissolved in dimethyl sulfoxide (DMSO), and tumor necrosis factor alpha (TNF-α) (Sigma) was dissolved in water and used at the concentrations indicated (see Fig. 6). Doxycycline (Sigma) was dissolved in water to a final concentration of 1 mg/ml.

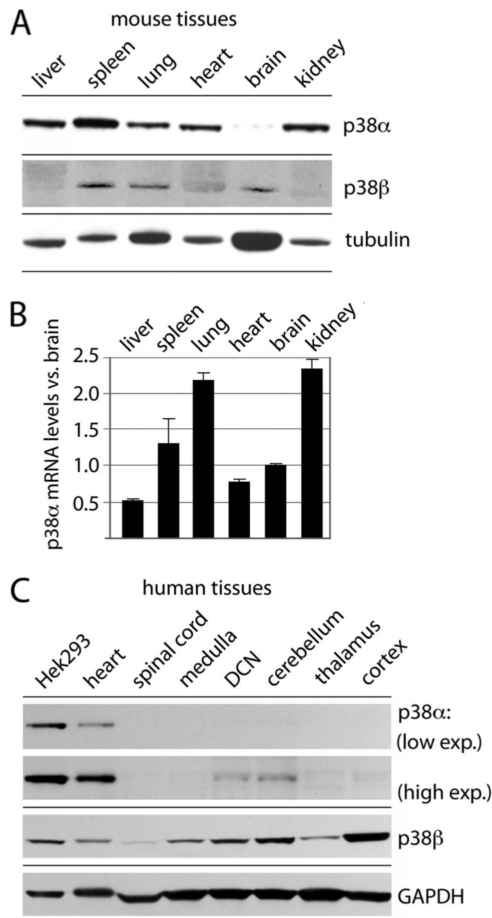

FIG 1 p38 MAPK isoform expression in mouse and human tissue samples. (A) Immunoblot of mouse tissue homogenates probed for p38α, p38β, and a tubulin loading control. (B) qRT-PCR of p38α mRNA in mouse tissues. p38α mRNA levels were normalized to 18S RNA levels. Values are means plus standard errors of the means (SEM) (error bars). (C) Expression of p38α/β MAPK isoforms and a GAPDH control in Hek293 cells, human heart, and diverse human CNS regions. DCN, deep cerebellar nuclei; exp., exposure.

RESULTS

Expression of the p38α MAPK is repressed in brain. There are two ubiquitously expressed p38 MAPK isoforms, p38α and p38β. Transcripts encoding both p38α and p38β proteins are present in all tissues, including brain (the only p38 MAPK isoforms in brain are p38α and p38β [25, 26]). However, immunoblot analysis of a mouse multitissue panel revealed that p38α protein expression is exceedingly low in normal brain compared to other tissues (Fig. 1A). This is in contrast to p38β expression, which is readily detected in brain and other tissues (Fig. 1A). All tissues were fresh-frozen immediately following euthanasia, dissected, and processed in parallel, which excludes postmortem protein stability as a factor in this phenomenon. qRT-PCR of p38α mRNA in total RNA isolated from mouse tissue lysates (Fig. 1B) confirmed roughly equivalent levels of p38α transcripts in all tissues (25, 26). Brain and heart contained similar amounts of p38α mRNA (Fig. 1B), but protein levels diverged significantly (Fig. 1A). Immunoblot analyses in (histologically confirmed normal) human central nervous system (CNS) tissues of diverse donors and regions confirmed this finding. In contrast to Hek293 cells and healthy human heart, p38α protein was substantially reduced in all human CNS samples (Fig. 1C). Meanwhile, p38β protein levels in the human CNS samples were similar compared to those in Hek293 cells and heart (Fig. 1C). This indicates that p38α depletion in brain is not due to low mRNA expression. Since p38α and -β transcripts are abundant in brain tissues but feature structurally distinct 3′UTRs, we tested whether differential expression of p38α in the brain may be due to posttranscriptional regulation of p38α mRNAs via miRNAs.

The p38α mRNA is targeted by two neuron-specific miRNAs. Analysis of the p38α mRNA 3′UTR with miRNA target site prediction algorithms revealed binding motifs for miR-124 and -128 (Fig. 2A) (27, 28). Both of these miRNAs were previously proposed to target p38α using *in silico* and *in vitro* methods (29–31). Both miR-124/-128 sites are highly conserved in the p38α mRNA 3′UTRs of vertebrates (from amphibians to humans) (Fig. 2B) and are not present in the 3′UTRs of other p38 isoforms. Both miR-124 (neuron specific) and miR-128 (neuron enriched) are intricately associated with the neuronal phenotype (32, 33), making them obvious candidates for p38α repression in the CNS. Since miR-124 and -128 are repressed in primary CNS tumors, such as medulloblastoma and glioblastoma (34–37) (Fig. 3B), we investigated whether this coincides with p38α upregulation in these tumor tissues. Immunoblot analyses of primary explant medulloblastoma and surgical glioblastoma samples revealed a p38α/p38β expression ratio comparable to those in nonneuronal tissues that lack miR-124/-128 (Fig. 3A and C). Since p38α expression was variable in the medulloblastoma xenografts, we tested the levels of miR-124/-128 in the two samples with the lowest p38α levels by qRT-PCR. Both miRNAs were repressed >10-fold compared to the levels in the cerebellum (Fig. 3B). This suggests that p38α expression in brain tumors is due to a loss of posttranscriptional repression, since published microarray data in the Oncomine database did not indicate significant changes in p38α/β message in brain tumors compared to normal brain (38).

To assess whether miR-124 and -128 repress p38α in cells, we expressed them in Hek293 cells, which do not naturally contain these miRNAs. Precursor RNA hairpins (pre-miRNA) of miR-124 and -128 were transfected into Hek293 cells, and lysates were tested for p38α expression. These assays suggest that p38α is depleted upon miR-124 or -128 expression and that enhanced depletion is achieved by cotransfection of both miRNAs (Fig. 4A).

To confirm the predicted miR-124 and -128 target sites in the p38α 3′UTR, we generated firefly *Luc* reporters containing the full-length p38α 3′UTR (Fig. 4B). Reporter plasmids were transfected into Hek293 cells along with plasmids expressing miR-124 or -128 either individually or combined. *Luc* expression of the wild-type p38α reporter decreased upon cotransfection with miR-124, -128, or both, indicating that it is targeted by these miRNAs (Fig. 4B). Reporters carrying mutations in the seed sequence for miR-124 or -128, or both combined, were no longer repressed by the corresponding miRNAs (Fig. 4B). We therefore conclude that p38α mRNA is a valid target of the neuron-specific microRNAs miR-124 and -128 upon their ectopic expression in Hek293 cells.

Derepression of p38α mRNA in neuronal cells with miR-124/-128 antagonists. The presence of confirmed miR-124/-128 target sites in the p38α 3′UTR and repression of the endogenous p38α transcript in Hek293 cells ectopically expressing miR-124/-128 does not necessarily mean that this mechanism is operational in mature, differentiated neuronal cells. To examine whether en-

A

p38-α 3′ UTR:

0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 2.0 2.1 2.2 2.3

x1,000 nt

miR-128 miR-124

```
32.....38                      604.....610
.cuguugaucccacuucacuguga....acuguaaauauguuugugccuua...
                 gugacacu                 cacggaau
miR-128      uuucucuggccaa    miR-124  agaugagucuucc
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B

```
                                           miR-128
H.sapiens        accugguuucuguucuguug..aucccacuucacugugaggggaaggccuuuucacggg
B.taurus         gu---------a--------..--------------------------------u-a-
G.gallus         -a-c-..c---c----c--u..g-------------------------.----gua--
M.musculus       ------------------cu..---u-----------------a----c---u---
C.familiaris     --------------u-----..--------------------------------u---
E.caballus       g----a----------c---..-----g----------------------.-------a-
L.africana       --------c-----------..----------------a--------------u---
O.anatinus       ---ca---c-.-c-------uuu-uu-c------------a--------------a--
A.carolinensis   -u-c-......u--g-c--u..g-u---ua-----------a------------cauaa

                                                      miR-124
H.sapiens        ca.gu...auauuugaaacug.u......aaauauguuugugccuuaaa...a.gga.g
B.taurus         --g--...---cc--g---cau-......--------------------...-.---.-
G.gallus         a-a-au..u-g-a--------.-......------------------uga-.u-g.-
M.musculus       --g--...--g--ca------.-......c-g-c-----------------...-.--gu-
C.familiaris     --g--...g-g-cc-------.-......-------c-----------g...-.---.-
E.caballus       --g--...g---c--------.-......--------------------...-.---.-
L.africana       --gc-...g---c--------.-......--------------------...-.---.-
O.anatinus       -cg--...u-g-c-a--c---.-......--------cc--------cg...uu---.-
A.carolinensis   aga-cuucu-g-c------a-.-guaaaa---g-a-------------uuac.ua-.-
```

FIG 2 Predicted binding sites for miR-124 and -128 in the p38α mRNA 3′UTR. (A) Mature miR-124 and -128 sequences are shown with complementarity to predicted seed sequences within the p38α 3′UTR. The numbers represent the positions of seed sequences within the 3′UTR, with the first nucleotide (nt) downstream of the stop codon set at 1. (B) The miR-124/-128 seed sequences in the p38α 3′UTR (shown on a gray background) are broadly conserved among vertebrates. Sequences from *Homo sapiens* (human), *Bos taurus* (cow), *Gallus gallus* (chicken), *Mus musculus* (mouse), *Canis familiaris* (dog), *Equus caballus* (horse), *Loxodonta africana* (African elephant), *Ornithorhynchus anotinus* (platypus), and *Anolis carolinensis* (an arboreal lizard) are shown.

dogenous miR-124 and -128 cause repression of p38α in neurons, we used lentiviral transduction of explanted mouse primary cerebellar granule neurons (CGNs) with antisense oligonucleotides to suppress either miR-124 or -128 (see Materials and Methods for details). It has been previously determined that both miR-124 and -128 are abundant in mouse cerebellum at postnatal day 5, which is the source of our explant CGNs (33). First, we tested whether p38α in mouse 5-day-old pup whole brain and cerebella (both the structure excised *in toto* and the CGN explant cultures) exhibit the same p38α versus p38β repression phenotype as adult mouse brain or human cerebellum (Fig. 1A and C). These tests revealed similarly suppressed p38α in 5-day-old mouse pup CNS structures and in CGNs as in adult CNS tissues (Fig. 5A). Moreover, p38α repression did not change upon prolonged culture of CGNs throughout 3 days (Fig. 5A). Last, p38α expression in cultured CGNs was far lower than in normal mouse embryonic fibroblasts (MEFs) and contrasted with approximately even expression of the p38β protein in all cells/tissues (Fig. 5A).

To evaluate the roles of miR-124 and -128 in p38α repression, we transduced CGN cultures with lentiviral vectors expressing antisense oligonucleotides complementary to miR-124 or -128 (see Materials and Methods for details) (Fig. 5B and C). The lentiviral vector stocks used contained ~1.5×10^6 transducing units/ml (determined by examining coexpression of GFP in Hek293 cells transduced with serially diluted vector stock). The addition of 100 μl vector stock to ~1.25×10^5 plated CGNs yielded transduction efficiencies of >90% (as judged by GFP expression in transduced CGNs). Transduction with either antisense construct moderately, but reproducibly, elevated p38α levels 24 to 72 h after lentiviral transduction (Fig. 5B and C). We also assessed expression of other, known targets of each miRNA and found that expression of these targets was also increased (Bmi1 for miR-128 [35] and integrin β1 [Itgb1] for miR-124 [39]) (Fig. 5B and C). These experiments were performed in 4 independent series, each involving CGN cultures established from 12 mouse pups, yielding 4 to 6 replicates for each lentiviral vector construct targeting miR-124 or -128 (Fig. 5B and C). Distinct vector preparations were used in each series.

The modest effects observed with our strategy are to be expected for several reasons. We targeted only a single miRNA in each assay, maintaining repression by the nonantagonized miRNA species. Both miRNAs implicated in p38α repression are abundant in neuronal cells. miR-124, for example, is the single most abundant miRNA in neurons and accounts for ~25 to 48% of the miRNA pool in such cells (40). It is therefore unlikely that strategies to neutralize such abundant miRNAs accomplish complete phenotypic reversals.

Depletion of p38α abolishes p38 MAPK signal transduction to Mnk1 and the translation apparatus. miRNA regulation of p38α in the brain may modulate signaling cascades with a poten-

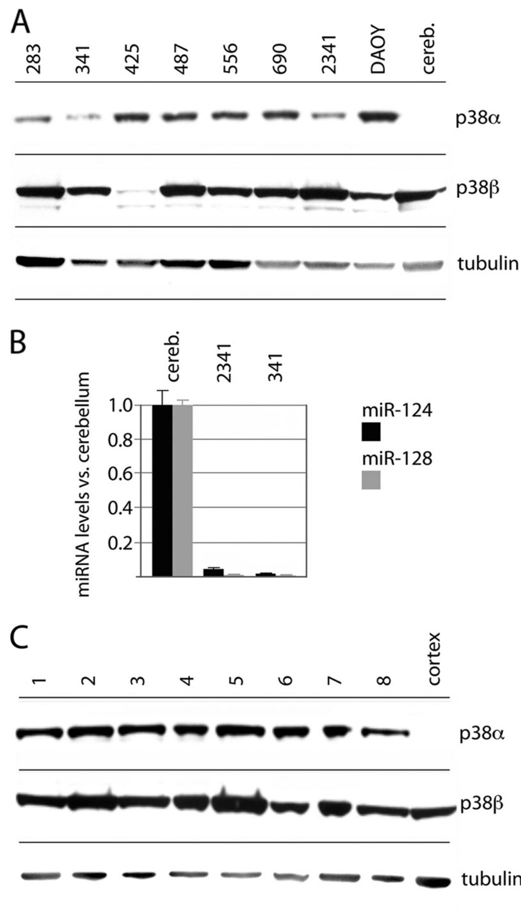

FIG 3 p38 MAPK isoform expression in primary CNS malignancies. (A) Expression of p38 isoforms in medulloblastoma primary explant xenografts and normal human cerebellum (cereb.) as a control. (B) qRT-PCR for miR-124 and -128 in normal cerebellum and medulloblastoma xenografts 2341 and 341 normalized to U6 snRNA. Values are means plus SEM (error bars). (C) Immunoblot for p38 isoforms in glioblastoma patient samples with human cortex used as a control.

tial for disruption or deregulation of neuronal processes. To gauge this possibility, we investigated the effect of p38α depletion on cytokine/stress-mediated signal transduction from p38 MAPKs to translation machinery via activation of Mnk1.

The only known direct link of p38 MAPK to translation machinery is via Mnk1 to eIF4G (Mnk1 binding to eIF4G) and eIF4E (phosphorylation of Ser209). Activation of the p38 MAPK-Mnk1 axis has been implicated in acute protein synthesis adjustment upon stress and inflammation, including rapid-onset cytokine induction (9). *In vitro*, both recombinant p38α and p38β can phosphorylate Mnk1 (41). It is unclear, however, whether this is true in the context of living cells. Indeed, it has been suggested that Mnk1 is no longer phosphorylated in p38α$^{-/-}$ MEFs stimulated with arsenic trioxide (42). Therefore, since p38α is selectively depleted in neuronal cells, it is compelling to investigate whether p38 MAPK signaling to protein synthesis machinery (via Mnk1) is retained in this context.

In order to determine whether p38α and p38β can both activate Mnk1 in living cells, we tested whether Mnk1 is phosphorylated upon p38 MAPK stimulation after siRNA-mediated knockdown of either p38 isoform. To do this, we utilized Hek293 cells with tetracycline (Tet)-inducible expression of hemagglutinin (HA)-tagged Mnk1 (Hek293$^{\text{HA-Mnk1}}$). Due to low levels of endogenous Mnk1 and limited avidity of the only available phospho-Mnk1 antibodies, it is difficult to reliably assay phosphorylation of endogenous Mnk1 upon MAPK activation (14). Tet-inducible HA-Mnk1 overexpression overcomes these limitations. Hek293$^{\text{HA-Mnk1}}$ cells were transfected with siRNAs targeting p38α or -β transcripts. siRNAs were used instead of miR-124/-128 to avoid off-target effects from depletion of other targets of these miRNAs in cells. siRNA-transfected cells were treated with the p38 MAPK activator anisomycin for subsequent analysis of phospho-Mnk1 by immunoblotting. To exclude inadvertent activation of Erk1/2 in our assay (Mnk1 is a convergent target of p38 and Erk1/2 MAPKs), we determined

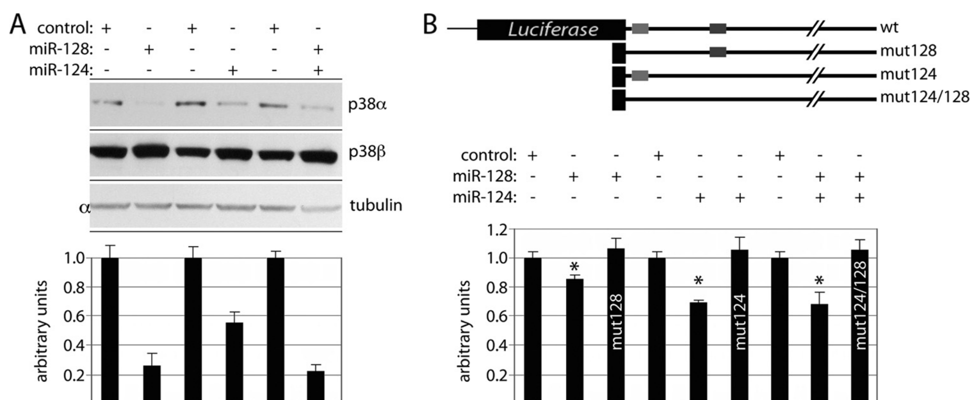

FIG 4 p38α is posttranscriptionally repressed by miR-124 and -128. (A) Hek293 cells were transfected (+) with 0.1 μM pre-miR-124, pre-miR-128, or a control, scrambled pre-miRNA and lysates were probed for p38α, p38β, and tubulin expression. (Top) An immunoblot representative of four independent assays. (Bottom) The mean densitometric quantification of p38α expression levels in four separate experiments is shown below the immunoblot. Expression of p38α in control samples was normalized to tubulin and set at 100 arbitrary units; values are means plus standard deviations (error bars). (B) (Top) Schematic of firefly *Luc* reporters containing wild-type (wt) and mutant p38α 3′UTRs, as indicated. Hek293 cells were transfected with the indicated p38α reporter along with pcDNA5 miR-124, pcDNA5 miR-128, or pcDNA5 as a control. Firefly luciferase (Luc) expression was normalized to *Renilla* Luc levels (used as a transfection control). (Bottom) Unlabeled black bars indicate reporter expression from constructs containing wild-type, endogenous seed sequences in the p38α 3′UTR. Expression levels of reporters containing mutant seed sequences are indicated. pcDNA5-transfected control samples were set at 100 arbitrary units for each reporter. Values are the means plus SEM for four independent experiments. Values that are significantly different ($P < 0.05$) from the value for the control are shown by an asterisk.

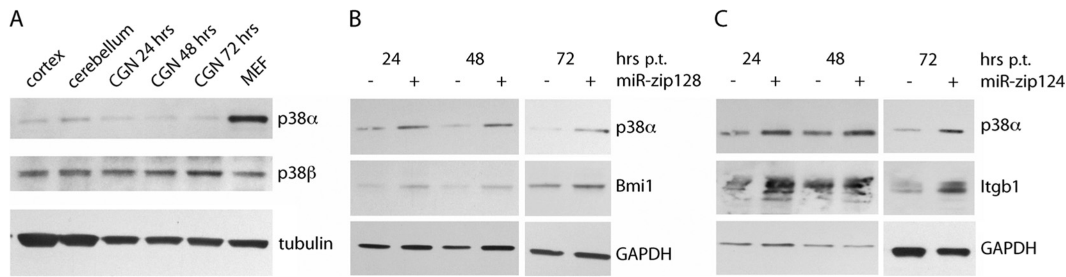

FIG 5 p38α is derepressed upon antagonizing miR-124 and -128 in primary explant mouse CGNs. (A) p38α and p38β expression in 5-day-old mouse pups. Cortex, cerebellum, and CGNs (analyzed throughout a culturing period of 3 days) exhibit p38α repression. The p38α/p38β expression ratio in normal mouse MEFs is shown as a control. (B and C) Lentiviral vector transduction of explant CGN cultures with miR-zip128 (B) and miR-zip124 (C) antisense oligonucleotides. The time (in hours) posttransduction (p.t.) is shown above the immunoblots. Immunoblots for p38α, the confirmed miR-128 target Bmi1 (B), the confirmed miR-124 target Itgb1 (C), and the loading control GAPDH are shown. The assays were performed in 4 independent series involving 4 to 6 replicates each of CGN cultures from the dissected cerebella of 48 5-day-old mouse pups. Representative results evaluating p38α/Bmi1 or p38α/Itgb1 levels at various intervals are shown.

that treatment with 10 μg/ml anisomycin for 10 min induced phosphorylation of p38α and its downstream target Mnk1, but not Erk1/2 (Fig. 6A). The p38 inhibitor SB203580 blocked anisomycin-mediated Mnk1 phosphorylation, indicating that anisomycin specifically works through p38 signaling (Fig. 6A). Two different sets of nonoverlapping siRNAs designed to target p38α mRNA decreased p38α protein levels (Fig. 6B to D). This generated a p38α/p38β ratio characteristic of CNS tissues in mice and humans (compare to Fig. 1). Compared to cells transfected with control siRNAs, targeted p38α knockdown drastically reduced p38 MAPK-mediated Mnk1 phosphorylation (Fig. 6B). p38α-depleted cells still responded to anisomycin with mild Mnk1 phosphorylation, likely reflecting residual p38α in siRNA-transfected cells. Both sets of siRNA targeting p38α had similar effects on Mnk1 activation (Fig. 6B). In contrast, targeted depletion of p38β did not alter Mnk1 phosphorylation patterns (Fig. 6B). This suggests that p38β cannot compensate for the loss of signal to Mnk1 and the translation apparatus due to p38α depletion.

Anisomycin is a commonly used p38 MAPK activator, but due to its toxicity, broad activation spectrum, and lack of physiological relevance, it may not accurately reflect authentic p38 responses. Therefore, we repeated our assay using the natural p38 MAPK activator tumor necrosis factor (TNF-α) as the stimulus. TNF-α is a classic proinflammatory cytokine that leads to activation of both p38α and p38β (25). Similar to anisomycin, treatment of Hek293$^{HA\text{-}Mnk1}$ cells with 100 ng/ml TNF-α for 25 min specifically activated p38α-Mnk1 without effects on Erk1/2 (Fig. 6A).

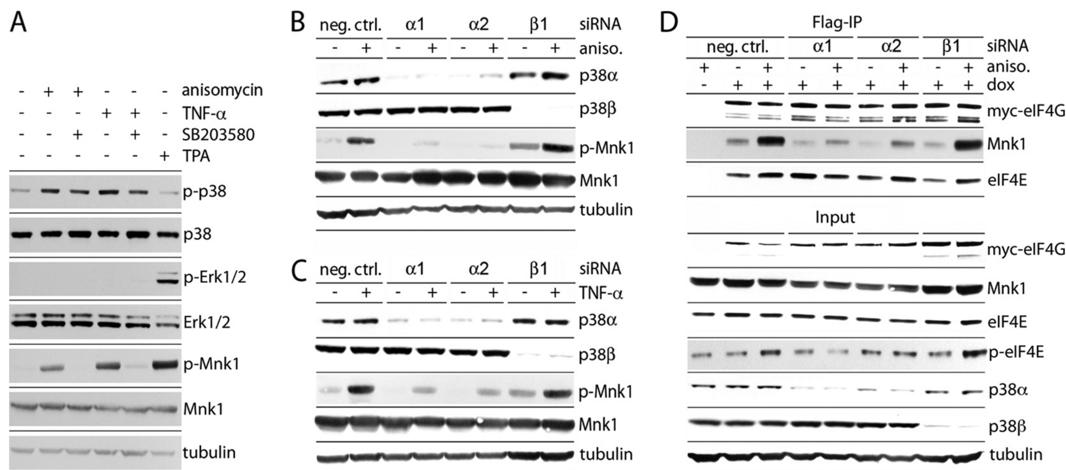

FIG 6 p38α alone is responsible for stress-induced activation of Mnk1. (A) Hek293$^{HA\text{-}Mnk1}$ cells, transfected with negative-control siRNA, were treated with anisomycin (10 μg/ml; 10 min), TNF-α (100 ng/ml; 25 min), or TPA (100 nM; 25 min) (+) with (+) and without (−) pretreatment with 10 μM SB203580 and processed for lysis and immunoblot analyses of MAPK/Mnk1 activation patterns. p-p38, phosphorylated p38. (B) Hek293$^{HA\text{-}Mnk1}$ cells transfected with control siRNA (negative control [neg. ctrl.]), siRNA to p38β (β1), or two different siRNA pairs to p38α (α1 and α2) were stimulated with 10 μg/ml anisomycin (aniso.) (+) or DMSO (−) for 10 min and lysed thereafter. The cell lysates were assayed for Mnk1 activation and knockdown of p38 isoforms. (C) Hek293$^{HA\text{-}Mnk1}$ cells transfected with siRNAs as shown in panel B were treated with 100 ng/ml TNF-α (+) or water (−) for 25 min. The lysates were analyzed for Mnk1 activation and p38 isoform expression levels. All experiments were performed in three independent series; representative results are shown. (D) Mnk1 binding to eIF4G upon stress is dependent on p38α. Hek293^{eIF4G} cells were transfected with control siRNA or paired siRNAs to p38α or p38β as shown in panels B and C. Cells were then uninduced (−) or induced with doxycycline (dox) (+) and serum starved (18 h), followed by stimulation with 10 μg/ml anisomycin (+) or DMSO (−) for 25 min and lysis. (Top) The resulting lysates were subjected to anti-Flag IP followed by immunoblotting with antibodies against myc-eIF4G and its binding partners Mnk1 and eIF4E. (Bottom) Immunoblot of the corresponding input samples. The assays were performed in three independent series; a representative assay is shown.

Hek293[HA-Mnk1] cells transfected with p38α-targeting siRNA and treated with TNF-α showed Mnk1 activation deficits similar to anisomycin-stimulated cells (Fig. 6C). Our observations indicate that p38α is the only upstream stress kinase for Mnk1 and that miRNA-mediated targeting of the p38α transcript suppresses Mnk1 activation.

To solidify our observations, we examined whether p38α depletion acts on events downstream of Mnk1 phosphorylation. Mnk1 does not interact directly with its prime substrate, eIF4E, but rather approaches it via binding to eIF4G. It has been shown previously that Mnk1 exists in an autoinhibitory conformation that prevents eIF4G binding, which is relaxed upon p38/Erk1/2 MAPK phosphorylation of Mnk1 (14). Thus, Mnk1-eIF4G binding is partly controlled through MAPK-mediated Mnk1 conformational activation (14). To document the effect of p38α depletion on Mnk1-eIF4G binding upon p38 MAPK activation, we used a Hek293 cell line with Tet-inducible expression of myc- and Flag-tagged eIF4G (Hek293[eIF4G]) followed by Flag IP of exogenous eIF4G and its binding partners (Fig. 6D). We tested whether knockdown of p38α or p38β with siRNAs would affect IP of Mnk1 with eIF4G after p38 stimulation. Hek293[eIF4G] cells were transfected with siRNA to p38α or -β and then stimulated with anisomycin before Mnk1-eIF4G binding was tested by Flag co-IP and immunoblotting (Fig. 6D). Anisomycin stimulation caused an increase in Mnk1-eIF4G binding in the presence of a control siRNA and when p38β protein levels were depleted (Fig. 6D). However, co-IP of Mnk1 with eIF4G was significantly diminished in cells transfected with either set of siRNAs to p38α, indicating that p38β was unable to compensate for the loss of p38α and to induce Mnk1-eIF4G binding (Fig. 6D). Additionally, Mnk1-directed eIF4E(Ser209) phosphorylation was increased upon anisomycin stimulation, but not in cells depleted of p38α (Fig. 6D). This agrees with our previous results showing that p38α is necessary and sufficient for Mnk1 activation in cells upon p38 MAPK activation (Fig. 6B and C).

p38α levels control phosphorylation of eIF4E(Ser209) upon stress in neuronal cells. Hek293 cells, although of neuronal lineage, are not a true representation of terminally differentiated, postmitotic neurons. Therefore, to establish whether p38α levels control the eIF4E(Ser209) phosphorylation response to stress in neurons, we examined whether ectopic p38α overexpression in such cells may elevate p38-Mnk1 activation (Fig. 7). Primary explant CGN cultures established as outlined for Fig. 5 were transduced with retroviral Flag-p38α expression vectors (featuring a cloning vector 3′UTR not targeted by miR-124 or miR-128) or a control vector expressing GFP (see Materials and Methods for details). Transduction enhanced p38α levels approximately 2-fold (Fig. 7). Anisomycin treatment of such cultures produced increased phospho-eIF4E(Ser209), suggesting that inherent neuronal p38α depletion limits the translational response to p38 activation.

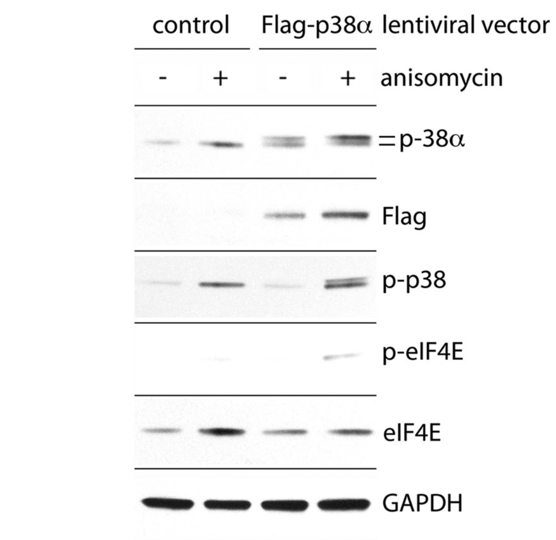

FIG 7 Ectopic overexpression of p38α in primary explant mouse CGNs enhances Mnk1-dependent phosphorylation of eIF4E(Ser209). CGNs from 5-day-old mouse pups were transduced with control lentiviral expression vectors (expressing GFP) or Flag-p38α-expressing vectors for 72 h. Then, they were mock treated (DMSO) or treated with anisomycin (10 μg/ml) for 1 h and harvested/lysed thereafter. Cell lysates were analyzed by immunoblotting as shown.

DISCUSSION

The most thoroughly studied MAPKs, p38, Jnk, and Erk1/2, coordinate cellular responses to extracellular stimuli associated with acute stress/inflammation or growth signals. In line with their pleiotropic activation spectrum and their powerful influence on many physiological processes in cells, the activity of MAPKs is carefully balanced at many levels. This typically involves control of the MAPK phosphorylation state (via either kinases or phosphatases), target substrate binding (via MAPK docking motifs) (43), physical integration of MAPK signaling modules in protein scaffolds (44), or intracellular localization (45).

We discovered that the abundance of p38α MAPK is controlled in the neuronal compartment through the action of two neuron-specific/enriched miRNAs, miR-124 and -128, resulting in significantly reduced kinase levels in normal brain tissues. miRNA control over p38α biosynthesis may suggest that p38α levels are not perpetually suppressed in neuronal tissues but that they must remain responsive to unforeseen, rapid-onset changes in conditions. For example, immediate increases of p38 MAPK levels occur in response to cerebral ischemia/reperfusion injury (46). Both miRNAs implicated in p38α regulation, miR-124 and -128, are closely linked to the neuronal phenotype (33). Indeed, miR-124 is the most abundant miRNA species in neurons and plays major, defining roles in neuronal differentiation and maintenance of neuronal function (47). miRNAs act by repressing translation initiation and inducing target template deadenylation and subsequent degradation (48). Since Northern blot data show that p38α and p38β mRNA expression is abundant in mouse brain, it appears that p38α depletion by miR-124/-128 in the brain primarily affects protein output but may have little influence on p38α transcript levels.

Our studies suggest that antagonizing miR-124 and -128 in terminally differentiated, nonproliferating CGNs elevates p38α levels. This indicates that these miRNAs play a role in posttranscriptional p38α regulation in the adult brain. For instance, CGN precursors induced to proliferate with the morphogen sonic hedgehog (shh) have elevated p38α protein levels and display heightened p38α activity (49). The increase of p38α protein upon shh exposure was a posttranscriptional event, since p38α mRNA levels were unchanged (49). Similar to our results and in accordance with a role for shh in proliferation control, elevated p38α

was also detected in medulloblastoma, tumors lacking miR-124/-128 (49). These observations support our hypothesis that posttranscriptional downregulation of p38α (via miR-124/-128) is associated with a terminally differentiated neuronal state. Additionally, p38α$^{-/-}$ embryonic stem cells spontaneously differentiate into neurons (50), indicating that p38α abundance and activity may be required for the proliferation of neuronal precursors but are downregulated upon neuronal differentiation and quiescence.

Our data show that p38α depletion ablates the p38 MAPK signal to the downstream substrate and crucial link to protein synthesis machinery, Mnk1. This suggests that only the p38α MAPK isoform signals to Mnk1 *in vivo*. Thus, miRNA-mediated p38α depletion in the neuronal compartment may restrict p38 MAPK activation mechanisms that result in acute-onset translation induction of susceptible mRNAs. Many studies show that the main class of mRNAs responding to the p38 MAPK-Mnk1 module encodes proinflammatory cytokines (8, 9, 51, 52). Therefore, miRNA-mediated p38α depletion in neurons may suppress proinflammatory cytokine expression in response to local or systemic stimuli. Disruption of this mechanism may contribute to the pathogenesis of degenerative disease with neuroinflammatory, behavioral, or cognitive components associated with cytokine signaling in the CNS, e.g., Alzheimer's disease.

Indeed, increased p38 MAPK activity in the brain is linked to Alzheimer's disease and is associated with progression of the disease (53). This is at least partially due to cytokine-mediated activation of p38 but may include other sources of p38 activity, such as β-amyloid fibrils (54). p38α may be the main p38 isoform implicated in Alzheimer's disease, since a p38α-specific inhibitor tested in a mouse model of Alzheimer's disease decreased the molecular signs of disease and improved behavioral symptoms (55). Translation suppression at p38α transcripts by miRNAs targeting p38α in neurons may be a mechanism to restrict p38α activity and its deleterious effects on brain physiology.

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