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

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Synopsis
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 if neurotrophins regulate the production of microRNAs, small RNA species that modulate protein translation, we used deep sequencing to identify BDNF induced microRNAs in cultured primary cortical mouse neurons. This revealed that the miR-212/132 cluster contained the microRNAs most responsive to BDNF treatment. This cluster was found to produce four microRNAs; miR-132, miR-132*, miR-212, miR-212*. Using specific inhibitors, mouse models and promoter analysis we have shown that the regulation of the transcription of the miR-212/132 microRNA cluster and the microRNAs derived from it are regulated by the ERK1/2 pathway, via both MSK dependent and independent mechanisms.

Keywords
miRNA, MSK1, MSK2, BDNF, neuron, MAPK

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, BDNF, NT3 and NT4 (reviewed in [1-5]). NGF functions by activating the TrkA receptor, while 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, a member of the TNF 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, PI 3-kinase and PLC 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. While 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 recently the gene encoding the microRNA miR-132 has been shown to be transcriptionally induced by neurotrophins [6].

microRNAs are small (21-24nt) regulatory RNAs that regulate gene expression post-transcriptionally by altering the translation of their target mRNAs. In the genome, microRNAs 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 microRNA production is the transcription and processing of a primary microRNA (pri-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 further processed by Dicer into a small double stranded intermediate [10-12]. One strand of a microRNA is loaded into RISC (RNA Induced Silencing Complex) of which a key component is an Argonaute protein [13-16]. Activated microRNA complexes can either cleave their target mRNA or regulate its translation, through a variety of mechanisms [17]. *In vivo*, microRNAs have been shown to modulate several processes including development, immunity and neuronal function. In addition microRNAs may have pathological roles, for instance in cancer and tumorogenesis [18].

It is possible that neurotrophins may induce other microRNAs in addition to miR-132 and that this could represent a mechanism of fine-tuning the protein expression following neurotrophic stimulation. We therefore looked to see what effect BDNF stimulation had on the overall expression of microRNAs in cultured cortical neurons. We have found that only the miR-212/132 cluster reproducibly produces microRNAs 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 this study: mmu-miR-132 - UAACAGUCUACAGCCAUGGUCG, mmu-miR-132 complementary sequence (used as a probe for Northern hybridization) - CGACCAUGGCUGUAGACUGUUA: mmu-miR-212 - UAACAGUCUCCAGUCACGGCC, mmu-miR-212 complementary sequence (used as a probe for Northern hybridization) - GGCCGUGACUGGAGACUGUUA.

Mice and cell culture

MSK1 and MSK2 knockout and Nestin-CRE transgenic mice have been described previously [19-21]. Macrophage specific knockin of CREB has been described previously [22], and CREB Ser133Ala mice described here were crossed onto a Nestin-CRE background to achieve neuronal mutation of CREB. All mice lines used had been backcrossed onto C57Bl/6 for at least 6 generations.

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

Where indicated cells were incubated with PD 184352 (2 μM), SB 203580 (5 μM), Kn93 30 μM), PI-103 (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) anisomycin (10 mg/ml). For RNA isolation, cells were lysed and RNA isolated using microRNeasy mini kits (Qiagen) in line with the manufacturer's protocol. For immunoblotting neurons were lysed directly into SDS sample buffer while 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, 0.1% (v/v) 2-mercaptoethanol and centrifuged at 13000g for 5 min at 4^{o}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 poly-acrylamide 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 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 dropped below 20. The quality filtered reads where 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 non-coding RNAs (ncRNAs) (Ensembl release 52) [26] and annotated microRNA 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 problematic. 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 $n^{1/2}$ [28] and the control and BDNF errors were propagated through to the BDNF/Control ratio (Table 2 and 3) with the following formula:

$$\alpha r = \sqrt{r^2\left[\left(\frac{\alpha C}{C}\right)^2 + \left(\frac{\alpha B}{B}\right)^2\right]}$$

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 data, respectively.

Q-PCR

For PCR of mRNA or intron sequences, total RNA was reverse transcribed with iScript (Biorad) in line with the manufacturer's protocols. Q-PCR was carried out using SYBERgreen detection. The PCR program consisted of an initial activation step of 3 min at 95°C, followed by 40 cycles of 20 sec at 95°C and 45 sec at 58°C. Fold induction was calculated relative to the unstimulated control (wild type) sample, using 18s levels to correct for loading [29]. Primer sequences used are given in supplementary table 2.

Q-PCR for mature microRNA 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

4.553 kb of the mouse miR212/132 promoter was amplified from mouse BAC 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 Corporation) in a three-way ligation using BamH1-Not1-Hind111. The resulting full-length clone was fully sequenced.

5' truncations of the promoter were created by introducing an Xho1 site within the promoter sequence by PCR mutagenesis using KOD Hot Start DNA polymerase (Novagen). The resulting Xho1 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 BamH1/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 3.

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. Two days after the transfection the cells were stimulated with BDNF (50 ng/ml) for 3 hours, then the cells were lysed with 1X passive lysis buffer. The luminescence of the samples was measured by a luminometer (Microlumat plus LB96, Berthold Technologies).

Immunoblotting

Samples were run on 10 % polyacrylamide gels and transfered onto nitrocellulose membranes using standard protocols. The antibodies against total ERK1/2 was from Cell Signalling and the phospho Ser133 CREB monoclonal antobody was from Millipore. Detection was acheived using horseradish peroxidase conjugated secondary antibodies (Pierce) and chemiluminescent substrate (Amersham).

Northern hybridizations

10 μg of total RNA was separated on 15% polyacrylamide gels containing 7M Urea and 20 mM MOPS/NaCl pH7.0. The RNAs were transferred onto Hybond N (Amersham) 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 that the filters were washed for two times for an hour at 65^{o}C with 0.1 % SDS, 2 X SSC. Detection and quantification of the signals were carried out as it was described above.

3′ RACE analysis

1 μg Qiazol (Qiagen) extracted total RNA was subjected to first strand synthesis with Superscript III (Invitrogen) reverse transcriptase at 50^{o}C for 1 hour. First and nested PCR reactions were carried out with KOD hot start DNA polymerase (NOVAGEN). Amplified DNA sequences were cloned and transformed using the Strataclone^{TM} Blunt PCR Cloning Kit (Stratagene). 8-8 positive clones were sequenced and the sequences were analyzed with Vector NTI software (Invitrogen).

Results

MicroRNA 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 24h, and microRNA expression analysed by Solexa sequencing. The output from the sequencing was filtered and collated into unique examples and then searched against known pre-microRNAs as well as other known RNAs as detailed in Table 1. This showed that the vast majority of the isolated RNA matched to pre-miRNA sequences in both samples. In the unstimulated sample, the matches corresponded to 330 individual pre-miRNA sequences from the 547 mouse pre-microRNAs annotated in miRBase [27]. Of these, 202 were represented by 10 or more hits. For the BDNF stimulated sample, there were 373 individual pre-miRNA matches with 243 having an abundance of at least 10 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 1.

Analysis of the normalised expression changes between the control and BDNF stimulated data showed that the expression of most of the microRNAs was similar between the two samples (Fig 1a, Supplementary Table 1). 12 pre-miRNAs had absolute fold changes of >4 and are shown in Table 2. However, most had fewer than 5 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 upregulation of these microRNAs following BNDF treatment (data not shown). Filtering out pre-miRNAs with fewer than 5 reads left 4 pre-miRNAs with a fold change of greater than 4: miR-98, miR-146a, miR-212 and miR-132 (Table 2).

To confirm the sequencing data, the expression of miR-98, miR-146a, miR-212 and miR-132, along with several other microRNAs that were not expected to change was examined by Q-PCR in a second set of samples, independent to those used for sequencing (Table 3 and Figure 1B). For each of the selected microRNAs, alignments of the sequencing hits to the hairpin sequence where examined to determine if 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 (* sequence) was also observed. In addition for miR-132, a sequence corresponding to the hairpin loop region was also sequenced (Supplementary Figure 1). Comparison between the Q-PCR data and sequencing data showed that while there was reasonable agreement between the two methods for the non–induced microRNAs (Fig 1A, table 3), only two of the 4 upregulated microRNAs from the sequencing data were reproduced in the Q-PCR analysis. Using Q-PCR, miR-98 and miR-146a were not significantly upregulated (p-value > 0.01) after 4 or 24h of BDNF treatment (Table 1, data not shown). The lack of miR-98 upregulation 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 for the sequencing data, which allowed up to 2 mismatches, could not always differentiate between miR-98 and other let-7 isoforms.

To examine this, the sequencing data was reanalysed allowing for 0 mismatches. Under this more stringent analysis, the number of miR-98 matches fell, while the fold change between the control and BDNF sample decreased to 1.22, a value similar to that obtained by Q-PCR. Both miR-132 and miR-212 were still induced using this analysis method.

Of the microRNAs tested only miR-132 and miR-212 were upregulated consistently by BDNF in both the sequencing and Q-PCR data (Fig 1B). Upregulation of both miR-132 and miR-212 by BNDF could also be demonstrated by Northern blotting (Fig 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 previously been shown to be upregulated in neuronal cells following neurotrophic stimulation [6], and has been suggested to be involved in the regulation of MeCP2 and p250Gap in neurons [31, 32]. The regulation of miR-212 expression has not however previously been studied 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 localise to the same region of chromosome 11. Both microRNAs are also found in an equivalent localisation 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 revealed evidence for a similar non-coding gene in mice, consisting of 3 exons with miR-132 and miR-212 located in intron 1 (Fig 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 exon1/2 or exon 2/3 fragments (Fig 2B). Cloning of these PCR products confirmed that the splicing predicted from the ESTs had occurred (data 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 (Fig 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 (Fig 2C). Analysis of the intron 2 sequence revealed 3 potential poly-adenylation sites. 3' RACE confirmed that termination could occur at least 2 of these sites in addition to the poly adenylation site 3' to exon 3 (supplementary figure 2). 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 (Fig 2C). This would suggest that the predominant form expressed following BDNF stimulation corresponded to termination at the poly-adenylation 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 and PI 3-kinase 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]. PI-103, a specific PI-3 kinase inhibitor, SB 203580, a p38α/β MAPK inhibitor, and KN 93, a CaMK inhibitor, did not affect the transcription of pri-miR-212/132 in response to BDNF (Fig 3A). In contrast PD 184352, a MEK1/2 inhibitor that blocks the activation of ERK1/2, completely blocked the induction of pri-miR-212/132 in response to BDNF (Fig 3A). The upregulation of the mature microRNA was also determined using a Taqman based PCR system. As star sequences were also identified in the sequencing data 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 upregulated by BNDF stimulation, and consistent with the data on the pri-miR-212/132, PD184352 blocked the accumulation of the mature sequences in response to BNDF (Fig 3B). In line with what was observed for miR-132 and miR-212, the * sequences of both microRNAs were also up regulated by BDNF in an ERK1/2 dependent manner (Fig 3B). To determine absolute levels for each microRNA, Q-PCR was carried out and levels determined relative to a standard curve prepared using a synthetic oligo corresponding to each microRNA 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 (Fig 3C). This was in contrast to the sequencing data which suggested that miR-212* might be expressed at a higher level than miR-212. This difference could possibly be due to a ligation basis 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 and MSK, that can in turn phosphorylate both transcription factors and chromatin [22, 35]. Bi-D1870, a specific RSK inhibitor [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 (Fig 3A). Analysis of cortical cultures from MSK1/2 double knockout mice showed that while 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 (Fig 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 (Fig 4B). MSKs have previously been shown to phosphorylate CREB on Ser133 downstream of ERK1/2 [19, 23], and CREB has previously been suggested 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 PD 184352 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 exon1 that was conserved in mouse and human, suggesting that this may contain the critical promoter elements. This 500bp 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 3105bp upstream of exon 1 to the start of exon 2 was cloned into a luciferase reporter (Fig 5A). This reporter was induced by BDNF in primary cortical neurons, and similar to the endogenous gene its induction was blocked by PD 184352 (Fig 5B). In addition two truncations of this promoter were also generated (Fig 5A). These showed that while the long form of the promoter has the highest activity, a short form starting 475 bp 5' to exon 1 also showed significant induction by BDNF (Fig 5C). This sequence contains all four of the potential CRE sites (Fig 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 (Fig 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, while mutation of all four CRE sites resulted in an even greater reduction in the induction of the reporter by BDNF (Fig 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 [6]. Classically CREB is regulated by phosphorylation on Ser133, which creates a binding site for the co-activator proteins CBP or p300 [37, 38]. We therefore also examined the role of CREB in miR-212/132 transcription using mice with an inducible Ser133Ala mutation in the endogenous CREB gene [39]. In this system, a Ser133Ala 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 nestin promoter sequence, that has previously been shown 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 Ser133Ala CREB but not the wild type protein [39]. BDNF induced pri-miR-212/132 transcription was reduced in Ser133Ala CREB knock-in neuronal cultures compared to wild type cells (Fig 6A). The reduction in pri-miR-212/132 transcription in CREB Ser133Ala knockin cells is in line with that seen in the MSK1/2 double knockout cells, the kinases responsible for BDNF induced CREB phosphorylation (compare Fig 6A and Fig 4A). It is however in contrast to the effect of the MEK inhibitor PD 184352 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 PD 184352. The residual induction of pri-miR-212/132 seen in these cells was blocked by PD184352 (Fig 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 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 (Fig 7A). Consistent with this, forskolin was also able to stimulate the induction of the miR-212/132 promoter construct. Mutation of the four Cre sites in this construct blocked induction (Fig 7B). NMDA, a mimic of the neurotransmitter glutamate, was also able to stimulate pri-miR-212/132 transcription (Fig 7C). Similar to BDNF and forskolin, NMDA could also stimulate the wild type but not CRE mutant miR-212/132 promoter construct (Fig 7D). NMDA stimulates CREB phosphorylation via both the CaMK and ERK1/2 pathways. Inhibition of either of these pathways reduced NMDA induced CREB phosphorylation, while inhibition of both pathways had an additive effect (Fig 7E). Consistent with this, inhibition of either the ERK1/2 or CaMK signalling cascades partially blocked pri-miR-212/132 induction by NMDA, while a combination of both inhibitors had an additive effect (Fig 7F). Both CREB phosphorylation and pri-miR-212/132 induction by NDMA were blocked by MK-801, an NDMA receptor antagonist (Fig 7E and F). 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 p38a MAPK pathway respectively, while pri-miR-212/132 transcription was also induced by LPS (which activates both ERK1/2 and p38) in THP-1, a human monocyte cells line, although unexpectedly not by LPS in primary murine macrophages (supplementary figure 3).

Discussion

The transcriptional up-regulation of specific microRNA 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 is reported to be responsible for the effect of IL-6 on BMPR2 expression [40] while the upregulation of miR-146a by TLRs is suggested to play a role in negative feedback loops involved in controlling TLR signalling [41]. BDNF has previously been shown to upregulate miR-132 expression, however the effect of BDNF on the expression of other microRNAs has not previously been addressed (38). Using Solexa sequencing, BDNF was found to upregulate four microRNAs 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 noncoding 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 previously shown the binding of CREB to the miR-212/132 locus (45). In addition ERK1/2 also regulated pri-miR-212/132 via a MSK/CREB Ser133 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 Ser133 phosphorylation, for instance by affecting the recruitment of the CREB co-activator TORC. 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). It is also possible however 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 microRNA cluster is processed independently and faster than the rest of the pri-miRNA. This finding supports the view that intronic microRNAs are processed by the Microprocessor co-transcriptionally *a priori* to splicing [33]. Additionally, it is very unusual that microRNAs with such a low basal expression like miR-212 and 132 (judging from the number of the reads obtained from the sequencing data and the intensity of Northern hybridizations) show the accumulation of the star sequences. Moreover, all strands derived from this microRNA 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 microRNAs. 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 "microRNA" 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. microRNA processing could be post-transcriptionally regulated and proteins that modulate pri- and pre-miRNA processing have been identified [42-45]. HnRNP A1 was shown to be required for the processing of miR-18 by binding to the terminal loop of the microRNA [46, 47]. In addition, common RNA binding proteins have been shown to bind to the loops of distinct microRNAs and predicted to have similar functions to hnRNP A1 [46]. The common characteristic of these microRNAs is that their loop sequences are extremely conserved throughout the vertebrates suggesting that it plays important role in microRNA biogenesis. The terminal loops of miR-212 and miR-132 are also extremely conserved throughout the vertebrates suggesting that they may bind proteins that regulate their processing. This could explain the accumulation of the star sequences of both microRNAs and the loop sequence of the pre-miR-132.

In summary we show here that pri-miR-212/132 is produced from the intron of a non-coding immediate early gene, and gives rise to 4 distinct microRNAs. 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.

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References

1 Reichardt, L. F. (2006) Neurotrophin-regulated signalling pathways. Philos Trans R Soc Lond B Biol Sci. **361**, 1545-1564

2 Chao, M. V., Rajagopal, R. and Lee, F. S. (2006) Neurotrophin signalling in health and disease. Clin Sci (Lond). **110**, 167-173

3 Segal, R. A. (2003) Selectivity in neurotrophin signaling: theme and variations. Annu Rev Neurosci. **26**, 299-330
4 Patapoutian, A. and Reichardt, L. F. (2001) Trk receptors: mediators of neurotrophin action. Curr Opin Neurobiol. **11**, 272-280
5 Friedman, W. J. and Greene, L. A. (1999) Neurotrophin signaling via Trks and p75. Exp Cell Res. **253**, 131-142
6 Vo, N., Klein, M. E., Varlamova, O., Keller, D. M., Yamamoto, T., Goodman, R. H. and Impey, S. (2005) A cAMP-response element binding protein-induced microRNA regulates neuronal morphogenesis. Proc Natl Acad Sci U S A. **102**, 16426-16431
7 Kim, V. N. (2005) MicroRNA biogenesis: coordinated cropping and dicing. Nat Rev Mol Cell Biol. **6**, 376-385
8 Gregory, R. I., Yan, K. P., Amuthan, G., Chendrimada, T., Doratotaj, B., Cooch, N. and Shiekhattar, R. (2004) The Microprocessor complex mediates the genesis of microRNAs. Nature. **432**, 235-240
9 Denli, A. M., Tops, B. B., Plasterk, R. H., Ketting, R. F. and Hannon, G. J. (2004) Processing of primary microRNAs by the Microprocessor complex. Nature. **432**, 231-235
10 Lee, Y., Ahn, C., Han, J., Choi, H., Kim, J., Yim, J., Lee, J., Provost, P., Radmark, O., Kim, S. and Kim, V. N. (2003) The nuclear RNase III Drosha initiates microRNA processing. Nature. **425**, 415-419
11 Hutvagner, G., McLachlan, J., Pasquinelli, A. E., Balint, E., Tuschl, T. and Zamore, P. D. (2001) A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. Science. **293**, 834-838
12 Lund, E., Guttinger, S., Calado, A., Dahlberg, J. E. and Kutay, U. (2004) Nuclear export of microRNA precursors. Science. **303**, 95-98
13 Meister, G., Landthaler, M., Patkaniowska, A., Dorsett, Y., Teng, G. and Tuschl, T. (2004) Human Argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs. Mol Cell. **15**, 185-197
14 Liu, J., Carmell, M. A., Rivas, F. V., Marsden, C. G., Thomson, J. M., Song, J. J., Hammond, S. M., Joshua-Tor, L. and Hannon, G. J. (2004) Argonaute2 is the catalytic engine of mammalian RNAi. Science. **305**, 1437-1441
15 Hutvagner, G. and Zamore, P. D. (2002) A microRNA in a multiple-turnover RNAi enzyme complex. Science. **297**, 2056-2060
16 Mourelatos, Z., Dostie, J., Paushkin, S., Sharma, A., Charroux, B., Abel, L., Rappsilber, J., Mann, M. and Dreyfuss, G. (2002) miRNPs: a novel class of ribonucleoproteins containing numerous microRNAs. Genes Dev. **16**, 720-728
17 Chekulaeva, M. and Filipowicz, W. (2009) Mechanisms of miRNA-mediated post-transcriptional regulation in animal cells. Curr Opin Cell Biol. **21**, 452-460
18 Sotiropoulou, G., Pampalakis, G., Lianidou, E. and Mourelatos, Z. (2009) Emerging roles of microRNAs as molecular switches in the integrated circuit of the cancer cell. Rna. **15**, 1443-1461
19 Wiggin, G. R., Soloaga, A., Foster, J. M., Murray-Tait, V., Cohen, P. and Arthur, J. S. (2002) MSK1 and MSK2 are required for the mitogen- and stress-induced phosphorylation of CREB and ATF1 in fibroblasts. Mol Cell Biol. **22**, 2871-2881
20 Arthur, J. S. and Cohen, P. (2000) MSK1 is required for CREB phosphorylation in response to mitogens in mouse embryonic stem cells. FEBS Lett. **482**, 44-48

21 Graus-Porta, D., Blaess, S., Senften, M., Littlewood-Evans, A., Damsky, C., Huang, Z., Orban, P., Klein, R., Schittny, J. C. and Muller, U. (2001) Beta1-class integrins regulate the development of laminae and folia in the cerebral and cerebellar cortex. Neuron. **31**, 367-379
22 Ananieva, O., Darragh, J., Johansen, C., Carr, J. M., McIlrath, J., Park, J. M., Wingate, A., Monk, C. E., Toth, R., Santos, S. G., Iversen, L. and Arthur, J. S. (2008) The kinases MSK1 and MSK2 act as negative regulators of Toll-like receptor signaling. Nat Immunol. **9**, 1028-1036
23 Arthur, J. S., Fong, A. L., Dwyer, J. M., Davare, M., Reese, E., Obrietan, K. and Impey, S. (2004) Mitogen- and stress-activated protein kinase 1 mediates cAMP response element-binding protein phosphorylation and activation by neurotrophins. J Neurosci. **24**, 4324-4332
24 Cole, C., Sobala, A., Lu, C., Thatcher, S. R., Bowman, A., Brown, J. W., Green, P. J., Barton, G. J. and Hutvagner, G. (2009) Filtering of deep sequencing data reveals the existence of abundant Dicer-dependent small RNAs derived from tRNAs. RNA
25 Abouelhoda, M. I., Kurtz, S. and Ohlebusch, E. (2004) Replacing suffix trees with enhanced suffix arrays. J. Discrete Algor. **2**, 53-56
26 Birney, E., Clamp, M. and Durbin, R. (2004) GeneWise and Genomewise. Genome Res. **14**, 988-995
27 Griffiths-Jones, S., Saini, H. K., van Dongen, S. and Enright, A. J. (2008) miRBase: tools for microRNA genomics. Nucleic Acids Res. **36**, D154-158
28 Rao, C. R. (1965) Linear Statistical Inference and its Applications. Wiley, New York
29 Darragh, J., Soloaga, A., Beardmore, V. A., Wingate, A. D., Wiggin, G. R., Peggie, M. and Arthur, J. S. (2005) MSKs are required for the transcription of the nuclear orphan receptors Nur77, Nurr1 and Nor1 downstream of MAPK signalling. Biochem J. **390**, 749-759
30 Pall, G. S. and Hamilton, A. J. (2008) Improved northern blot method for enhanced detection of small RNA. Nat Protoc. **3**, 1077-1084
31 Wayman, G. A., Davare, M., Ando, H., Fortin, D., Varlamova, O., Cheng, H. Y., Marks, D., Obrietan, K., Soderling, T. R., Goodman, R. H. and Impey, S. (2008) An activity-regulated microRNA controls dendritic plasticity by down-regulating p250GAP. Proc Natl Acad Sci U S A. **105**, 9093-9098
32 Klein, M. E., Lioy, D. T., Ma, L., Impey, S., Mandel, G. and Goodman, R. H. (2007) Homeostatic regulation of MeCP2 expression by a CREB-induced microRNA. Nat Neurosci. **10**, 1513-1514
33 Morlando, M., Ballarino, M., Gromak, N., Pagano, F., Bozzoni, I. and Proudfoot, N. J. (2008) Primary microRNA transcripts are processed co-transcriptionally. Nat Struct Mol Biol. **15**, 902-909
34 Bain, J., Plater, L., Elliott, M., Shpiro, N., Hastie, C. J., McLauchlan, H., Klevernic, I., Arthur, J. S., Alessi, D. R. and Cohen, P. (2007) The selectivity of protein kinase inhibitors: a further update. Biochem J. **408**, 297-315
35 Roux, P. P. and Blenis, J. (2004) ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. Microbiol Mol Biol Rev. **68**, 320-344

36 Sapkota, G. P., Cummings, L., Newell, F. S., Armstrong, C., Bain, J., Frodin, M., Grauert, M., Hoffmann, M., Schnapp, G., Steegmaier, M., Cohen, P. and Alessi, D. R. (2007) BI-D1870 is a specific inhibitor of the p90 RSK (ribosomal S6 kinase) isoforms in vitro and in vivo. Biochem J. **401**, 29-38
37 Johannessen, M., Delghandi, M. P. and Moens, U. (2004) What turns CREB on? Cell Signal. **16**, 1211-1227
38 Mayr, B. and Montminy, M. (2001) Transcriptional regulation by the phosphorylation-dependent factor CREB. Nat Rev Mol Cell Biol. **2**, 599-609
39 Wingate, A. D., Martin, K. J., Hunter, C., Carr, J. M., Clacher, C. and Arthur, J. S. (2009) Generation of a conditional CREB Ser133Ala knockin mouse. Genesis **47**, 688-696
40 Brock, M., Trenkmann, M., Gay, R. E., Michel, B. A., Gay, S., Fischler, M., Ulrich, S., Speich, R. and Huber, L. C. (2009) Interleukin-6 modulates the expression of the bone morphogenic protein receptor type II through a novel STAT3-microRNA cluster 17/92 pathway. Circ Res. **104**, 1184-1191
41 Taganov, K. D., Boldin, M. P., Chang, K. J. and Baltimore, D. (2006) NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. Proc Natl Acad Sci U S A. **103**, 12481-12486
42 Suzuki, H. I., Yamagata, K., Sugimoto, K., Iwamoto, T., Kato, S. and Miyazono, K. (2009) Modulation of microRNA processing by p53. Nature. **460**, 529-533
43 Davis, B. N., Hilyard, A. C., Lagna, G. and Hata, A. (2008) SMAD proteins control DROSHA-mediated microRNA maturation. Nature. **454**, 56-61
44 Trabucchi, M., Briata, P., Garcia-Mayoral, M., Haase, A. D., Filipowicz, W., Ramos, A., Gherzi, R. and Rosenfeld, M. G. (2009) The RNA-binding protein KSRP promotes the biogenesis of a subset of microRNAs. Nature. **459**, 1010-1014
45 Viswanathan, S. R., Daley, G. Q. and Gregory, R. I. (2008) Selective blockade of microRNA processing by Lin28. Science. **320**, 97-100
46 Michlewski, G., Guil, S., Semple, C. A. and Caceres, J. F. (2008) Posttranscriptional regulation of miRNAs harboring conserved terminal loops. Mol Cell. **32**, 383-393
47 Guil, S. and Caceres, J. F. (2007) The multifunctional RNA-binding protein hnRNP A1 is required for processing of miR-18a. Nat Struct Mol Biol. **14**, 591-596
48 Impey, S., Davare, M., Lasiek, A., Fortin, D., Ando, H., Varlamova, O., Obrietan, K., Soderling, T. R., Goodman, R. H. and Wayman, G. A. (2009) An activity-induced microRNA controls dendritic spine formation by regulating Rac1-PAK signaling. Mol Cell Neurosci

Figure legends

Figure 1 microRNA profiling following BDNF stimulation.

A) microRNAs were sequenced from unstimulated and BDNF (50 ng/ml, 24 h) stimulated cortical neurons. The sequencing data was analysed as described in the methods, and the numbers of sequences that mapped to each pre-microRNA were plotted. MicroRNAs present in only one sample were excluded from the plot.

B) Comparison of Q-PCR and sequencing results of selected microRNAs (listed in table 3). Error bars for Q-PCR represents the standard error of the mean of 3 independent stimulations. Error bars for sequencing data represent estimated errors that were calculated as described in the methods.

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 analysed by Northern blotting as described in the methods. Fold induction of the mature microRNAs is given above the blots.

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

A) Mir-132 and miR-212 map to the 1st intron of a non-coding mRNA gene on chromosome 11 in the mouse genome. The presence of the three exons predicted by from mouse ESTs are indicated (white boxes). The positions of miR-132 and miR-212 in the 1st intron of this gene are shown (vertical lines) and potential poly-adenylation sites (AATAAA) are shown (grey vertical lines). The position of the various primer sets used for Q-PCR are marked. Sequences for these primers are given in supplementary table 2.

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 noncoding transcript determined by Q-PCR. Primers amplifying mRNA derived from splicing at the exon 1/2 boundary are shown in the left panel, primers detecting splicing at the exon 2/3 boundary in the right panel. 18s RNA levels were used to correct for total RNA concentrations. Error bars represent the standard deviation from 3 independent stimulations.

C) As (B), but Q-PCR was used to determine absolute levels of sequences from intron1, exon2, intron 3 and exon 4. All 4 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 standard deviation from 3 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 determined by Q-PCR. Error bars represent the s.e.m. of three independent stimulations.

B) Primary cortical neurons were incubated for 1 h in the presence of 2 μM PD184352 were indicated and then stimulated for 4 or 24 h with 50 ng/ml BDNF. Induction of both mature and star microRNA sequences for miR-132 and miR-

212 determined by Q-PCR. Error bars represent the s.e.m. of three independent stimulations.

C) The absolute levels of the mature and * microRNA 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 oligo corresponding to the microRNA sequences. Quantification was carried out for the 4 and 24h BDNF stimulated samples described in (B).

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 determined by Q-PCR. For both wild type and knockout samples, fold change was calculated relative to the wild type control samples. Errors bars represent the s.e.m. of 4 independent stimulations.

B) As (A) but mature and * microRNA sequences for both miR-132 and miR-212 were measured.

Figure 5 miR-212/132 promoter analysis.

A) Schematic 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 pretreated 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 methods. Error bars represent the standard deviation of 3 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 measured. Values are expressed as fold change relative to the unstimulated -1306 luciferase vector. Error bars represent the standard deviation of 6 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 standard deviation of 6 independent stimulations.

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+ve (black bars) and CREB Ser133Ala Nestin-Cre^{+ve} (grey bars) mice. Cultures were stimulated with 50 ng/ml BDNF for 1h and the levels of pri-miR-212/132 determined by Q-PCR. Error bars represent the s.e.m. of independent cultures from 4 (wild type) or 5 (CREB Ser133Ala) mice.

B) Primary cortical cultures were established from MSK1/2 double knockout mice. Cells were preincubated with 2 μM PD 184352 where indicated and then stimulated with 50 ng/ml BDNF for 1 h and the levels of pri-miR-212/132

determined by Q-PCR. Error bars represent the s.e.m. of independent cultures from 4 mice.

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 determine by Q-PCR Error bars represent the standard deviation of stimulations from 4 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 4 potential Cre sites had been mutated (see Fig 4A). Cells were stimulated with 10 μM forskolin for 3 h then lysed and luciferase activity measured. Error bars represent the standard deviation of 6 stimulations.

C) As (A) except that the neurons were stimulated for the indicated times with 20 μM NMDA.

D) As (B) except that cells were stimulated with 20 μM NMDA.

E) Primary cortical neuronal cultures were pretreated with 2 μM PD 184352, 30 μM Kn 93 or 10 μM MK 801 as indicated. Cells were stimulated for 20 min with 20 μM NMDA lysed and phospho-CREB and total ERK1/2 levels determined by immunoblotting.

F) Primary cortical neuronal cultures were pretreated with 10 mM PD 184352, 30 uM Kn 93 or 10 μM MK 801 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. of 4 stimulations.

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.

	Read Abundance	
RNA species[+]	Unstimulated control	BDNF Stimulated
microRNA	2,101,986	3,426,272
Misc. RNA	3,547	8,373
tRNA	3,069	12,925
snRNA	2,218	5,996
rRNA	1,082	3,167
snoRNA	807	4,184
Mt rRNA	572	1,494
Mt tRNA	143	362

[+] RNA species names are as annotated in miRBase and Ensembl.

Table 2 microRNAs showing potential induction in the sequencing data.
pre-miRNA sequences showing the largest fold change in expression when stimulated by BDNF. Raw read abundance for each miRNA was normalised 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 normalised abundance of BDNF data/Control data. Estimated errors, calculated as described in the methods, are shown in brackets.

	Read Abundance		Normalised Abundance		Fold Change in Expression
miRNA	Control	BDNF	Control	BDNF	(BDNF/Control) (Error)
mir-712	1	19	4.76E-07	5.55E-06	11.66 (11.96)
mir-194-1	1	14	4.76E-07	4.09E-06	8.59 (8.89)
mir-98	5753	74250	2.74E-03	2.17E-02	7.92 (0.11)
mir-146a	12	139	5.71E-06	4.06E-05	7.11 (2.14)
mir-212	30	276	1.43E-05	8.06E-05	5.64 (1.09)
mir-207	1	9	4.76E-07	2.63E-06	5.52 (5.82)
mir-381	1	9	4.76E-07	2.63E-06	5.52 (5.82)
mir-200b	1	8	4.76E-07	2.33E-06	4.91 (5.21)
mir-483	2	15	9.51E-07	4.38E-06	4.60 (3.46)
mir-758	1	7	4.76E-07	2.04E-06	4.29 (4.59)
mir-218-1	3	21	1.43E-06	6.13E-06	4.29 (2.65)
mir-132	52	353	2.47E-05	1.03E-04	4.16 (0.62)
mir-1197	4	1	1.90E-06	2.92E-07	0.15 (0.17)

Table 3 Comparison and sequencing and Q-PCR data.
Selected microRNAs from the sequencing results are shown. Total number of hits indicated the number of times a sequence for the pre-microRNA was obtained in the RNA sample from the 24h BDNF stimulated neurons. For each microRNA, the % of hits for the potential 5p and 3p microRNA 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 data and Q-PCR analysis. For the sequencing data estimated errors, calculated as described in the methods, are shown in brackets. The Q-PCR was carried out on 3 independent samples, and p-values (students t-test) are given for the difference between control and BDNF stimulated samples for the PCR results.

		Sequencing abundance in BDNF sample			fold induction		
	miRbase				Normalised Sequencing		
	annotation	% 5p	% 3p	total	(Error)	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

Figure 1

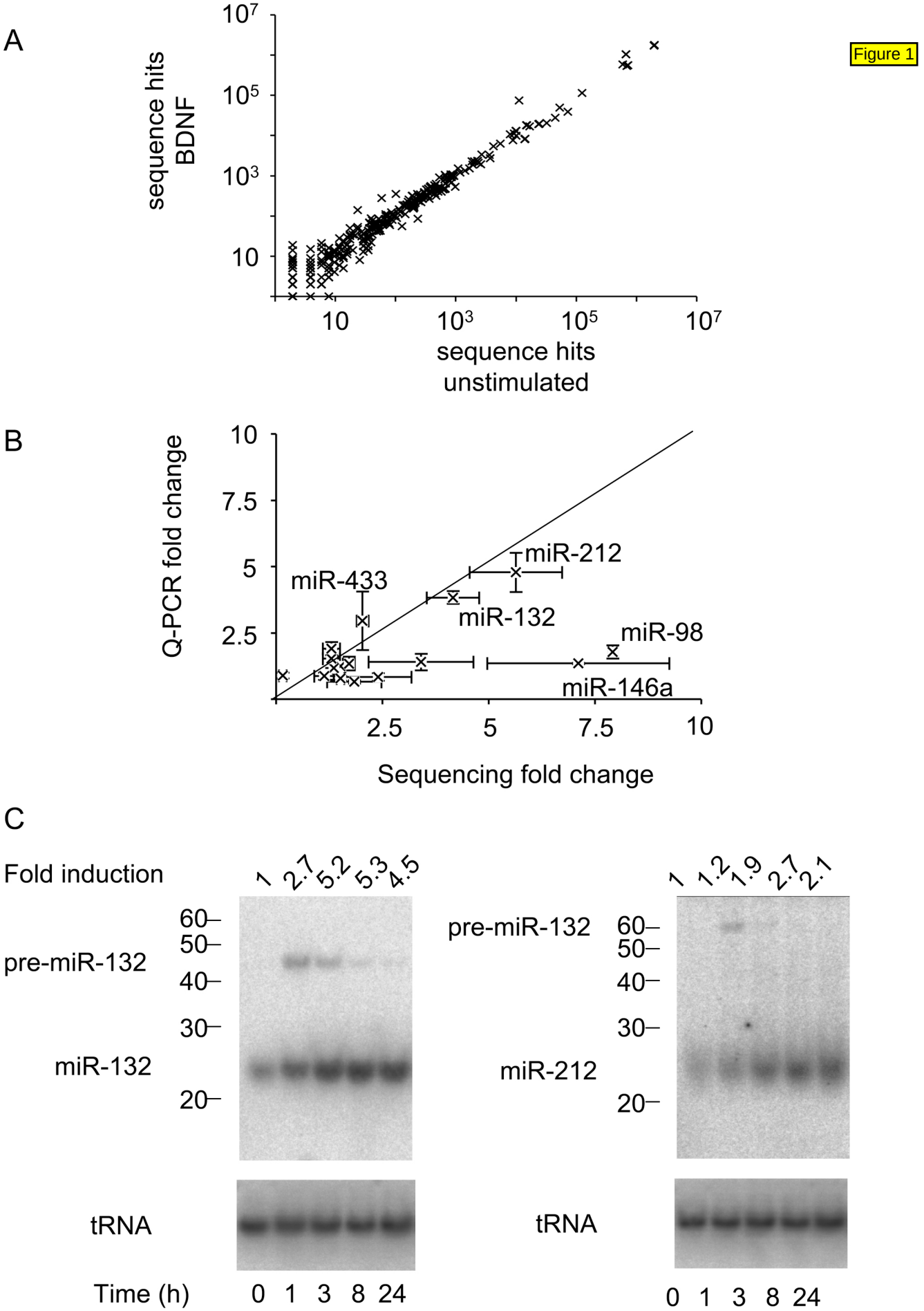
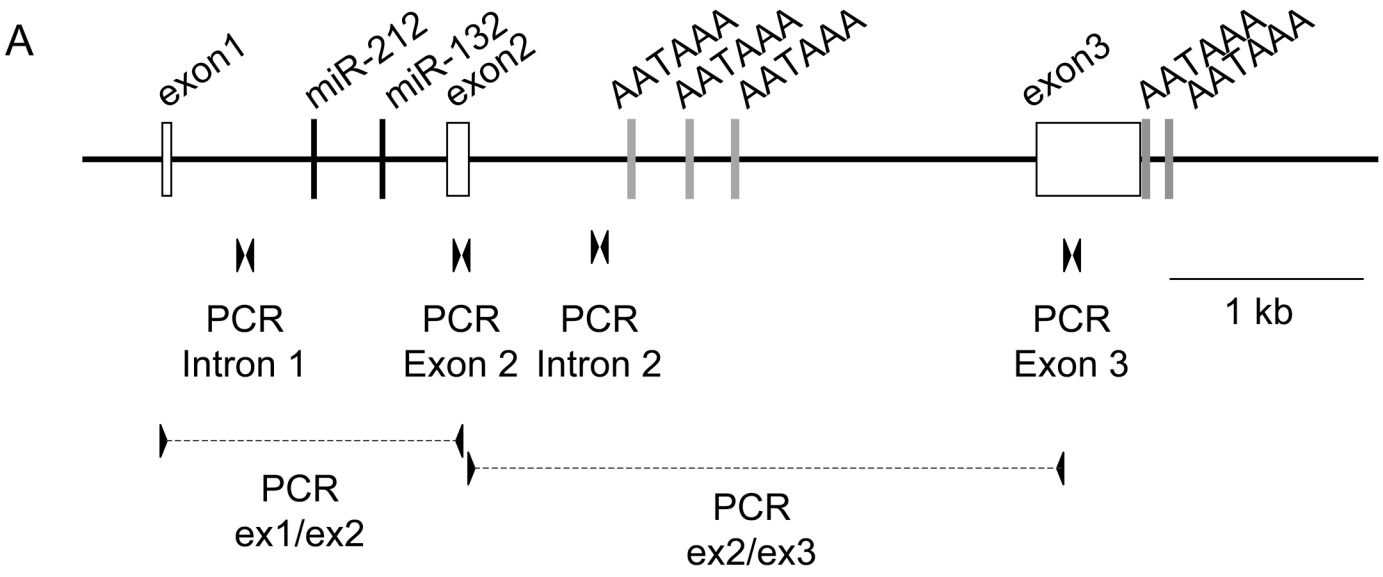

A
sequence hits BDNF
10^7
10^5
10^3
10
10
10^3
10^5
10^7
sequence hits unstimulated
B
Q-PCR fold change
10
7.5
5
2.5
miR-212
miR-433
miR-132
miR-98
miR-146a
2.5
5
7.5
10
Sequencing fold change
C
Fold induction
1 2.7 5.2 5.3 4.5
pre-miR-132
miR-132
60
50
40
30
20
tRNA
Time (h)
0 1 3 8 24
pre-miR-132
1 1.2 1.9 2.7 2.1
miR-212
60
50
40
30
20
tRNA
0 1 3 8 24

Figure 2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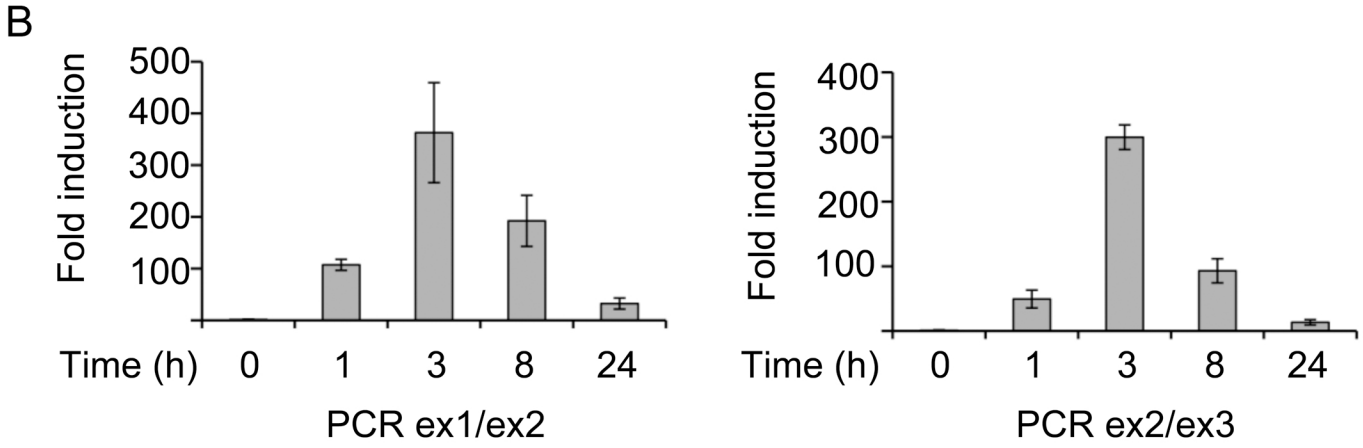
exon1
miR-212
miR-132
exon2
AATAAA
AATAAA
AATAAA
exon3
AATAAA
AATAAA
PCR
Intron 1
PCR
Exon 2
PCR
Intron 2
PCR
Exon 3
1 kb
PCR
ex1/ex2
PCR
ex2/ex3


B
Fold induction
500
400
300
200
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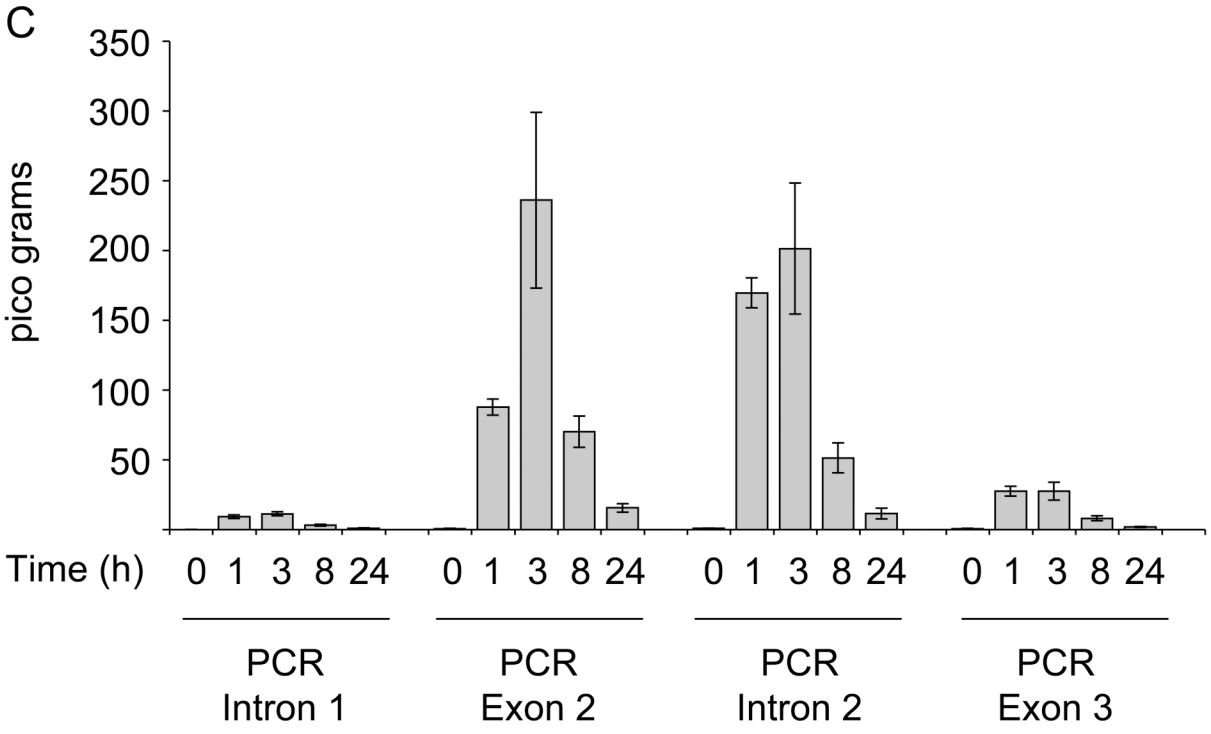
PCR ex1/ex2
Fold induction
400
300
200
100
Time (h) 0 1 3 8 24
PCR ex2/ex3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300
250
200
150
100
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PCR
Intron 1
PCR
Exon 2
PCR
Intron 2
PCR
Exon 3

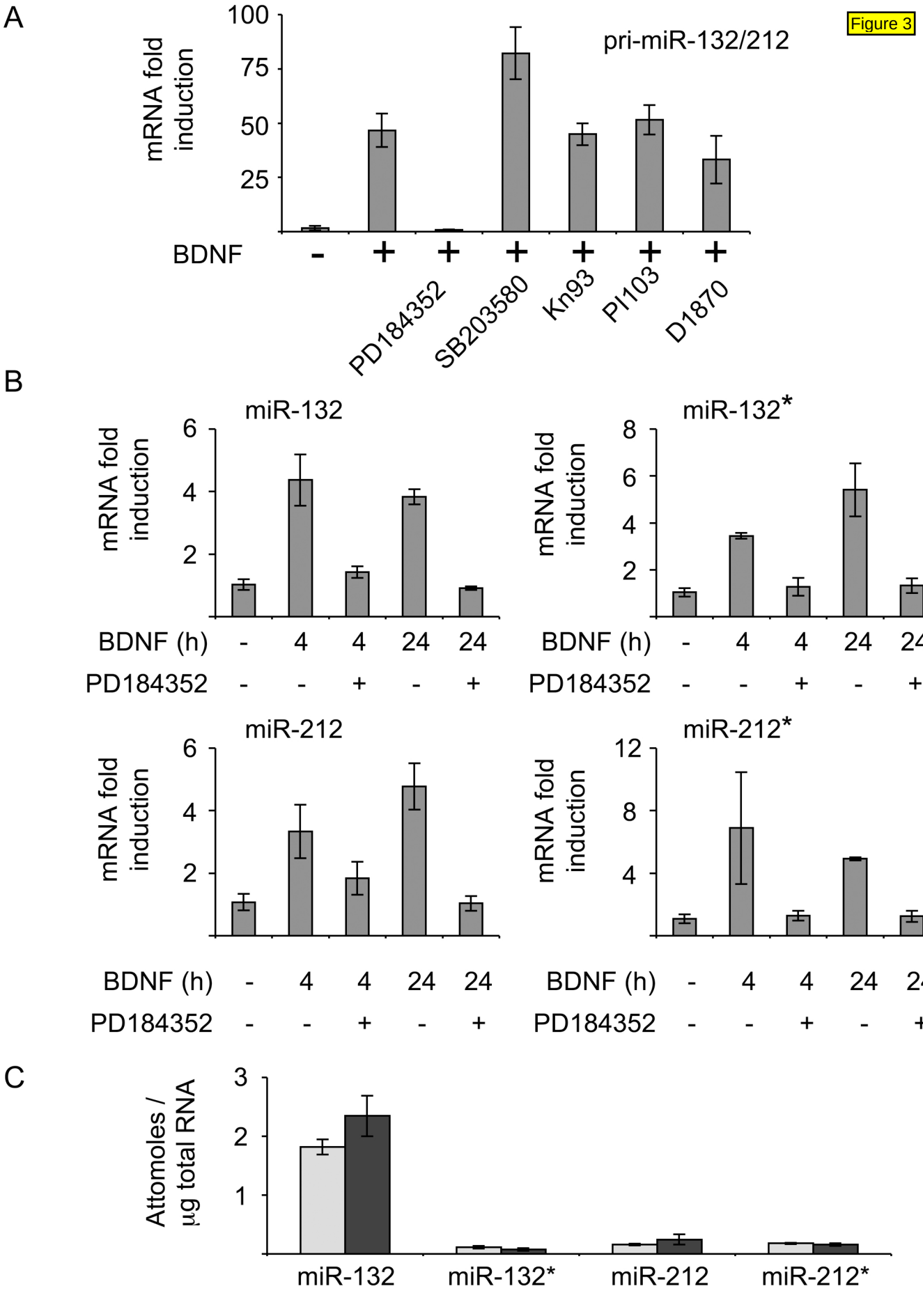
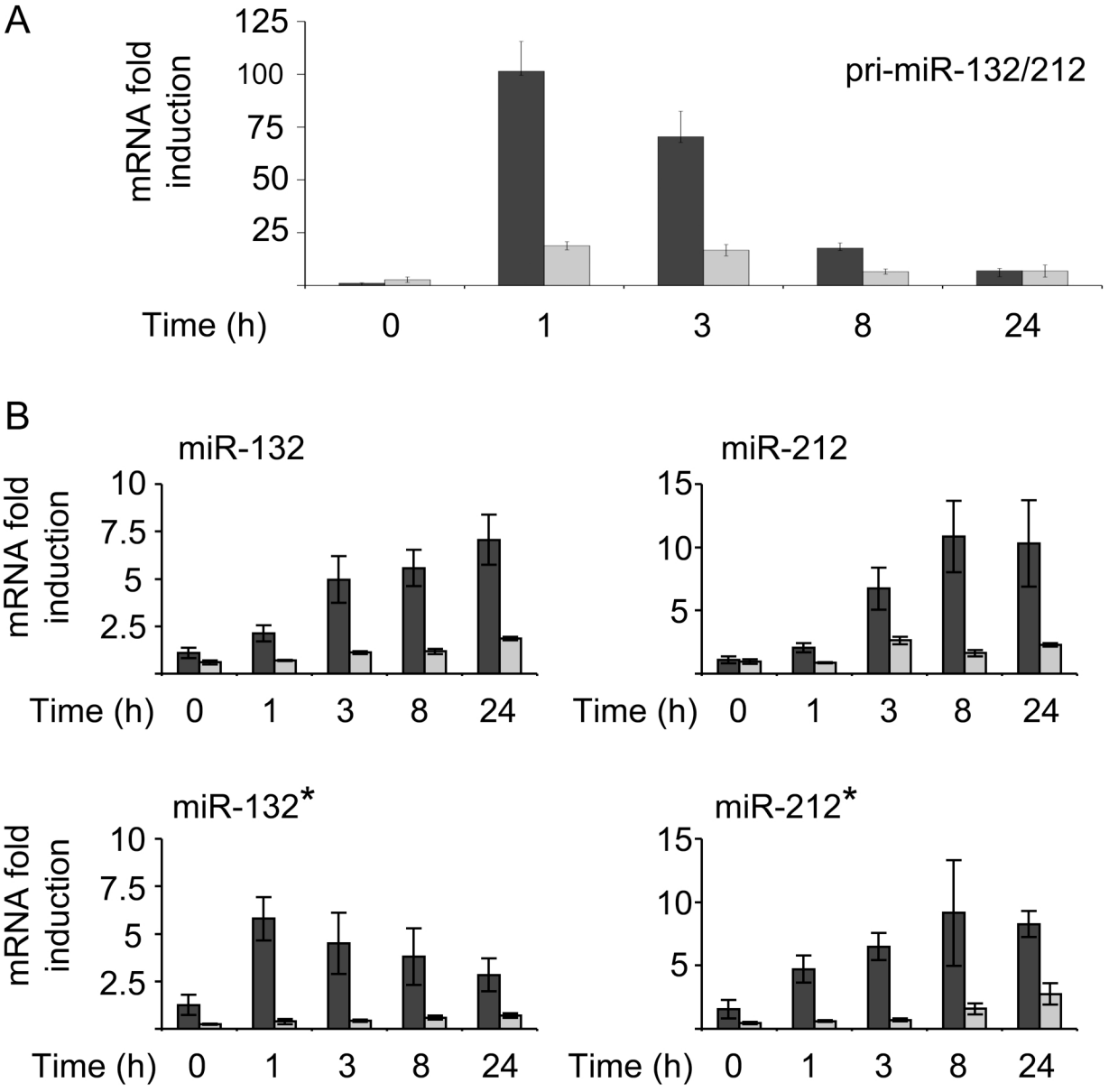


Figure 3
A
pri-miR-132/212
mRNA fold induction
100
75
50
25
BDNF
-
+
+
+
+
+
+
PD184352
SB203580
Kn93
PI103
D1870
B
miR-132
miR-132*
miR-212
miR-212*
mRNA fold induction
BDNF (h)
PD184352
C
Attomoles / µg total RNA
miR-132
miR-132*
miR-212
miR-212*

Figure 4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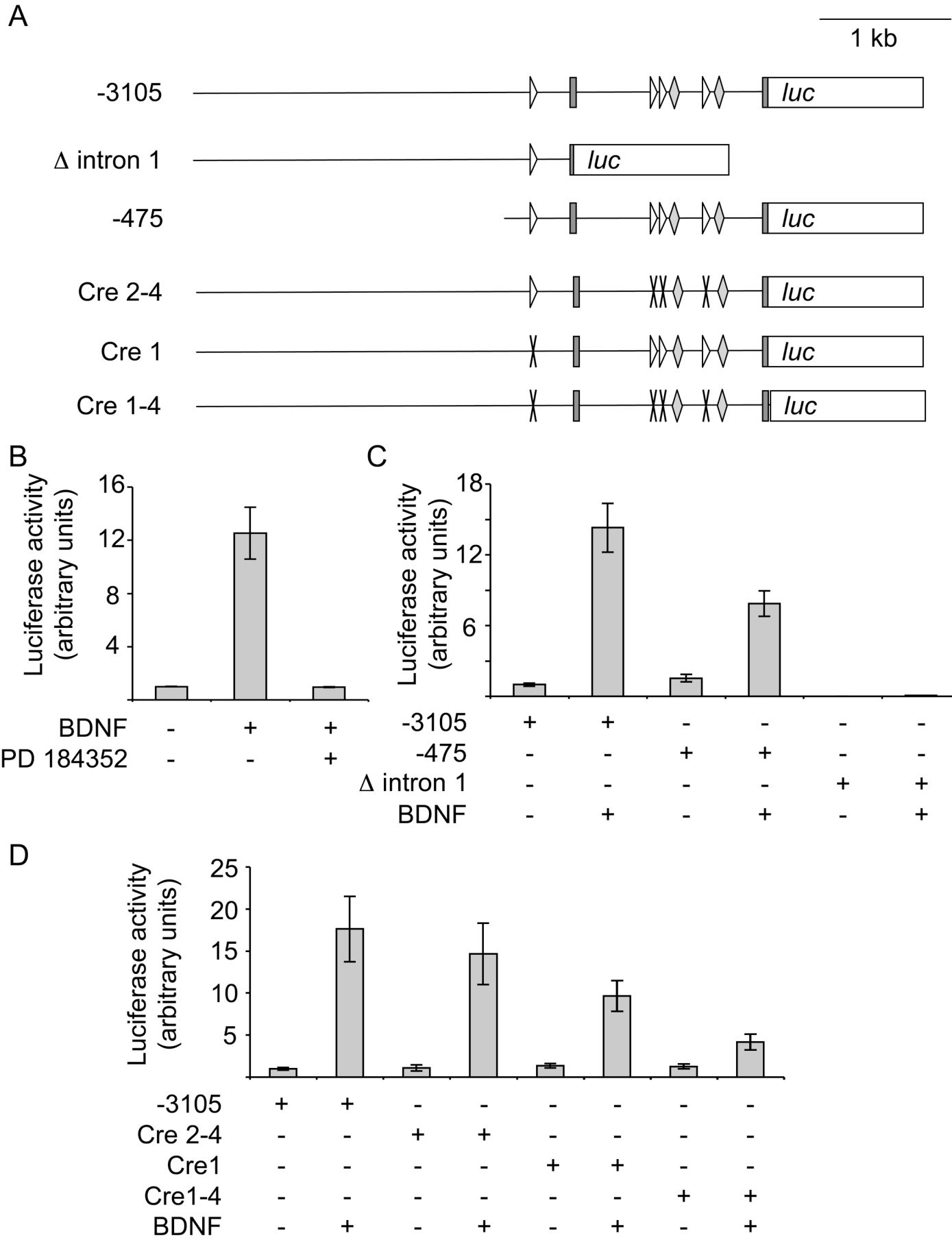
mRNA fold induction
125
100
75
50
25
pri-miR-132/212
Time (h)
0
1
3
8
24
B
miR-132
miR-212
miR-132*
miR-212*
mRNA fold induction
10
7.5
5
2.5
15
10
5
Time (h)
0
1
3
8
24

Figure 5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-3105
luc
Δ intron 1
luc
-475
luc
Cre 2-4
luc
Cre 1
luc
Cre 1-4
luc
B
Luciferase activity (arbitrary units)
16
12
8
4
BDNF - + +
PD 184352 - - +
C
Luciferase activity (arbitrary units)
18
12
6
-3105 + + - - - -
-475 - - + + - -
Δ intron 1 - - - - + +
BDNF - + - + - +
D
Luciferase activity (arbitrary units)
25
20
15
10
5
-3105 + + - - - - - -
Cre 2-4 - - + + - - - -
Cre1 - - - - + + - -
Cre1-4 - - - - - - + +
BDNF - + - + - + - +

Figure 6

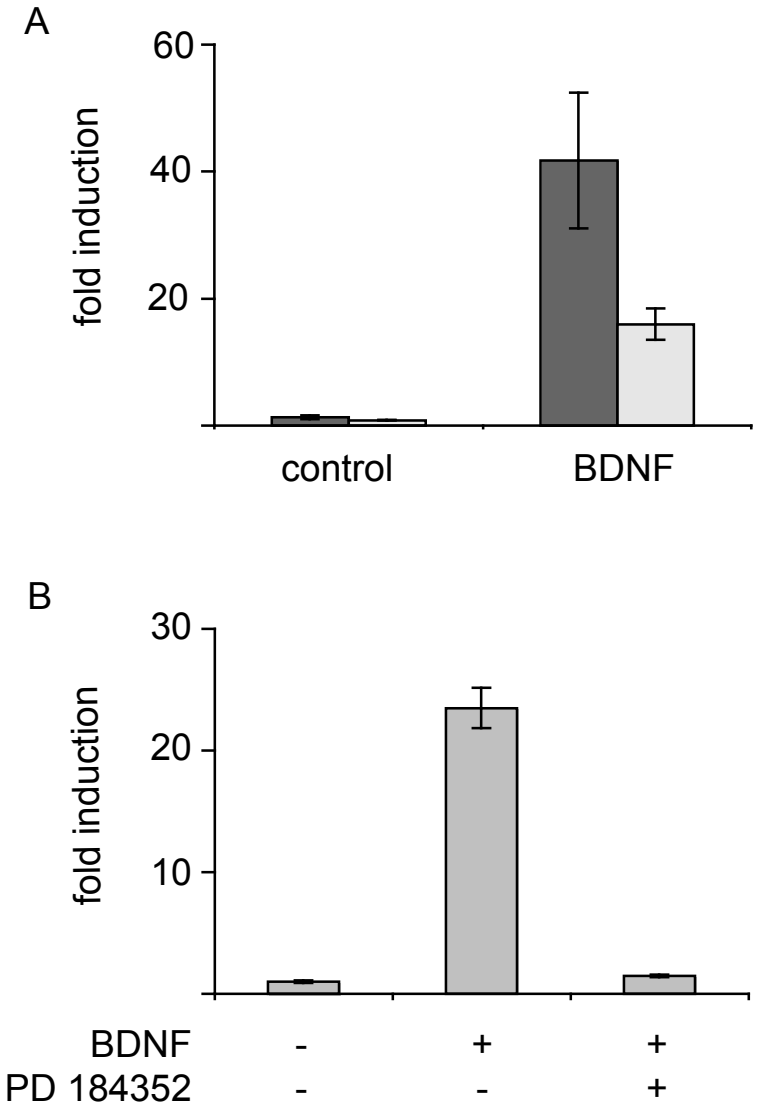
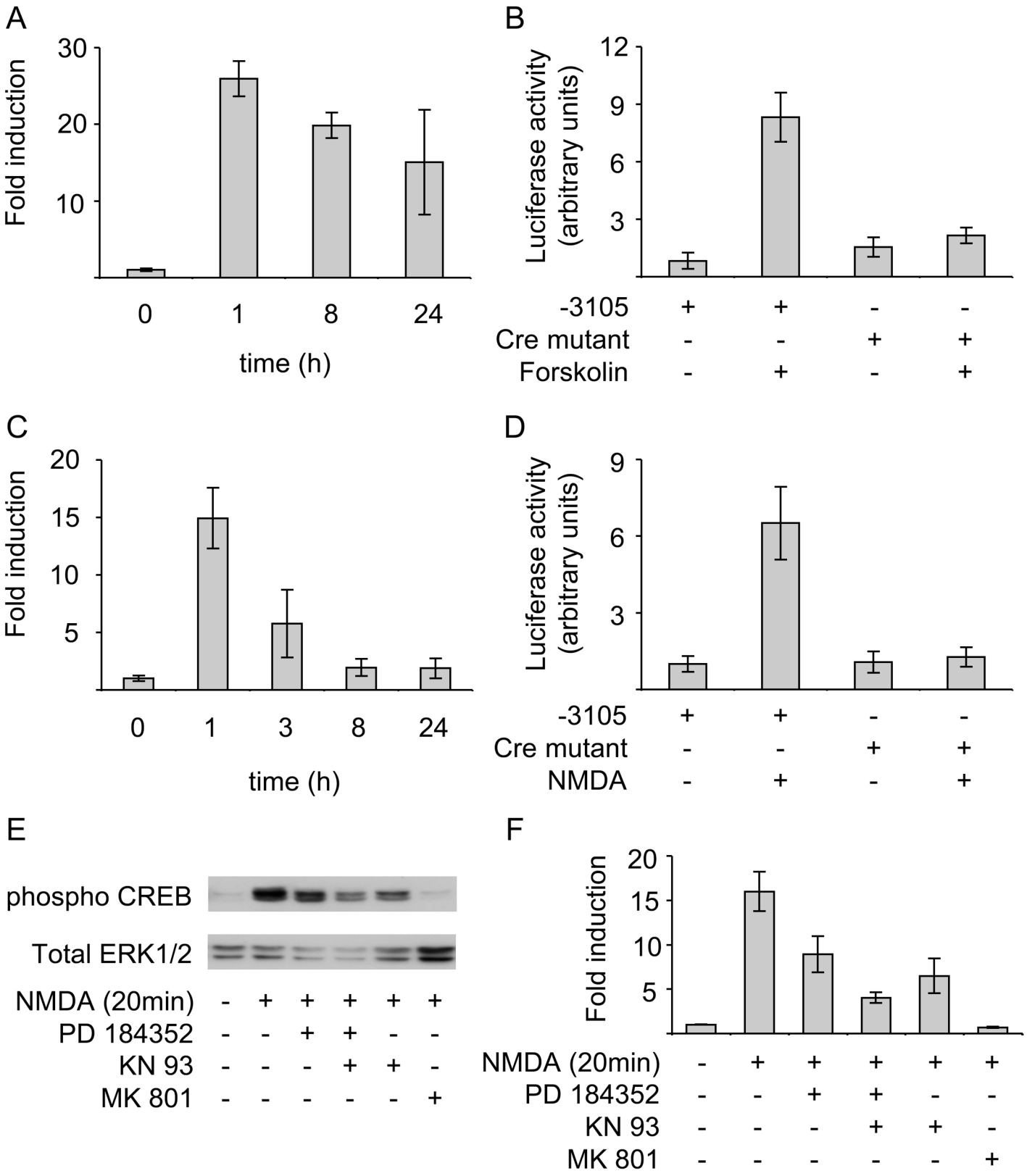

A
60
40
20
fold induction
control
BDNF
B
30
20
10
fold induction
BDNF - + +
PD 184352 - - +

Figure 7


A
Fold induction
30
20
10
0
1
8
24
time (h)
B
Luciferase activity (arbitrary units)
12
9
6
3
-3105 + + - -
Cre mutant - - + +
Forskolin - + - +
C
Fold induction
20
15
10
5
0
1
3
8
24
time (h)
D
Luciferase activity (arbitrary units)
9
6
3
-3105 + + - -
Cre mutant - - + +
NMDA - + - +
E
phospho CREB
Total ERK1/2
NMDA (20min) - + + + + +
PD 184352 - - + + - -
KN 93 - - - + + -
MK 801 - - - - - +
F
Fold induction
20
15
10
5
NMDA (20min) - + + + + +
PD 184352 - - + + - -
KN 93 - - - + + -
MK 801 - - - - - +