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Regulation of the miR-212/132 locus by MSK1 and CREB in response to neurotrophins

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Neurotrophins are growth factors that are important in neuronal development and survival as well as synapse formation and plasticity. Many of the effects of neurotrophins are mediated by changes in protein expression as a result of altered transcription or translation. To determine whether neurotrophins regulate the production of microRNAs (miRNAs), small RNA species that modulate protein translation or mRNA stability, we used deep sequencing to identify BDNF (brain-derived neurotrophic factor)-induced miRNAs in cultured primary cortical mouse neurons. This revealed that the miR-212/132 cluster contained the miRNAs most responsive to BDNF treatment. This cluster was found to produce four miRNAs: miR-132, miR-132*, miR-212 and miR-212*. Using specific inhibitors, mouse models and promoter analysis we have shown that the regulation of the transcription of the miR-212/132 miRNA cluster and the miRNAs derived from it are regulated by the ERK1/2 (extracellular-signal-regulated kinase 1/2) pathway, via both MSK (mitogen and stress-activated kinase)-dependent and -independent mechanisms.

Key words: brain-derived neurotrophic factor (BDNF), microRNA (miRNA), mitogen-activated protein kinase (MAPK), mitogen- and stress-activated kinase 1 (MSK1), mitogen- and stress-activated kinase 2 (MSK2), neuron.

INTRODUCTION

Neurotrophins comprise a family of growth factors that are important for both neuronal development and survival as well as synapse formation and plasticity. Four neurotrophins have been identified in mammalian cells: NGF (nerve growth factor), BDNF (brain-derived neurotrophic factor), NT3 (neurotrophin 3) and NT4 (neurotrophin 4) (reviewed in [1–5]). NGF functions by activating the TrkA receptor, whereas BDNF and NT4 act via TrkB. NT3 binds most strongly to TrkC; however, under some circumstances, it may also be able to act via TrkA and TrkB. In addition, all four neurotrophins may be able to stimulate cells via p75NTR (p75 neurotrophin receptor), a member of the TNF (tumour necrosis factor) receptor superfamily.

Binding of neurotrophins to Trk receptors results in the activation of the tyrosine kinase domain in the receptor. This leads to activation of several intracellular signalling pathways, including the ERK1/2 (extracellular-signal-regulated kinase 1/2), PI3K (phosphoinositide 3-kinase) and PLC (phospholipase C) pathways [1–5]. These signalling cascades promote the effects of neurotrophins via multiple mechanisms; however, an important aspect of their function is to regulate specific changes in gene transcription. These transcriptional changes are critical for many of the effects of neurotrophins. Although neurotrophic stimulation can affect the expression of hundreds of genes, less is understood about the roles of many of the individual genes in mediating the downstream effect of neurotrophins. To date, most studies have focused on the neurotrophin-induced expression of protein-encoding genes; however, the gene encoding the miRNA (microRNA) miR-132 has been shown to be transcriptionally induced by neurotrophins [6].

miRNAs are small (21–24 nt) regulatory RNAs that regulate gene expression post-transcriptionally by altering the translation of their target mRNAs. In the genome, miRNAs can be located in either the introns of protein-coding and non-coding genes, exons of non-coding genes or in intragenic regions [7]. The first step in miRNA production is the transcription and processing of a pri-miRNA (primary miRNA). The majority of pri-miRNAs are first processed by the Microprocessor complex in the nucleus releasing a hairpin-structured pre-miRNA [8–10]. The pre-miRNAs are then exported into the cytoplasm where they are processed further by Dicer into a small double-stranded intermediate [10–12]. One strand of a miRNA is loaded into RISC (RNA-induced silencing complex) of which a key component is an Argonaute protein [13–16]. Activated miRNA complexes can either cleave their target mRNA or regulate its translation, through a variety of mechanisms [17]. *In vivo*, miRNAs have been shown to modulate several processes, including development, immunity and neuronal function. In addition miRNAs may have pathological roles, for instance in cancer and tumorigenesis [18].

It is possible that neurotrophins may induce other miRNAs in addition to miR-132 and that this could represent a mechanism

Abbreviations used: BDNF, brain-derived neurotrophic factor; CaMK, Ca^{2+}/calmodulin-dependent protein kinase; ChIP, chromatin immunoprecipitation; CREB, cAMP-response-element-binding protein; ERK, extracellular-signal-regulated kinase; EST, expressed sequence tag; GAP, GTPase-activating protein; hnRNPA1, heterogeneous nuclear ribonucleoprotein A1; IL-6, interleukin-6; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MeCP2, methyl CpG-binding protein 2; MEK, MAPK/ERK kinase; miRNA, microRNA; MSK, mitogen- and stress-activated kinase; ncRNA, non-coding RNA; NGF, nerve growth factor; NMDA, *N*-methyl-D-aspartate; NT3, neurotrophin 3; NT4, neurotrophin 4; PI3K, phosphoinositide 3-kinase; pri-miRNA, primary miRNA; Q-PCR, quantitative PCR; RACE, rapid amplification of cDNA ends; RSK, ribosomal S6 kinase; TLR, Toll-like receptor.

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of fine-tuning the protein expression following neurotrophic stimulation. We therefore looked to see what effect BDNF stimulation had on the overall expression of miRNAs in cultured cortical neurons. We have found that only the miR-212/132 cluster reproducibly produces miRNAs upon BDNF induction. Analysis of the signalling cascades required for the induction of the miR-212/132 cluster revealed a critical role for the ERK1/2 pathway.

MATERIALS AND METHODS

Oligonucleotides

The following RNA oligonucleotides (Dharmacon) were used in the present study: mmu-miR-132, UAACAGUCUACAGCCAUGGUCG; mmu-miR-132 complementary sequence (used as a probe for Northern hybridization), CGACCAUGGCUGUAGACUGUUA; mmu-miR-212, UAACAGUCUCCAGUCACGGCC; and mmu-miR-212 complementary sequence (used as a probe for Northern hybridization), GGCCGUGACUGGAGACUGUUA.

Mice and cell culture

MSK (mitogen- and stress-activated kinase) 1- and MSK2-knockout and nestin-Cre transgenic mice have been described previously [19–21]. Conditional knockin of CREB (cAMP-response-element-binding protein) has been described previously [22], and, to generate the neuronal-specific knockin, CREB S133A mice were crossed on to a nestin-Cre background. All mice lines used had been back-crossed on to C57Bl/6 for at least six generations. All experiments involving mice were subject to local ethical review and were carried out in accordance with U.K. Home Office regulations.

Cortical cultures were isolated as described previously [23], and were cultured in Neurobasal A medium supplemented with 2 % (v/v) B27, 1 mM L-glutamine, 100 units/ml penicillin and 0.1 mg/ml streptomycin and plated on to poly-D-lysine-coated plates (100 μg/ml). HeLa cells were maintained in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10 % (v/v) FBS (fetal bovine serum), 5 mM L-glutamine, 100 units/ml penicillin and 0.1 mg/ml streptomycin. HeLa cells were serum-starved for 16 h before stimulation.

Where indicated, cells were incubated with PD184352 (2 μM), SB203580 (5 μM), Kn93 (30 μM), PI103 (10 μM) Bi-D1870 (5 μM) or MK 801 (10 μM) for 1 h before stimulation. Cells were stimulated with BNDF (50 ng/ml), PMA (400 ng/ml) or anisomycin (10 mg/ml). For RNA isolation, cells were lysed and RNA was isolated using microRNeasy mini kits (Qiagen) in line with the manufacturer's protocol. For immunoblotting, neurons were lysed directly into SDS sample buffer, whereas HeLa cells were lysed into 50 mM Tris/HCl (pH 7.5), 1 mM EGTA, 1 mM EDTA, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 1 mM sodium pyrophosphate, 0.27 M sucrose, 1 % (v/v) Triton X-100 and 0.1 % 2-mercaptoethanol and centrifuged at 13 000 ***g*** for 5 min at 4 °C to remove insoluble material.

Sequencing and analysis

Purified total RNA from unstimulated primary cortical neurons or neurons stimulated with 50 ng/ml BDNF was separated on polyacrylamide gels and the band corresponding to small RNAs was excised. cDNA libraries were generated containing small RNA sequences by ligation of adapters attached to both ends, and sequencing was carried out using Solexa technology. A total of 2 217 376 and 3 696 184 raw sequence reads were obtained for the unstimulated and BDNF-stimulated samples. The native RNA sequences were retrieved by removing the 3′ 'TCGTATGCCGTCTTCTGCTTG' adaptor tag. Removal of the adaptor tag was performed with a perl script allowing for inexact matching. Searching from the 5′ end of each read, a minimum length match of four bases was required which was then extended to the end of the reads allowing for up to three correctly matched tag bases for every one mismatched. Any reads containing A_{13} poly-A bases or unknown bases were removed.

Quality filtering was then performed similar to that previously described [24], where, starting from the 5′ end of each read, a minimum quality score of 20 was required over a sliding window of four bases. Sequence reads were clipped at the first position where the mean quality fell below 20. The quality-filtered reads were then collated to their unique examples, but only including those longer than 16 bases in length and with an abundance count of >1. This filtering resulted in 62 613 unique sequences totaling 2 213 730 reads for the control dataset, and 127 734 unique sequences totaling 3 692 981 reads for the BDNF-stimulated dataset.

Sequence matching the collated reads was performed with the Vmatch algorithm [25] against annotated ncRNAs (non-coding RNAs) (Ensembl release 52) [26] and annotated miRNA hairpin sequences (miRBase release 12) [27]. The full complement of tRNA sequences were added to the downloadable ncRNA dataset from Ensembl via a perl script accessing the Ensembl API. All Vmatch runs were performed allowing for up to two mismatches and only matches with the fewest mismatches were retained for each read.

The read abundance for the two samples is significantly different making comparisons problematical. Therefore the absolute read numbers were normalized relative to the total abundance of pre-miRNA sequences in each dataset, thus making comparisons of changes between the samples more straightforward. Simple counts of sequence reads have an estimated error of $n^{\frac{1}{2}}$ [28] and the control and BDNF errors were propagated through to the BDNF/Control ratio (see Tables 2 and 3) with the following formula:

$$\alpha r = \sqrt{r^2 \frac{\alpha C^2}{C} + \frac{\alpha B^2}{B}}$$

where r, B and C are the values for BDNF/Control ratio, BDNF and Control data respectively. αr, αB and αC are the errors for BDNF/Control ratio, BDNF and Control results respectively.

Q-PCR (quantitative PCR)

For PCR of mRNA or intron sequences, total RNA was reverse-transcribed with iScript (Bio-Rad Laboratories) in line with the manufacturer's protocols. Q-PCR was carried out using SYBR green detection. The PCR programme consisted of an initial activation step of 3 min at 95 °C, followed by 40 cycles of 20 s at 95 °C and 45 s at 58 °C. Fold induction was calculated relative to the unstimulated control (wild-type) sample, using 18S levels to correct for loading [29]. The primer sequences used are given in Supplementary Table S2 (at http://www.BiochemJ.org/bj/428/bj4280281add.htm).

Q-PCR for mature miRNA was carried out using TaqMan MicroRNA assays from Applied Biosystems, according to the manufacturer's protocols. miR-16 levels were used to correct for total RNA levels.

Mir-212/132 promoter luciferase reporter constructs

A total of 4.553 kb of the mouse miR212/132 promoter was amplified from a mouse BAC (bacterial artificial chromosome) clone (RP23-142A14) in two halves using oligonucleotides 5′-gaggatccggaaggttctgtcttcaaatgaggaactc-3′ and 5′-catgcggccgcggctcctacac-3′ for the 5′ portion and oligonucleotides 5′-gccgcggccgcatgaatgagc-3′ and 5′-ctaagcttctcgccaccttaggcagcgatac-3′ for the 3′ portion using KOD Hot Start DNA polymerase (Novagen). The purified PCR products were cloned into BglII/HindIII of pGL4.11(luc2P) (Promega) in a three-way ligation using BamHI/NotI/HindIII. The resulting full-length clone was fully sequenced.

5′ Truncations of the promoter were created by introducing an XhoI site within the promoter sequence by PCR mutagenesis using KOD Hot Start DNA polymerase (Novagen). The resulting XhoI fragment was then removed by digestion and gel purification, followed by re-ligation of the vector. A 3′ truncated promoter was made by re-amplifying the shorter promoter sequence from the full-length clone and cloning as a BamHI/HindIII fragment into BglII/HindIII of pGL4.11(luc2P). Mutations of the consensus Cre sites were also generated by PCR mutagenesis and changed the GCAG core region of the Cre consensus to TAAA. Primer sequences used for mutagenesis are given in Supplementary Table S3 (at http://www.BiochemJ.org/bj/428/bj4280281add.htm).

Transfection

Cortical cultures were transfected with promoter-firefly (0.25 μg/well) and control *Renilla* (pRL-TK; Promega) luciferase plasmids (0.1 μg/well) using Lipofectamine™ 2000 (Invitrogen) transfection reagent. At 2 days after transfection, the cells were stimulated with BDNF (50 ng/ml) for 3 h, then the cells were lysed with 1× passive lysis buffer. The luminescence of the samples was measured using a luminometer (Microlumat plus LB96; Berthold Technologies).

Immunoblotting

Samples were run on 10 % (w/v) polyacrylamide gels and transfered on to nitrocellulose membranes using standard protocols. The antibodies against total ERK1/2 was from Cell Signaling Technology, and the anti-phospho-CREB (Ser^{133}) monoclonal antobody was from Millipore. Detection was achieved using HRP (horseradish peroxidase)-conjugated secondary antibodies (Pierce) and chemiluminescent substrate (Amersham Biosciences).

Northern hybridizations

A portion (10 μg) of total RNA was separated on 15 % (w/v) polyacrylamide gels containing 7 M urea and 20 mM Mops/NaCl (pH 7.0). The RNAs were transferred on to a Hybond N (Amersham Biosciences) nylon membrane by semi-dry blotting. For the detection of the small RNAs, we followed the published protocol for sensitive Northern blotting using chemical cross-linking [30] with the only modification being that the filters were washed for twice for 1 h at 65 °C with 0.1 % SDS and 2× SSC (1× SSC is 0.15 M NaCl/0.015 M sodium citrate). Detection and quantification of the signals were carried out as described above.

3′ RACE (rapid amplification of cDNA ends) analysis

A portion (1 μg) of Qiazol (Qiagen)-extracted total RNA was subjected to first-strand synthesis with Superscript III (Invitrogen) reverse transcriptase at 50 °C for 1 h. First and nested PCRs were carried out with KOD Hot Start DNA polymerase (Novagen). Amplified DNA sequences were cloned and transformed using the Strataclone™ Blunt PCR Cloning Kit (Stratagene). A total of 88 positive clones were sequenced and the sequences were analysed with Vector NTI software (Invitrogen).

Table 1 Small RNA species identified in the sequencing data

RNA species were identified by matching sequence reads to miRBase and Ensembl non-coding RNA data. Matching was performed with Vmatch allowing for up to two mismatches per read. RNA species names are as annotated in miRBase and Ensembl. Misc., miscellanous; Mt, mitochondrial; snRNA, small nuclear RNA; snoRNA, small nucleolar RNA.

	Read abundance	
RNA species	Unstimulated control	BDNF-stimulated
miRNA	2 101 986	3 426 272
Misc. RNA	3547	8373
tRNA	3069	12925
snRNA	2218	5996
rRNA	1082	3167
snoRNA	807	4184
Mt rRNA	572	1494
Mt tRNA	143	362

RESULTS

miRNA expression in cortical neurons

Primary cortical neuronal cultures were established from C57Bl/6 mice. Total RNA was isolated from both unstimulated cells and cells that had been stimulated with BDNF for 24 h, and miRNA expression was analysed by Solexa sequencing. The output from the sequencing was filtered and collated into unique examples and then searched against known pre-miRNAs as well as other known RNAs as detailed in Table 1. This showed that the vast majority of the isolated RNA matched pre-miRNA sequences in both samples. In the unstimulated sample, the matches corresponded to 330 individual pre-miRNA sequences from the 547 mouse pre-miRNAs annotated in miRBase [27]. Of these, 202 were represented by ten or more hits. For the BDNF-stimulated sample, there were 373 individual pre-miRNA matches with 243 having an abundance of at least ten reads. In both samples, the vast majority of sequences (>90 %) corresponded to let-7 isoforms. The detailed breakdown of the pre-miRNA matches can be found in Supplementary Table S1 (at http://www.BiochemJ.org/bj/428/bj4280281add.htm).

Analysis of the normalized expression changes between the control and BDNF-stimulated results showed that the expression of most of the miRNAs was similar between the two samples (Figure 1A and Supplementary Table S1). Twelve pre-miRNAs had absolute fold changes of >4 and are shown in Table 2. However, most had fewer than five reads in the control sample, meaning that the changes were unlikely to be statistically meaningful. Consistent with this, Q-PCR for miR-712 and miR-194-1 showed no up-regulation of these miRNAs following BNDF treatment (results not shown). Filtering out pre-miRNAs with fewer than five reads left four pre-miRNAs with a fold change of >4: miR-98, miR-146a, miR-212 and miR-132 (Table 2).

To confirm the sequencing results, the expression of miR-98, miR-146a, miR-212 and miR-132, along with several other miRNAs that were not expected to change, was examined by Q-PCR in a second set of samples, independent of those used

Table 2 miRNAs showing the potential induction in the sequencing results

Pre-miRNA sequences showing the largest fold change in expression when stimulated by BDNF. Raw read abundance for each miRNA was normalized by dividing by the total number of identified miRNA matches (see Table 1). The change in expression is then determined as the ratio of the normalized abundance of BDNF/Control results. Estimated errors, calculated as described in the Materials and methods section, are shown in parentheses.

	Read abundance		Normalized abundance		
miRNA	Control	BDNF	Control	BDNF	Fold change in expression (BDNF/control)
miR-712	1	19	4.76×10^{-7}	5.55×10^{-6}	11.66 (11.96)
miR-194-1	1	14	4.76×10^{-7}	4.09×10^{-6}	8.59 (8.89)
miR-98	5753	74 250	2.74×10^{-3}	2.17×10^{-2}	7.92 (0.11)
miR-146a	12	139	5.71×10^{-6}	4.06×10^{-5}	7.11 (2.14)
miR-212	30	276	1.43×10^{-5}	8.06×10^{-5}	5.64 (1.09)
miR-207	1	9	4.76×10^{-7}	2.63×10^{-6}	5.52 (5.82)
miR-381	1	9	4.76×10^{-7}	2.63×10^{-6}	5.52 (5.82)
miR-200b	1	8	4.76×10^{-7}	2.33×10^{-6}	4.91 (5.21)
miR-483	2	15	9.51×10^{-7}	4.38×10^{-6}	4.60 (3.46)
miR-758	1	7	4.76×10^{-7}	2.04×10^{-6}	4.29 (4.59)
miR-218-1	3	21	1.43×10^{-6}	6.13×10^{-6}	4.29 (2.65)
miR-132	52	353	2.47×10^{-5}	1.03×10^{-4}	4.16 (0.62)
miR-1197	4	1	1.90×10^{-6}	2.92×10^{-7}	0.15 (0.17)

Table 3 Comparison and sequencing and Q-PCR results

Selected miRNAs from the sequencing results are shown. Total number of hits indicated the number of times a sequence for the pre-miRNA was obtained in the RNA sample from the 24 h BDNF-stimulated neurons. For each miRNA, the percentage hits for the potential 5p and 3p miRNA sequences from the hairpin is given, and the sequences already annotated in miRBase are also indicated. In the final columns the fold induction between the control and stimulated samples for the sequencing results and Q-PCR analysis are given. For the sequencing results, estimated errors, calculated as described in the Materials and methods section, are shown in parentheses. Q-PCR was carried out on three independent samples, and *P* values (Student's *t* test) are given for the difference between control and BDNF-stimulated samples for the PCR results. Asterisks indicate that the annotated miRNA star sequence was identified in the sequencing data.

		Sequencing abundance in BDNF sample			Fold induction		
miRNA	miRBase annotation	5p (%)	3p (%)	Total	Normalized sequencing	Q-PCR	*P* value
miR-652	3p	0	100	70	1.13 (0.23)	0.87	0.433
miR-369-3p	5p and 3p	63	37	129	1.30 (0.20)	1.53	0.217
miR-369-5p			1.90	0.055			
miR-181d	5p	100	0	6302	1.36 (0.03)	1.18	0.719
miR-146b	5p and *	100	0	571	1.51 (0.12)	1.25	0.118
miR-669c	5p	100	0	899	1.51 (0.09)	0.78	0.194
miR-30d	5p	100	0	1087	1.57 (0.09)	0.89	0.552
miR-30e	5p	100	0	553	1.71 (0.14)	1.34	0.368
miR-425	5p and *	90	10	33	1.84 (0.64)	0.65	0.264
miR-433	3p and *	3	97	910	2.03 (0.14)	2.95	0.286
miR-668	3p	0	100	47	2.40 (0.78)	0.83	0.448
miR-709	3p	0	100	50	3.41 (1.23)	1.40	0.369
miR-132	3p	9	84	353	4.16 (0.62)	3.83	0.001
miR-212	3p	86	14	276	5.64 (1.09)	4.78	0.009
miR-146a	5p	100	0	139	7.11 (2.14)	1.35	0.034
miR-98	5p	100	0	74250	7.92 (0.11)	1.78	0.057

for sequencing (Table 3 and Figure 1B). For each of the selected miRNAs, alignments of the sequencing hits to the hairpin sequence were examined to determine whether the 5p or 3p sequences were expressed. In most cases, the sequences found matched the main species annotated in miRBase; however, not all of the annotated star sequences were observed (Table 3). The exceptions to this were miR-132 and miR-212, where in addition to the annotated 3p sequence, the 5p sequence (star sequence) was also observed. In addition, for miR-132 a sequence corresponding to the hairpin loop region was also sequenced (Supplementary Figure S1 at http://www.BiochemJ.org/bj/428/bj4280281add.htm). Comparison between the Q-PCR and sequencing results showed that, although there was reasonable agreement between the two methods for the non-induced miRNAs (Figure 1A and Table 3), only two of the four up-regulated miRNAs from the sequencing results were reproduced in the Q-PCR analysis. Using Q-PCR, miR-98 and miR-146a were not significantly up-regulated ($P > 0.01$) after 4 or 24 h of BDNF treatment (Table 1 and results not shown). The lack of miR-98 up-regulation in the Q-PCR was unexpected, given the high abundance (up to 74 250 reads) in the sequencing experiment would suggest that this should be one of the more reproducible results. miR-98 is part of the let-7 family and, given the very high expression of other let-7 isoforms in the neurons, it is possible that either the Q-PCR was not sufficiently specific for miR-98 relative to the other let-7 isoforms or that the read matching the sequencing results, which allowed up to two mismatches, could not always differentiate between miR-98 and other let-7 isoforms. To examine this, the sequencing results was re-analysed allowing for zero mismatches. Under this more stringent analysis, the number of miR-98 matches fell, whereas the fold change between the control and BDNF sample decreased to 1.22, a value similar to that obtained by

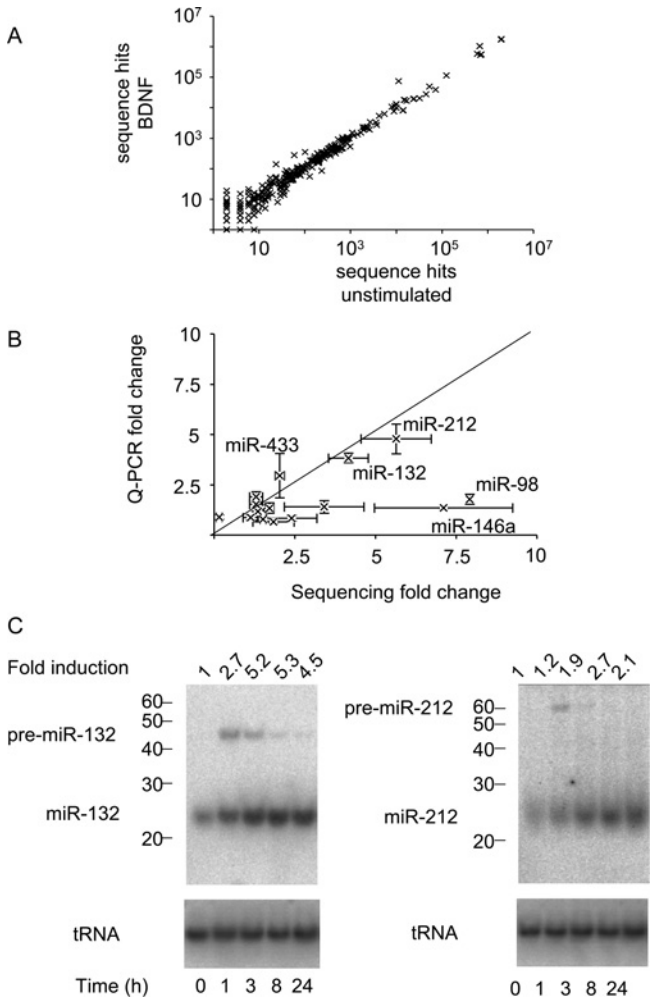

Figure 1 miRNA profiling following BDNF stimulation.

(**A**) miRNAs were sequenced from unstimulated and BDNF (50 ng/ml, 24 h)-stimulated cortical neurons. The sequencing results were analysed as described in the Materials and methods section, and the numbers of sequences that mapped to each pre-miRNA were plotted. miRNAs present in only one sample were excluded from the plot. (**B**) Comparison of Q-PCR and sequencing results of selected miRNAs (listed in Table 3). Error bars for Q-PCR represents the S.E.M. for three independent stimulations. Error bars for sequencing results represent the estimated errors that were calculated as described in the Materials and methods section. (**C**) Primary cortical neuronal cultures were left unstimulated or stimulated with 50 ng/ml BDNF for the indicated times and miR-132 and miR-212 levels were analysed by Northern blotting as described in the Materials and methods section. Fold induction of the mature miRNAs is given above the blots. Sizes are indicated in bases to the left of the blots.

Q-PCR. Both miR-132 and miR-212 were still induced using this analysis method.

Of the miRNAs tested, only miR-132 and miR-212 were up-regulated consistently by BDNF in both the sequencing and Q-PCR results (Figure 1B). Up-regulation of both miR-132 and miR-212 by BNDF could also be demonstrated by Northern blotting (Figure 1C). Interestingly, both miR-132 and miR-212 map to the same genomic locus, suggesting that their expression may be co-regulated. miR-132 has been shown previously to be up-regulated in neuronal cells following neurotrophic stimulation [6], and has been suggested to be involved in the regulation of MeCP2 (methyl CpG-binding protein 2) and p250GAP (GTPase-activating protein) in neurons [31,32]. The regulation of miR-212 expression has not, however, been studied previously in neuronal cells. The regulation of miR-132 and miR-212 transcription was therefore examined in more detail.

BDNF promotes transcription of the miR-212/132 locus in murine cortical neurons

In mice, both miR-132 and miR-212 localize to the same region of chromosome 11. Both miRNAs are also found in an equivalent localization in other species, including the human and rat genomes. In rats, miR-132 has been shown to be processed from the intron of a small non-coding RNA gene [6]. Analysis of mouse ESTs (expressed sequence tags) revealed evidence for a similar non-coding gene in mice, consisting of three exons with miR-132 and miR-212 located in intron 1 (Figure 2A). To examine the transcription of this gene, PCRs were designed to amplify regions from intron 1, exon 2, intron 2 and exon 3. In addition, primers were also made to amplify PCR products from exons 1 to 2 and from 2 to 3. Using Q-PCR, it was found that stimulation of primary cortical neurons with BDNF was able to induce the transcription of this non-coding mRNA using primer sets that amplified either the exon 1/2 or exon 2/3 fragments (Figure 2B). Cloning of these PCR products confirmed that the splicing predicted from the ESTs had occurred (results not shown).

The induction of exons 2 and 3 as well as introns 1 and 2 was also examined by Q-PCR using the primer sets internal to these regions (Figure 2). In this case, the absolute amounts of the products were determined for each PCR using a standard curve prepared from a plasmid containing a subclone of the miR-212/132 genomic locus. All four regions were induced by BDNF; however, the absolute levels of intron 1 were lower than exon 2, consistent with the rapid processing of this intron by the microprocessor pathway [33]. Unexpectedly, the absolute levels of exon 3 were much lower than exon 2 (Figure 2C). Analysis of the intron 2 sequence revealed three potential polyadenylation sites. 3′ RACE confirmed that termination could occur in at least two of these sites in addition to the polyadenylation site 3′ to exon 3 (Supplementary Figure S2 at http://www.BiochemJ.org/bj/428/bj4280281add.htm). This would suggest that an alternative transcript, with a longer version of exon 2 but lacking exon 3, was also produced. Consistent with this, Q-PCR using primers in the 5′ region of intron 2 gave similar results to primers for exon 2 (Figure 2C). This would suggest that the predominant form expressed following BDNF stimulation corresponded to termination at the polyadenylation signals in intron 2.

ERK1/2 regulates pri-miR-212/132 transcription

BDNF is known to stimulate a number of intracellular signalling cascades, leading to the activation of the ERK1/2, CaMK (Ca^{2+}/calmodulin-dependent protein kinase) and PI3K pathways. To determine which of these pathways were involved in miR-212/132 transcription, small-molecule inhibitors of these proteins were used. Profiling of the specificity of these inhibitors has been reported previously [34]. PI103, a specific PI3K inhibitor, and Kn93, a CaMK inhibitor, did not affect the transcription of pri-miR-212/132 in response to BDNF (Figure 3A). In some experiments, SB203580, a p38α/β MAPK (mitogen-activated protein kinase) inhibitor, resulted in an increase in pri-miR-212/132 transcription; however, this did not reach statistical significance ($P = 0.056$ in Figure 3A) and was not reproduced with Birb 0796, a structurally unrelated p38 MAPK inhibitor (Supplementary Figure S3A at http://www.BiochemJ.org/bj/428/bj4280281add.htm). In contrast PD184352, a MEK1/2

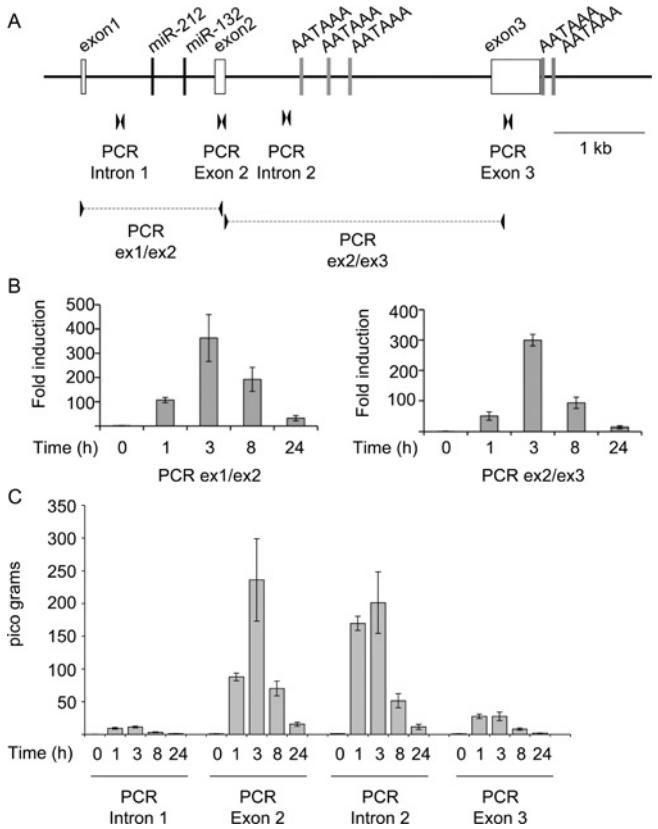

Figure 2 BDNF regulates the miR-212/132 genomic locus

(**A**) miR-132 and miR-212 map to the first intron of a non-coding mRNA gene on chromosome 11 in the mouse genome. The presence of the three exons predicted from mouse ESTs are indicated (white boxes). The positions of miR-132 and miR-212 in the first intron of this gene (vertical lines) and potential poly-adenylation sites (AATAAA) (grey vertical lines) are shown. The position of the various primer sets used for Q-PCR are marked. Sequences for these primers are given in Supplementary Table S2 (at http://www.BiochemJ.org/bj/428/bj4280281add.htm). (**B**) Primary cortical neuronal cultures were stimulated for the indicated times with 50 ng/ml BDNF. Total RNA was isolated, and the fold induction of the processed non-coding transcript was determined by Q-PCR. Primers amplifying mRNA derived from splicing at the exon 1/2 (ex1/ex2) boundary are shown in the left-hand panel; primers detecting splicing at the exon 2/3 (ex2/ex3) boundary are shown in the right-hand panel. 18S RNA levels were used to correct for total RNA concentrations. Error bars represent the S.D. for three independent stimulations. (**C**) As in (**B**), but Q-PCR was used to determine the absolute levels of sequences from intron 1, exon 2, intron 2 and exon 3. All four Q-PCRs were quantified relative to a standard curve derived from a plasmid that contained a subclone of miR-212/132 genomic region. 18S RNA levels were used to correct for total RNA concentrations. Error bars represent the S.D. for three independent stimulations.

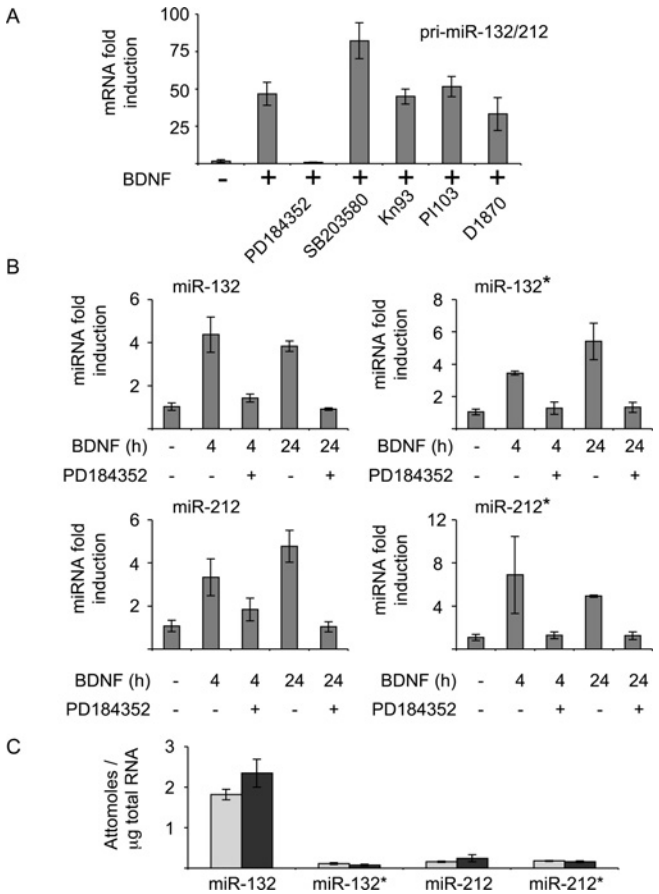

Figure 3 ERK1/2 signalling is required for pri-miR-212/132 transcription

(**A**) Primary cortical neurons were incubated for 1 h in the presence of 2 μM PD184352, 5 μM SB203580, 10 μM Kn93, 10 μM PI103 or 5 μM Bi-D1870 as indicated. Cells were then stimulated for 1 h with 50 ng/ml BDNF and pri-miR-212/132 levels were determined by Q-PCR. Error bars represent the S.E.M. for three independent stimulations. (**B**) Primary cortical neurons were incubated for 1 h in the presence of 2 μM PD184352 where indicated and then stimulated for 4 or 24 h with 50 ng/ml BDNF. Induction of both mature and star miRNA sequences for miR-132 and miR-212 were determined by Q-PCR. Error bars represent the S.E.M. for three independent stimulations. (**C**) The absolute levels of the mature and star miRNA sequences for both miR-132 and miR-212 were determined by Q-PCR calibrated against a concentration curve generated using Q-PCR against a synthetic oligonucleotide corresponding to the miRNA sequences. Quantification was carried out for the 4 h (grey bars) and 24 h (black bars) BDNF-stimulated samples described in (**B**).

(MAPK/ERK kinase 1/2) inhibitor, that blocks the activation of ERK1/2, completely blocked the induction of pri-miR-212/132 in response to BDNF (Figure 3A). The up-regulation of the mature miRNA was also determined using a TaqMan-based PCR system. As star sequences were also identified in the sequencing results for both miR-132 and miR-212, the induction of these sequences was also examined by Q-PCR. Both miR-132 and miR-212 were up-regulated by BNDF stimulation and, consistent with the results on the pri-miR-212/132, PD184352 blocked the accumulation of the mature sequences in response to BNDF (Figure 3B). In line with what was observed for miR-132 and miR-212, the star sequences of both miRNAs were also up-regulated by BDNF in an ERK1/2-dependent manner (Figure 3B). To determine absolute levels for each miRNA, Q-PCR was carried out and levels were determined relative to a standard curve prepared using a synthetic oligonucleotide corresponding to each miRNA sequence. This showed that miR-132 was expressed at much higher levels than miR-132*. Both the miR-212 and miR-212* sequences were found at similar levels; however, these levels were significantly lower than that found for miR-132 (Figure 3C). This was in contrast with the sequencing results which suggested that miR-212* might be expressed at a higher level than miR-212. This difference could possibly be due to a ligation bias in the sequencing library that could result in an over-representation of miR-212* in the library.

MSK1/2 regulate pri-miR-212/132 transcription via the phosphorylation of CREB

ERK1/2 is able to regulate transcription both by the direct phosphorylation of specific transcription factors and by the activation of downstream kinases, such as RSK (ribosomal S6 kinase) and MSK, that can in turn phosphorylate both transcription factors and chromatin [22,35]. Bi-D1870, a specific RSK inhibitor

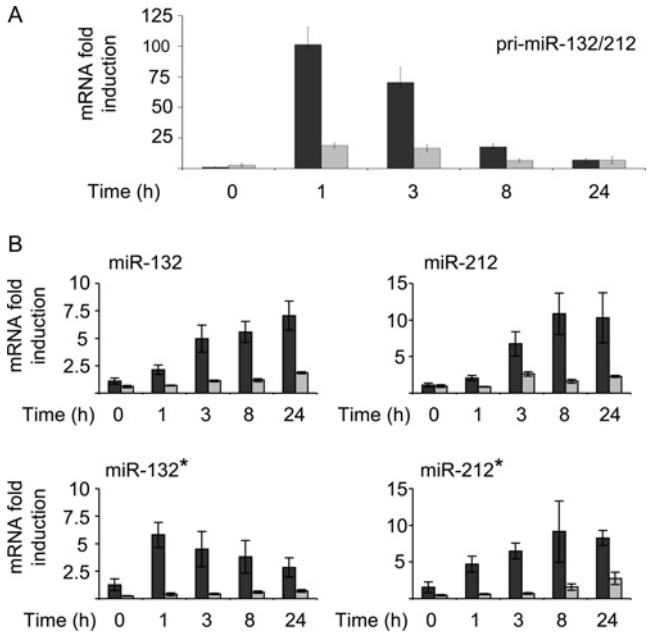

Figure 4 MSKs regulate miR-212/132 transcription

(**A**) Primary cortical neurons were cultured from wild-type (black bars) or MSK1/2-knockout (grey bars) mice. Cells were stimulated for the indicated times with 50 ng/ml BDNF and the fold induction of pri-miR-212/132 was determined by Q-PCR. For both wild-type and knockout samples, the fold change was calculated relative to the wild-type control samples. Error bars represent the S.E.M. for four independent stimulations. (**B**) As in (**A**) but mature and star miRNA sequences for both miR-132 and miR-212 were measured.

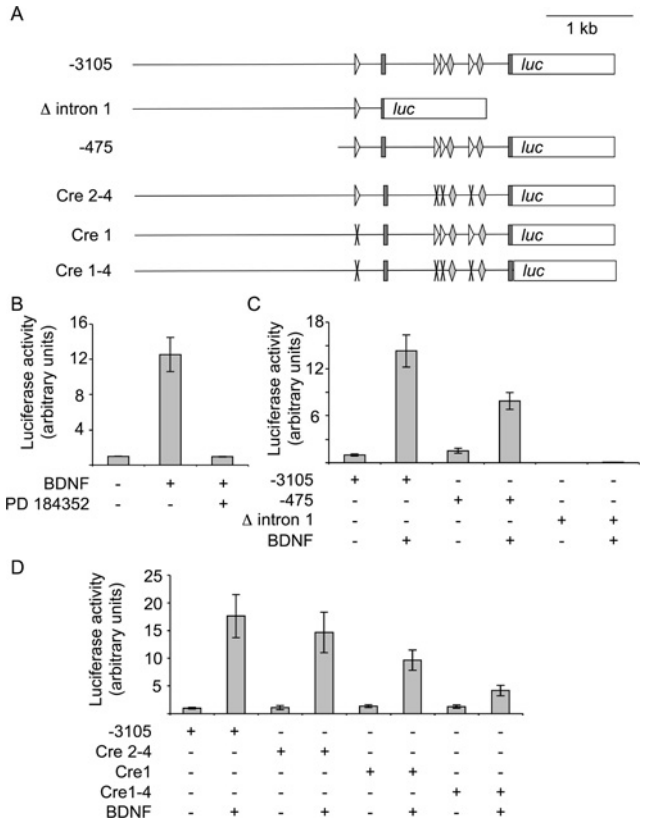

Figure 5 miR-212/132 promoter analysis

(**A**) Schematic diagram showing the luciferase promoter constructs used. Exon 1 is shown by a grey box, miR-132 and miR-212 by diamonds and Cre sites by triangles. Mutated Cre sites are indicated by crosses. (**B**) The −3105 promoter was transfected into primary cortical neurons. Where indicated, cells were pre-treated for 1 h with 2 μM PD184352. Cells were then stimulated for 3 h with 50 ng/ml BDNF. Luciferase activity was then measured as described in the Materials and methods section. Error bars represent the S.D. for three independent stimulations. (**C**) The −3105, −475 and Δ intron 1 constructs were transfected into primary cortical cultures. Where indicated, cells were stimulated with 50 ng/ml BDNF for 3 h and luciferase activity was measured. Values are expressed as the fold change relative to the unstimulated −3105 luciferase vector. Error bars represent the S.D. for six independent stimulations. (**D**) The −3105, Cre 2-4, Cre 1 and Cre 1-4 constructs were transfected into primary cortical cultures. Where indicated, cells were stimulated with 50 ng/ml BDNF for 3 h and luciferase activity measured. Error bars represent the S.D. for six independent stimulations.

[36], did not affect the induction in pri-miR-212/132 transcription in response to BDNF, indicating that ERK1/2 controlled pri-miR-212/132 induction independently of RSK (Figure 3A). Analysis of cortical cultures from MSK1/2-double-knockout mice showed that, although BDNF was able to induce pri-miR-212/132 transcription in these cells, the levels were consistently lower than those seen in wild-type cells (Figure 4A), suggesting that MSKs were partially, but not completely, responsible for regulating pri-miR-212/132 transcription downstream of ERK1/2. Consistent with this, MSK1/2 knockout also reduced the induction of the mature and star sequences for both miR-132 and miR-212 (Figure 4B). MSKs have been shown previously to phosphorylate CREB on Ser^{133} downstream of ERK1/2 [19,23], and CREB has been suggested previously to regulate miR-132 transcription [6]. Previous studies in primary cortical neuronal cultures have shown that CREB phosphorylation in response to BDNF is blocked by the MEK1/2 inhibitor PD184352 and is greatly reduced by MSK1/2 knockout [23]. Analysis of the genomic sequence around miR-132 and miR-212 revealed a 500 bp region upstream of exon 1 that was conserved in mouse and humans, suggesting that this may contain the critical promoter elements. This 500 bp region contains a potential Cre site and, in addition, three further potential Cre consensus sites could be identified in intron 1. To analyse this further, the region from 3105 bp upstream of exon 1 to the start of exon 2 was cloned into a luciferase reporter (Figure 5A). This reporter was induced by BDNF in primary cortical neurons and, similar to the endogenous gene, its induction was blocked by PD184352 (Figure 5B). In addition, two truncations of this promoter were also generated (Figure 5A). These showed that, although the long form of the promoter had the highest activity, a short form starting 475 bp 5′ to exon 1 also showed significant induction by BDNF (Figure 5C). This sequence contains all four of the potential Cre sites (Figure 5A). Deletion of the intron 1 region (which contains three of the four Cre sites) was sufficient to greatly reduce expression of the luciferase vector (Figure 4C). Mutation of the three intronic Cre sequences, however, was not enough to abolish promoter activity. In contrast, mutation of the 5′ Cre site did reduce the induction of the reporter, whereas mutation of all four Cre sites resulted in an even greater reduction in the induction of the reporter by BDNF (Figure 5D). The requirement for these Cre sites is consistent with a previous report showing that A-CREB, a dominant-negative inhibitor of CREB, could block miR-132 induction and that CREB could bind to this region as judged by ChIP (chromatin immunoprecipitation) [6]. Classically, CREB is regulated by phosphorylation on Ser^{133}, which creates a binding site for the co-activator proteins CBP (CREB-binding protein) or p300 [37,38]. We therefore also examined the role of CREB in miR-212/132 transcription using mice with an inducible S133A mutation in the endogenous CREB gene [39]. In this system, a S133A mutation in the CREB gene is induced by the excision of a wild-type floxed minigene from the endogenous CREB gene by Cre recombinase. To specifically induce this mutation in neuronal cells, Cre expression was driven from a

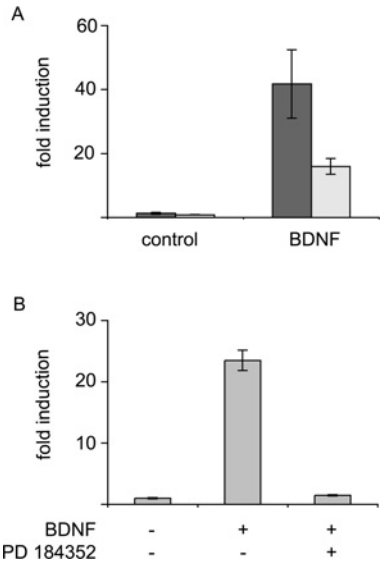

Figure 6 ERK1/2 regulate miR-212/132 transcription via CREB-phosphorylation-dependent and -independent mechanisms

(**A**) Primary cortical cultures were established from wild-type CREB nestin-Cre^{+} (black bars) and CREB S133A nestin-Cre^{+ve} (grey bars) mice. Cultures were stimulated with 50 ng/ml BDNF for 1 h and the levels of pri-miR-212/132 were determined by Q-PCR. Error bars represent the S.E.M. for independent cultures from four (wild-type) or five (CREB S133A) mice. (**B**) Primary cortical cultures were established from MSK1/2-double-knockout mice. Cells were pre-incubated with 2 μM PD184352 where indicated, and then stimulated with 50 ng/ml BDNF for 1 h and the levels of pri-miR-212/132 were determined by Q-PCR. Error bars represent the S.E.M. for independent cultures from four mice.

nestin promoter sequence that has been shown previously to express Cre in neuronal progenitor cells during development [21]. Previous studies have confirmed that primary cortical neuronal cultures from these mice express the mutated CREB S133A, but not the wild-type protein [39]. BDNF-induced pri-miR-212/132 transcription was reduced in CREB S133A-knockin neuronal cultures compared with wild-type cells (Figure 6A). The reduction in pri-miR-212/132 transcription in CREB S133A-knockin cells is in line with that seen in the MSK1/2-double-knockout cells, the kinases responsible for BDNF-induced CREB phosphorylation (compare Figure 6A with Figure 4A). It is, however, in contrast with the effect of the MEK inhibitor PD184352 on miR-212/132 transcription, as the inhibitor completely blocked the induction of this gene by BDNF. This suggests that ERK1/2 may also regulate miR-212/132 transcription via a second mechanism independent of MSK1/2 and CREB phosphorylation. To confirm this, MSK1/2-knockout cells were treated with PD184352. The residual induction of pri-miR-212/132 seen in these cells was blocked by PD184352 (Figure 6B).

Regulation of pri-miR-212/132 transcription is not restricted to neurotrophic signalling

Several other stimuli are known to induce CREB phosphorylation in neurons. Forskolin elevates cAMP levels, resulting in PKA (protein kinase A) activation and CREB phosphorylation in a number of cell types, including cortical neurons, independently of ERK1/2 and MSK1/2 [22]. Forskolin stimulation resulted in a sustained increase in pri-miR-212/132 in cortical neurons (Figure 7A). Consistent with this, forskolin was able to stimulate the induction of the miR-212/132 promoter construct. Mutation of the four Cre sites in this construct blocked induction (Figure 7B). NMDA (*N*-methyl-D-aspartate), a mimic of the neurotransmitter glutamate, could also stimulate pri-miR-212/132 transcription (Figure 7C). Similar to BDNF and forskolin, NMDA was able to stimulate the wild-type but not Cre mutant miR-212/132 promoter construct (Figure 7D). NMDA stimulates CREB phosphorylation via both the CaMK and ERK1/2 pathways. Inhibition of either of these pathways reduced NMDA-induced CREB phosphorylation, whereas inhibition of both pathways had an additive effect (Figure 7E). In line with this, inhibition of either the ERK1/2 or CaMK signalling cascades partially blocked pri-miR-212/132 induction by NMDA, whereas a combination of both inhibitors had an additive effect on pri-miR-212/132 transcription (Figure 7F). Both CREB phosphorylation and pri-miR-212/132 induction by NDMA were blocked by MK-801, an NDMA receptor antagonist (Figures 7E and 7F). Induction of miR-132 and miR-212 was not restricted to neuronal lineages. Treatment of HeLa cells with either PMA or anisomycin was able to induce pri-miR-212/132 transcription via the ERK1/2 or p38α MAPK pathway respectively, whereas pri-miR-212/132 transcription was also induced by LPS (lipopolysaccharide) which activates both ERK1/2 and p38 MAPK, in THP-1, a human monocyte cells line, although unexpectedly not by LPS in primary murine macrophages (Supplementary Figures 3B–3E).

DISCUSSION

The transcriptional up-regulation of specific miRNA genes is a potential mechanism by which signal transduction cascades could mediate their cellular functions. Some examples of this have already been reported; for instance, the induction of the miR-17/92 cluster by IL-6 (interleukin-6) is reported to be responsible for the effect of IL-6 on BMPR2 (bone morphogenetic protein receptor 2) expression [40], whereas the up-regulation of miR-146a by TLRs (Toll-like receptors) is suggested to play a role in negative-feedback loops involved in controlling TLR signalling [41]. BDNF has been shown previously to up-regulate miR-132 expression; however, the effect of BDNF on the expression of other miRNAs has not been addressed previously [6]. Using Solexa sequencing, BDNF was found to up-regulate four miRNAs with significant abundances, of which two, miR-132 and miR-212, could be confirmed by TaqMan-based Q-PCR. Both miR-132 and miR-212 are encoded by a single non-coding gene. The transcription of pri-miR-212/132 in response to BDNF was dependent on the activation of ERK1/2. In addition, it was found that the phosphorylation of CREB by MSKs downstream of ERK1/2 was also involved in pri-miR-212/132 transcription. Consistent with a role for CREB, ChIP experiments have shown previously the binding of CREB to the miR-212/132 locus [6]. In addition, ERK1/2 also regulated pri-miR-212/132 via a MSK/CREB Ser^{133}-phosphorylation-independent mechanism. The identity of this mechanism is not clear. It is possible that ERK1/2 could affect the activity of CREB in a manner independent of Ser^{133} phosphorylation, for instance by affecting the recruitment of the CREB co-activator TORC (transducer of regulated CREB-binding protein). This would be consistent with the observation that A-CREB, a dominant-negative form of CREB that prevents CREB binding to DNA, inhibits pri-miR-212/132 transcription in response to BDNF [38]. However, it is also possible that ERK1/2 may regulate pri-miR-212/132 transcription via the phosphorylation of a different transcription factor that has not yet been found to be associated with the miR-212/132 promoter.

The processing and maturation of the miR-212/132 cluster also bear some remarkable characteristics. The intron that encodes the miRNA cluster is processed independently and faster than

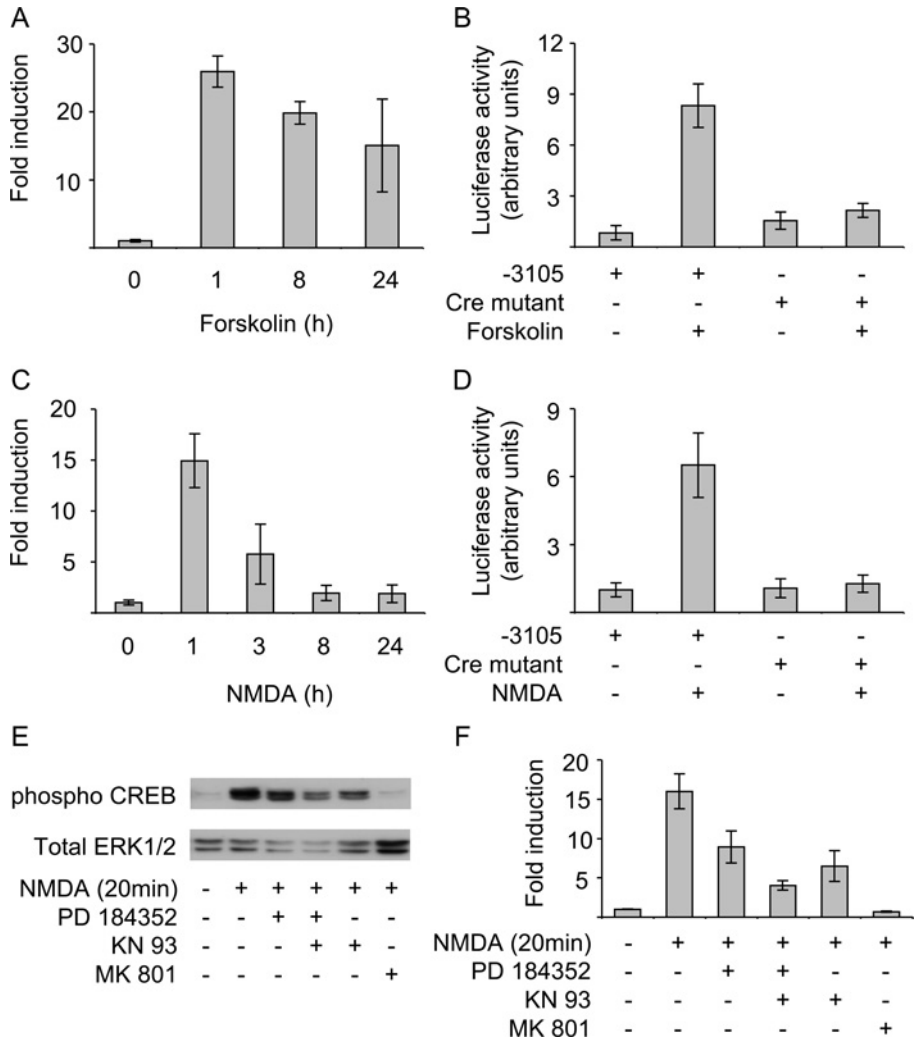

Figure 7 Forskolin and NMDA stimulate pri-miR-212/132 transcription

(**A**) Primary cortical neuron cultures were stimulated with 10 μM forskolin for the indicated times and pri-miR-212/132 levels were determined by Q-PCR. Error bars represent the S.D. for stimulations from four independent cultures. (**B**) Primary cortical neuron cultures were transfected with either a wild-type miR-212/132 promoter luciferase construct or one in which all four potential Cre sites had been mutated (see Figure 4A). Cells were stimulated with 10 μM forskolin for 3 h, lysed and luciferase activity was measured. Error bars represent the S.D. for six stimulations. (**C**) As in (**A**), except that the neurons were stimulated for the indicated times with 20 μM NMDA. (**D**) As in (**B**), except that cells were stimulated with 20 μM NMDA. (**E**) Primary cortical neuronal cultures were pre-treated with 2 μM PD184352, 30 μM Kn93 or 10 μM MK801 as indicated. Cells were stimulated for 20 min with 20 μM NMDA and lysed, and phospho-CREB and total ERK1/2 levels were determined by immunoblotting. (**F**) Primary cortical neuronal cultures were pre-treated with 2 μM PD184352, 30 μM Kn93 or 10 μM MK801 as indicated. Cells were stimulated for 3 h with 20 μM NMDA. Pri-miR-212/132 levels were determined by Q-PCR. Error bars represent the S.E.M. for four stimulations.

the rest of the pri-miRNA. This finding supports the view that intronic miRNAs are processed by the Microprocessor co-transcriptionally *a priori* to splicing [33]. Additionally, it is very unusual that miRNAs like miR-212 and miR-132 with such a low basal expression (judging from the number of the reads obtained from the sequencing results and the intensity of Northern hybridizations) show the accumulation of star sequences. Moreover, all strands derived from this miRNA cluster respond to BDNF treatment. This may suggest that there is an active mechanism that facilitates the processing of all strands from the miR-212/132 cluster generating four functional miRNAs. In addition, a 19 nt sequence corresponding to the loop sequence of pre-miR-132 was shown to be increased upon BDNF treatment. It is unlikely that this sequence is an additional 'miRNA' product from pre-miR-132, but it may accumulate because it is protected by protein that binds to the loop when miR-132 is processed. miRNA processing could be post-transcriptionally regulated and proteins that modulate pri- and pre-miRNA processing have been identified [42–45]. hnRNPA1 (heterogeneous nuclear ribonucleoprotein A1) was shown to be required for the processing of miR-18 by binding to the terminal loop of the miRNA [46,47]. In addition, common RNA-binding proteins have been shown to bind to the loops of distinct miRNAs and are predicted to have similar functions to hnRNPA1 [46]. The common characteristic of these miRNAs is that their loop sequences are extremely conserved throughout vertebrates, suggesting that it plays important role in miRNA biogenesis. The terminal loops of miR-212 and miR-132 are also extremely well conserved throughout vertebrates, suggesting that they may bind proteins that regulate their processing. This could explain the accumulation of the star sequences of both miRNAs and the loop sequence of the pre-miR-132.

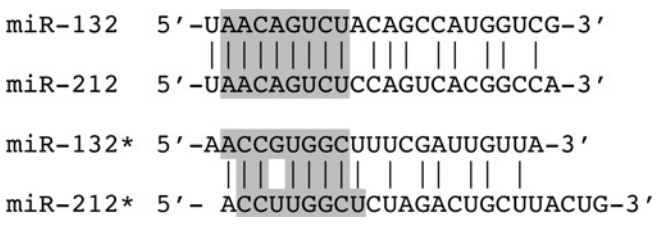

Figure 8 Alignment of miR-132 and miR-212

The mature and star sequences for murine m0069R-212 and niR-132 were aligned. Seed regions are highlighted.

The miR-212/132 cluster produces four miRNAs; however, only the function of miR-132 has been studied in neurons. miR-132 has been implicated previously in regulating neuronal morphogenesis, in part via the regulation of p250GAP [6,31,48]. Overexpression of miR-132 was found to promote neurite outgrowth and spine formation, whereas miR-132 inhibitors reduced these processes [6,31,48,49]. In addition MeCP2 and SirT1 (sirtuin 1), both of which can act as transcriptional regulators, have been suggested as targets for miR-132 [50,51]. The seed regions of miR-132 and miR-212 are identical (Figure 8), suggesting that the two miRNAs will target similar mRNAs. Analysis of the top 35 hits for miR-132 showed that 33 were also predicted as miR-212 targets, whereas 32 of the top 35 hits for miR-212 were also predicted as miR-132 targets. However, although it is widely considered that miRNAs with identical seed sequences regulate similar targets, there is increasing evidence suggesting that perfect seed sequence complementarity is not always a good predictor for miRNA target recognition [52,53]. In addition, some studies have now shown that complementarity in the 3′ region could also play role in target specificity [54]. It is therefore possible that miR-132 and miR-212 may have some distinct targets *in vivo*; however, it is likely that miR-212, like miR-132, plays a role in regulating neuronal morphology or transcription in response to neurotrophins or synaptic activity. The two star sequences carry a mismatch in their seed region and are also diverse in the 3′ region. No experimentally validated targets have been reported for miR-132* or miR-212*. Predictions using seed sequences in TargetScan suggest that the two star sequences will target different mRNAs, although experimental validation would be required for these targets before predictions could be made about the functions of these miRNAs.

In summary, we show in the present study that pri-miR-212/132 is produced from the intron of a non-coding immediate early gene and gives rise to four distinct miRNAs. The transcription is strongly regulated by ERK1/2 signalling in neurons, in part through the downstream kinase MSK1 and the phosphorylation of CREB. Previous studies have suggested roles for both ERK1/2 and miR-132 in the regulation of neuronal morphology [6,31,48]. It will therefore be of interest in future studies to examine the *in vivo* roles of both miR-132 and miR-212 in neuronal development.

AUTHOR CONTRIBUTION

Judit Remenyi and Christopher Hunter carried out all of the experimental work, with the exception of the miRNA sequencing (Hideaki Ando and Soren Impey), bioinformatics analysis (Christian Cole and Geoffrey Barton), CREB knockins (Kristy Martin), and HeLa and THP-1 cell work (Claire Monk). Gyorgy Hutvaagner and Simon Arthur helped to plan the study. All authors were involved in writing the manuscript.

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