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Role of 3′UTR in the Regulation of Neurotrophic Factors BDNF and GDNF

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Role of 3'UTR in the regulation of neurotrophic factors BDNF and GDNF

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”छोटे-से जीवन में कितना प्यार करूँ, पी लूँ हाला,
आने के ही साथ जगत में कहलाया 'जानेवाला',
स्वागत के ही साथ विदा की होती देखी तैयारी,
बंद लगी होने खुलते ही मेरी जीवन-मधुशाला।”

-हरिवंशराय बच्चन (मधुशाला)

मेरे प्रिये बाबा और पिता को समर्पित...

Dedicated to memory of my beloved Grandfather and Father ...

Table of Contents

Abbreviations

AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
ARE	AU rich element
ARE-BP	AU rich element binding protein
ARTN	Artemin
BDNF	Brain-derived neurotrophic factor
BDNF-L	BDNF transcript with long 3′UTR
BDNF-S	BDNF transcript with short 3′UTR
BRF 1	Butyrate response factor 1
BRF2	Butyrate response factor 2
cKO	Conditional knock out
CDNF	Cerebral dopamine neurotrophic factor
CNS	Central nervous system
ELISA	Enzyme linked immunosorbent assay
EMSA	Electrophoretic mobility shift assay
GDNF	Glial cell line-derived neurotrophic factor
Gdnf 3′UTR-crR	Conditionally reversible replacement of GDNF 3′ UTR
GFL	GDNF Family Ligands
GFRα	GDNF family receptor alpha
HSCR	Hirschsprung disease
KO	Knock out
LC	Locus coeruleus
mRNA	Messenger RNA
miR	MicroRNA
MANF	Mesencephalic astrocyte-derived neurotrophic factor
NGF	Nerve growth factor
NTF	Neurotrophic factor
NTRN	Neurturin
PCD	Programmed cell death
PD	Parkinson's disease
PRPN	Persephin
RNA-IP	RNA immunoprecipitation assay
SN	Substantia nigra
SNpc	Substantia nigra pars compacta
TH	Tyrosine hydrolase
TTP	Tristetraprolin
UTR	Untranslated region
VTA	Ventral tegmental area

List of original publications

I. miR-1, miR-10b, miR-155 and miR-191 are novel regulators of BDNF
Kärt Varendi, Anmol Kumar, Mari-Anne Härma and Jaan-Olle Andressoo
Cell Mol Life Sci. (CMLS) 2014, PMID: 24804980

II. Tristetraprolin is a novel regulator of BDNF
Anmol Kumar, Kärt Varendi, Johan Peränen and Jaan-Olle Andressoo
SpringerPlus 2014, 3:502, PMID: 25279294

III. In vivo replacement of 3′UTR as a versatile method to study gene function – the case of GDNF
Jaan-Olle Andressoo, Anmol Kumar*, Jakko Kopra*, Lauriina Porokuokka, Kärt Varendi, Pepin Marshall, Nina Nevalainen, Mari-Anne Härma, Carolina Vilenius, Kersti Lilleväli, Triin Tekko, Jelena Mijatovic, Madis Jakobson, Roxana Ola, Erik Palm, Maria Lindahl, Ingrid Strömberg, Vootele Voikar, T. Petteri Piepponen, Mart Saarma
*Equal contribution
Submitted manuscript

The publications are referred in the text by their Roman numerals.
In addition, unpublished data is presented.

Contributions:

I. The author initiated the project and contributed to experiments leading to Figures 2B, 4C, 4D and conducted experiments along with MA to answer reviewer's questions for revision.
II. The author conceived the idea, planned and executed experiments, analyzed the results and wrote 1st draft of manuscript and also 1st draft of response letter to editor addressing reviewer's comments.
III. The author conducted and analyzed experiments for Figures 1A, 1C, 3B, 3D, 3E, 3F and Supplementary Figures 1B, 1C, 1D, 2G, 3A, 3D in the manuscript. The author also contributed in the experiments leading to Figure 5C and Supplementary Figures 2 B, 2D, 2E, and 2F in the manuscript.

Abstract

During the past decade, 3′ untranslated regions (3′UTRs) of mRNAs continue to emerge as important sites for gene regulation due to the binding of microRNAs (miRs), RNA-binding proteins (RBPs) and possibly other trans-acting factors. Brain derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) play important roles inside and outside the central nervous system (CNS). Whilst precise spatiotemporal regulation of BDNF and GDNF levels are crucial in determining biological outcome, mechanisms involved in controlling their levels are not fully understood. In this thesis, we investigated the 3′UTR mediated regulation of BDNF and GDNF. We demonstrate the presence of regulatory elements in the 3′UTR of BDNF and GDNF and, show that BDNF is regulated by 4 different miRs, namely miR-1, miR-10b, miR-155 and miR-191, and by RBP tristetraprolin (TTP) in different cell lines. Further, we show that GDNF is regulated by multiple miRs in cell lines and identify binding sites for miR-146 and miR-96 in the GDNF 3′UTR. We demonstrate that *in vivo* replacement of GDNF 3′UTR with a 3′UTR with reduced responsiveness to negative regulators including miRs leads to elevated level of endogenous GDNF mRNA and protein in various organs with profound effects on the brain dopaminergic system in mice. We conclude that 3′UTR mediated regulation of BDNF and GDNF is biologically important and propose that 3′UTR replacement is a highly informative way to study gene function *in vivo*.

1 Review of the literature

1.1 The concept of neurotrophic factors

Development of vertebrate nervous system is a complex and highly coordinated process that requires expression of various intracellular and extracellular factors in a controlled manner. Neurotrophic factors (NTFs) are a group of secreted proteins which support the survival of neurons, promote neuritic growth and branching, and regulate the number of neurons and density of innervation; in particular during the time of target innervation in the development. Nerve growth factor (NGF) was the first growth factor and NTF discovered more than half a century ago by Italian scientist Rita Levi-Montalcini (Levi-Montalcini and Cohen, 1956, Levi-Montalcini, 1982). The classical target derived NTF hypothesis states that secreted NTFs from target neurons/tissues act extracellularly as a chemoattractants and survival factors for innervating neurons and facilitating synapse formation with the target cell. Without neurotrophic support from target neurons/tissues during development, innervating neurons die via PCD (Figure 1.1). The underlying concept here is that during embryonic development of the peripheral nervous system, neurons are produced in excess and overproduced neurons are removed by PCD due to limited access to neurotrophic support. Only neurons innervating the correct NTF producing targets in time will receive sufficient trophic support to survive. Neurons innervating incorrect targets or displaying delayed development will die via PCD. This process is believed to be crucial to ensure optimal neuronal connections in the nervous system (Oppenheim, 1991).

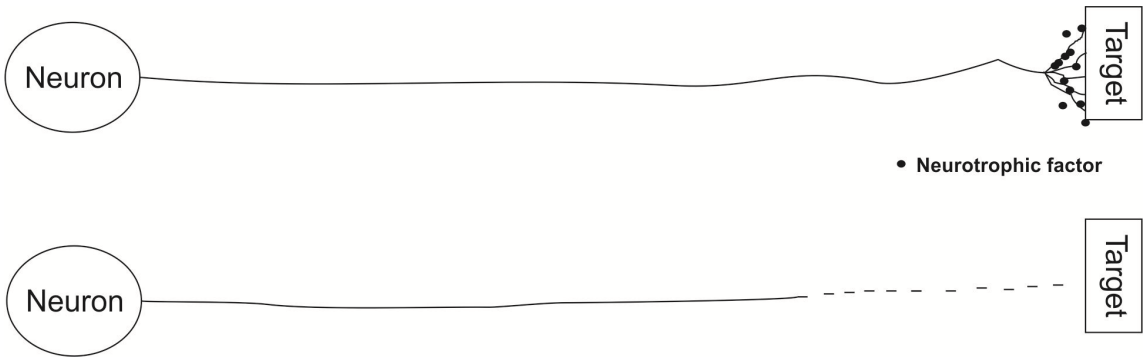


Figure 1.1: Sketch presenting the classical neurotrophic factor hypothesis. If a neurotrophic factor is secreted from a target tissue then innervating neuron expressing a cognate receptor makes connection and survives otherwise the innervating neuron dies through PCD.

Originally proposed for peripheral neurons, this classical model was extended to the CNS as well, where target neurons synthesizes NTFs and provide trophic support to their afferent neurons (Ernfors et al., 1990). However, the role and mechanism of above the target field NTF theory is still matter of debate in the context of the CNS.

1.2 Classification of NTFs

NTFs are classified broadly into four major families: 1. Neurotrophins 2. GDNF family ligands (GFLs) 3. Neurokines 4. MANF/CDNF family.

NGF, BDNF, neurotrophin-3 (NT-3) and neurotrophin – 4 (NT-4) are the members of the mammalian neurotrophin family. Neurotrophin mediated signaling is critically important for the development and maintenance of the CNS (Skaper, 2012).

GDNF, Neurturin (NRTN), Artemin (ARTN) and Persephin (PSPN) are the GDNF family members. GDNF and GFLs are structurally related to the transforming growth factor beta (TGF-β) superfamily and play a crucial role in the development, differentiation and maintenance of a variety of neurons (Airaksinen and Saarma, 2002).

Neurokines or neuropoietic cytokines are neurally active cytokines and may signal via common cytokine receptor components. Ciliary neurotrophic factor(CNTF), interleukin 6(IL-6), cardiotrophin 1 (CT-1), cardiotrophin 2 (CT-2) and leukemia inhibitory factor (LIF) are well known members of this family (Nathanson, 2012).

MANF and CDNF are the only two members of this recently founded family (Petrova et al., 2003, Lindholm et al., 2007). MANF and CDNF are also involved in inhibiting ER stress-induced cell death (Apostolou et al., 2008, Cheng et al., 2013, Lindahl et al., 2014). Intracranial applications of ectopic MANF and CDNF are shown to have beneficial effects in rodent models of Parkinson's disease (Lindholm et al., 2007, Voutilainen et al., 2009, Airavaara et al., 2012, Back et al., 2013).

1.3 BDNF and GDNF biology

1.3.1 BDNF

In the year 1982, Yves Barde and his colleagues discovered BDNF only the second neurotrophic factor found after the discovery of NGF (Barde et al., 1982). BDNF plays an important role in a wide spectrum of brain functions which include the development of neuronal networks, the plasticity of synapses and the survival of neurons (Kirschenbaum and Goldman, 1995, Bamji et al., 2006, Loeliger et al., 2008, Caputo et al., 2011). Mice overexpressing BDNF exhibit enhanced learning and memory (Nakajo et al., 2008). BDNF has acute effects on synaptic transmission and plays a role in long-term potentiation. It changes the properties of ionotropic receptors (e.g. NMDA receptor, AMPA receptor, $GABA_A$ receptor) which rapidly affect both excitatory and inhibitory synaptic transmission (Rose et al., 2004).

BDNF's biological function is mediated via multiple intracellular signaling pathways triggered by its binding to two classes of cell surface receptors namely Tropomyosin related kinase B (TrkB) and $p75^{NTR}$ (Numakawa et al., 2010). By binding to TrkB, BDNF activates a broad range of signaling cascades such as MAP kinase, PI3-kinase and PLC-gamma that lead to variety of biological outcomes in different cell types (Huang and Reichardt, 2003). p75 is a receptor for all neurotrophins and it can bind neurotrophin precursors or their mature forms (Figure 1.2). For example, the BDNF precursor (pro-BDNF) binds to p75 and leads to a decrease in synaptic efficacy followed by a retraction of presynaptic terminals of neuromuscular synapses (Roux and Barker, 2002, Yang et al., 2009). Pro-BDNF can also signal via p75 by binding to sortilin (Teng et al., 2005).

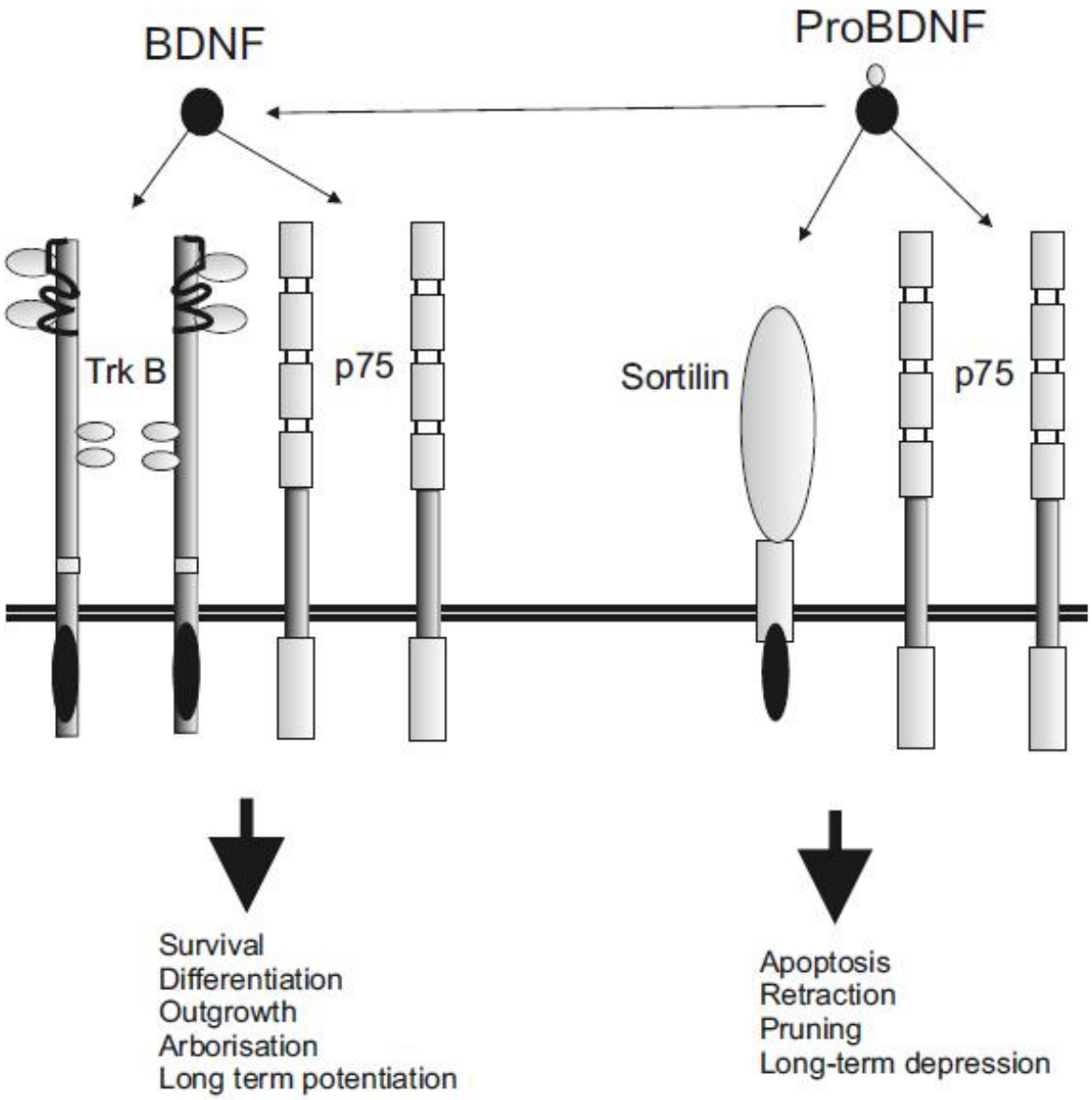


Figure 1.2: Both proBDNF and mature BDNF are biologically active but lead to opposite biological outcomes. Mature BDNF can signal via TrkB in the presence or absence of p75, whereas proBDNF can signal through sortilin and p75. Adapted from review by Deinhardt and Chao (2014).

BDNF is also involved in a wide range of functions outside the CNS. BDNF is expressed in skeletal muscle precursors termed as satellite cells and inhibits myogenic differentiation. Mousavi and Jasmin (2006) showed using rat myoblast cell line L6 that a reduction of BDNF levels is required for myoblasts to exit the cell cycle and to initiate myogenic differentiation. In line with the functions of BDNF in muscle growth and renewal, BDNF knockdown specifically from mouse skeletal muscle contributes to compromised regeneration after injury (Clow and Jasmin, 2010). BDNF also promotes angiogenesis both *in vivo* and *in vitro* (Sun et al., 2006). Furthermore, activated microglia release BDNF which changes the anion gradient in neurons; this may have a role in neuropathic pain (Siniscalco et al., 2011, Coull et al., 2005).

BDNF mRNA has at least 22 isoforms; the structurally complex BDNF gene (Figure 1.3 A) contains multiple 5' untranslated exons, each having independent promoters conjugated with a coding exon (exon IX) composed of two alternate polyadenylation (PolyA) sites that leads to generation of mature mRNA pools containing two distinct short (0.35 kb, BDNF-S) or long 3'UTR (2.8 kb, BDNF-L) (Timmusk et al., 1993, Pruunsild et al., 2007). Multiple promoters facilitate sophisticated transcriptional regulation by distinct stimulation cues. For example, there are several Ca^{2+} responsive elements in BDNF promoters I, III and IV that allow modulation of promoter activity based on Ca2+ ionic cues (Tabuchi et al., 2000, Takeuchi et al., 2002). This somewhat demonstrates the complexity of the regulatory elements determining the functions of BDNF in biological processes.

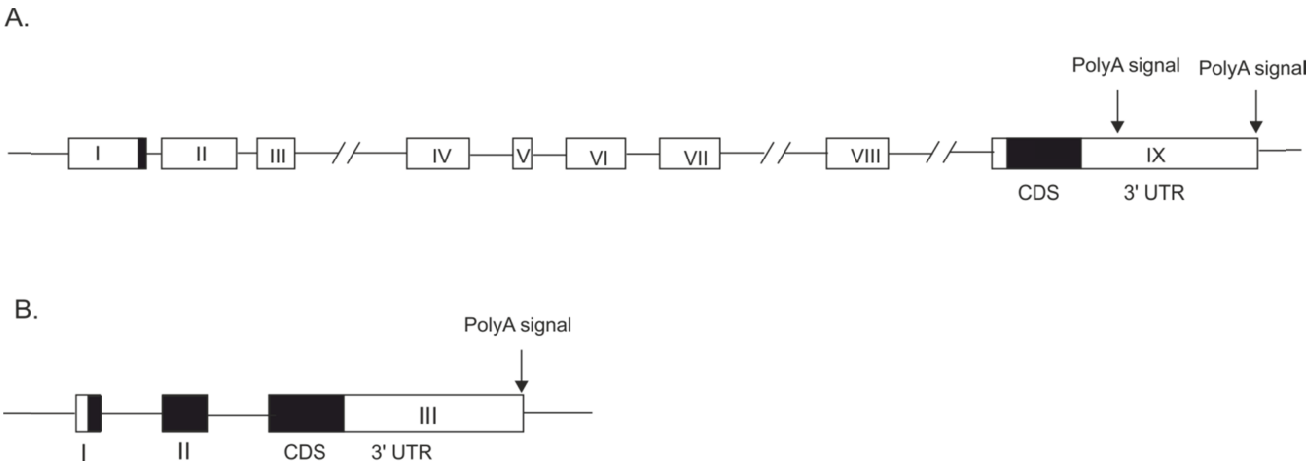


Figure 1.3: A. Schematic diagram of mouse BDNF gene B. Schematic diagram of mouse GDNF gene. Straight line represents introns, boxes represent exons and dark boxes indicate coding sequence (CDS). Arrows show PolyA signal site. Roman numerals represent exon number. Based on information in original publication by Matsushita et al. (1997), Aid et al. (2007).

1.3.2 GDNF

GDNF was first discovered as a factor promoting *in vitro* survival of embryonic dopaminergic (DA) neurons in primary midbrain dopaminergic cultures (Lin et al., 1993). Apart from the dopaminergic neuronal subpopulation, the trophic role of GDNF in the survival of spinal motor neurons (Henderson et al., 1994) and noradrenergic neurons (Arenas et al., 1995) has been shown. Mice lacking GDNF have 20-40% less spinal and cranial motor neurons (Airaksinen and Saarma, 2002).

Pascual et al. (2008) reported an indispensable role of GDNF in the survival of catecholaminergic neurons including complete loss of noradrenergic neurons and massive loss of DA neurons in ventral tegmental area (VTA) and substantia nigra (SN). GDNF also regulates the development of enteric, sensory, sympathetic and parasympathetic neurons (Airaksinen and Saarma, 2002). Transgenic mice overexpressing of GDNF in the CNS in astrocytes on the other hand leads to increased number of motor neurons due to rescue from PCD and promotion of long term motor neuron survival after axotomy (Zhao et al., 2004). Outside the nervous system, GDNF is also a critical regulator of kidney development and spermatogenesis (Sariola and Saarma, 2003).

GDNF requires the GPI-anchored co-receptor protein GFRα 1 and transmembrane receptor tyrosine kinase RET (Rearranged during Transfection) to form a receptor complex to exert its biological activity (Airaksinen and Saarma, 2002, Sariola and Saarma, 2003). See Figure 1.4 for summary of GDNF family ligand signaling.In addition, GDNF can signal independently from RET via neural adhesion molecule (NCAM)(Paratcha et al., 2003) or directly without GFRα or RET through syndecan-3 (Bespalov et al., 2011) .

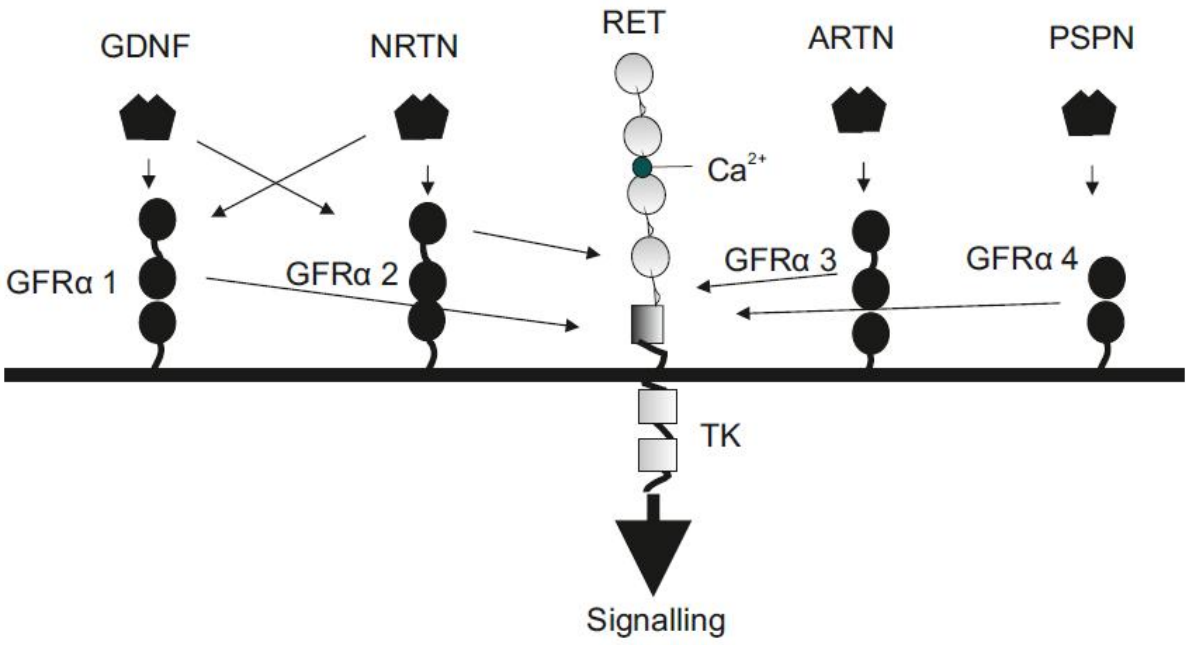


Figure 1.4: Schematics showing receptors and signaling for GDNF and it's family members. GFLs specificity to GFRα receptors; GDNF binds to GFRα1, NRTN to GFRα2, ARTN to GFRα3 and PSPN to GFRα 4 . GDNF and NRTN can cross-bind to GFRα2 and GFRα1 respectively (Cik et al., 2000). PSPN also appears to activate RET also via GFRα1 (Sidorova et al., 2010). GFLs are homodimers and bring together two molecules of GFRα receptors to form a signaling complex with two molecules of RET resulting in transphophorylation of their tyrosine residues in the kinase domains leading to downstream signaling. Alternatively, GDNF can also bind to the preformed GFRα1-RET complex (Bespalov and Saarma, 2007). Scheme adapted from Airaksinen and Saarma (2002).

GDNF transcripts are detected outside the CNS in kidney, lung, bone, heart, liver, spleen, sciatic nerve and blood (Suter-Crazzolara and Unsicker, 1994). It has been shown that Sertoli cells in mouse testis secrete GDNF and regulate the fate and lineage determination of undifferentiated spermatogonia (Meng et al., 2000). Skeletal muscles produce GDNF facilitating innervation of the neuromuscular junction in rats (Wehrwein et al., 2002). Despite the detection of GDNF transcripts in various tissues and its known or presumed role in many biological processes, the mechanisms regulating endogenous GDNF expression are not well understood. BDNF and GDNF levels are linked to human diseases and have been widely studied in the context of various pathological conditions in humans.

1.4 BDNF and GDNF in human disease

1.4.1 BDNF in human disease

BDNF mis-expression has been reported in several human disease conditions. AD is characterized by progressive impairment of memory and learning due to loss of neurons in cerebral cortex and certain subcortical areas of the brain (Berchtold and Cotman, 1998). BDNF mRNA levels are reduced by approximately 2 fold in the hippocampal formation of postmortem brains of AD patients (Phillips et al., 1991). A recent association study performed with 140 participants linked reduced BDNF plasma levels to AD and dementia (Aisen, 2014).

Specific loss of DA neurons in the SNpc is the cause of motor symptoms in PD patients and BDNF expression is reported to be approximately 55% reduced compared to the control group in the melanized dopaminergic neurons of the SNpc in PD patients (Parain et al., 1999). HD is another neurodegerative disease which leads to loss of motor coordination and cognitive functions (Papoutsi et al., 2014). BDNF protein expression is reported to be reduced by 50% to 80% in the caudate and putamen of HD patients (Ferrer et al., 2000).

Reduced levels of BDNF are linked to AD, PD and HD, but it is only HD in which multiple experimental approach using *in vitro* cell systems, animal models and systematic analysis of postmortem tissues from human have provided a strong link between the impaired production and transport of BDNF and the manifestation of disease (Zuccato and Cattaneo, 2009). On the other hand, mechanisms attributing reduced BDNF level to AD and PD are not well understood (Zuccato and Cattaneo, 2009). In humans, increasing BDNF levels has been proposed to be one of the potential therapeutic approaches to cure HD (Zuccato and Cattaneo, 2014). BDNF is also linked to psychiatric disorders like schizophrenia and depression (Muglia et al., 2003, D'Addario et al., 2013).

The congenital Val66Met polymorphism where methionine (Met) substituted for valine (Val) at codon 66 in the pro-region of BDNF affects activity- dependent secretion of BDNF is associated with a poor episodic memory and abnormal hippocampal activation in humans (Egan et al., 2003). However, there have been conflicting reports on the role of the Val66Met polymorphism as a risk factor for developing AD,PD ,HD and schizophrenia (Zuccato and Cattaneo, 2009, Guan et al., 2014).

These studies highlight the role of aberrant BDNF levels in different human diseases. As the mechanisms of regulation of BDNF levels are still poorly understood there is a need for basic research in this direction. Studies on mechanisms controlling BDNF expression is one of the objectives of this thesis.

1.4.2 GDNF in human disease

ALS is a neurodegenerative disease attributed to preferential loss of motor neurons of the motor cortex, brain stem and spinal cord, resulting in progressive muscle atrophy, paralysis and eventually death (Ajroud-Driss and Siddique, 2014). Grundstrom et al. (2000) found elevated level of GDNF in the cerebrospinal fluid of 13 patients with ALS suggesting that GDNF expression may be enhanced to stall the loss of motor neurons. There is an ongoing clinical trial for treatment of ALS using progenitor cells secreting GDNF.

HSCR, also known as congenital megacolon or intestinal aganglionosis, is a congenital abnormality characterized by an absence of enteric neurons from variable lengths of the hind gut (McKeown et al., 2013). Mutation of the GDNF receptor component RET is found in in about 50% of familial and about 20% of sporadic HSCR cases (Attie et al., 1995, Martucciello et al., 2000). Mutations in GDNF coding sequence

are associated with about 4.6% of all HSCR cases (Martucciello et al., 2000). Mutations in the GDNF signaling component RET is involved in multiple endocrine neoplasia type 2 (MEN2), an autosomal-dominant cancer syndrome (Lodish, 2013). Additionally, it is reported that activating mutations in GDNF receptor RET cause familial medullary thyroid cell carcinoma (FMTC) syndromes (Airaksien and Saarma, 2002).

Intracranial GDNF protein delivery has been tested in two phase 2 clinical trials for therapy of PD with inconclusive outcomes. In the first trial GDNF was injected to the ventricle and did not reach to the SNpc and the second trial had some problems with connection between the delivery pump and catheter (Nutt et al., 2003, Lang, 2006, Penn et al., 2006, Patel and Gill, 2007). Despite two failed clinical trials, GDNF continues to be one of the best candidates among NTFs for neurorestorative therapy in PD due to its ability to protect and restore dopaminergic function *in vitro* and *in vivo* in animal models of PD (Tarazi et al., 2014). Highlighting the clinical and economic importance of GDNF, currently four Phase II clinical trials with GDNF in PD are ongoing. Interestingly, Backman et al. (2006) found significantly elevated endogenous GDNF but not RET or GFRα1 mRNA expression in patients diagnosed with idiopathic PD indicating a compensatory mechanism involving GDNF attempting to rescue dying neurons at late stage of disease progression.

Despite the use of GDNF in clinical trials for PD and its known or presumed role in human diseases, the basic mechanisms regulating GDNF *in vivo* in particular in aging and diseased brain are largely unexplored stressing a need for basic research in this direction. Studies on mechanisms of GDNF levels regulation is one of the objectives of this thesis.

1.5 Mouse models to study the functions of BDNF and GDNF

1.5.1 Mouse models of BDNF

Mouse models are a central tool to study the functions of genes, including those involved in human diseases. There are several mouse models to study BDNF functions *in vivo.* Since BDNF homozygous KO mice die within 2-4 postnatal weeks, most studies on BDNF deficiency in mice are done with heterozygous KO mice where one BDNF allele is intact (Korte et al., 1995). Such mice are viable and fertile with an approximate 50% reduction in the BDNF mRNA and protein level. BDNF heterozygous KO mice show phenotypes such as severe defects in coordination and balance, impairment of long term potentiation in the hippocampus and learning difficulties (Ernfors et al., 1994, Korte et al., 1995, Linnarsson et al., 1997, Bartoletti et al., 2002).

Using cKO technology, BDNF was deleted specifically in hippocampal area CA3 and to some extent in dentate gyrus, cerebellum and facial nerve nucleus. These mice exhibited increased aggressive behavior with additional behavior changes like anxiety and deficient cognition (Ito et al., 2011). In another study with conditional approach, Monteggia et al. (2004) used Cre- line with tetracycline inducible forebrain specific promoter to delete BDNF in the forebrain of young and adult mice and, found hippocampal dependent memory impairment and hyperactivity during early stages of development in young mice in addition to the antidepressant role of BDNF in adult mice. Later the same group also studied specific BDNF deletion in the forebrain during pre and post natal development and found gender specificity in depression-related phenotypes in these conditional BDNF KO mice (Monteggia et al., 2007). Clow and Jasmin (2010) conditionally deleted BDNF in the muscles of mice using Cre-line specific to skeletal muscles and showed a role of BDNF in the regulation of satellite cell differentiation and skeletal muscle regeneration.

Chen et al. (2006) created Val66Met polymorphic mice that have impaired BDNF secretion from neurons despite normal expression of BDNF. These mice showed an increased anxiety related phenotype under

stressful conditions that could not be rescued by administration of fluoxetine, an antidepressant drug thought to act via BDNF signaling. There are at least two different BDNF overexpressing mice generated under constitutive human β-actin and α Ca^{2+}/calmodulin-dependent kinase II (αCaMKII) promoters respectively. Mice overexpressing BDNF under human β-actin showed learning deficits, increased sensitivity to seizures and elevated mental excitability (Croll et al., 1999). αCaMKII promoter driven BDNF overexpression was mainly restricted to the forebrain and these mice showed early development of visual acuity and shortening of critical period for ocular dominance plasticity (Huang et al., 1999). Overall, these studies stress the importance of animal models in understanding BDNF function and its regulation in various biological processes.

1.5.2 Mouse models of GDNF

GDNF homozygous KOs die soon after birth due to complete kidney agenesis and lack of enteric nerves distal to stomach limiting their usefulness to study the role of endogenous GDNF in adults (Sanchez et al., 1996, Pichel et al., 1996, Moore et al., 1996). There have been multiple studies with GDNF heterozygous KO mice that suggest a role of GDNF in nigrostriatal dopaminergic transmission (Gerlai et al., 2001, Airavaara et al., 2004, Boger et al., 2006, Griffin et al., 2006, Airavaara et al., 2006, Boger et al., 2007, Littrell et al., 2013).

There is a single study using a cKO approach where by tamoxifen inducible Esr1-cre is used to delete GDNF by administering tamoxifen (TMX) in 2 month old mice resulting in the complete loss of noradrenergic neurons in LC and massive loss of DA neurons in VTA and SN (Pascual et al., 2008). This study by Pascual et al. (2008) concluded that GDNF is essential for survival of catecholaminergic neurons in the adult mice. However, the Saarma and Andressoo group has shown with three independent GDNF conditional ablation approaches including step-wise repetition of experiments presented in Pascual et al.(2008) study that GDNF deletion has no consequence on midbrain dopaminergic system survival and function (Kopra et al., in press). Additionally, Saarma and Andressoo group has found comparable up-regulation of several NTFs and their receptors in the brain of GDNF KO heterozygous mice and in animals where GDNF is conditionally deleted from the brain indicating that reduction or lack of GDNF in the brain is compensated for by the elevation of alternative growth factors and their receptors (Varendi et al., unpublished).

Transgenic mice overexpressing GDNF have been widely used to study GDNF in various contexts using different constitutive promoters such as human translation elongation factor–1α (EF-1α), myosin light chain-1 (MLC1) and glial fibrillary acidic protein (GFAP). Testicular overexpression of GDNF under EF-1α promoter demonstrated a role of GDNF in spermatogenesis and resulted in testicular tumors in 100% of mice by 12 months of age (Meng et al., 2000). Overexpression of GDNF in muscles under the MLC1 promoter resulted in hyperinnervation of muscle fibers (Zwick et al., 2001). Zhao et al. (2004) overexpressed GDNF in CNS under the GFAP promoter demonstrating a potent effect of GDNF on motor neuron survival. Skeletal muscle-specific overexpression of GDNF resulted in a slight but significant delay in motor neuron loss in an ALS mouse model (Li et al., 2007). Kholodilov et al. (2004) used a double transgenic approach with CaMKII promoter and tTA-response promoter to overexpress GDNF in the principal target areas of the mesencephalic dopaminergic projections and concluded that the GDNF has a role in the development of the mesencephalic dopaminergic system. However, it should be noted that the CaMKII promoter drives expression in excitatory neurons (Klug et al., 2012), whereas native GDNF is expressed in a specific subset of striatal inhibitory neurons which control striatal output (Hidalgo-Figueroa et al., 2012). It remains unclear that to what extent transgenic overexpression reflects the function of endogenous GDNF.

Apart from transgenic and KO approach, exogenous GDNF has been delivered to intact rodent brain. These studies with exogenous GDNF demonstrated various effects on brain dopaminergic system function (See table 1.2).

Procedure	Increase	Decrease	References
Single intracranial dose of GDNF protein	TH staining TH+ neurite sprouting DA release DA turnover DA and metabolite level TH phosphorylation ERK1/2 phosphorylation Motor activity Amphetamine turning	Food intake Water Intake Body weight	Hudson et al. (1995) Hebert et al. (1996) Hebert and Gerhardt (1997) Lapchak et al. (1997) Xu and Dluzen (2000) Salvatore et al. (2004)
Virally mediated continuous expression of GDNF in the midbrain	Early: DA turnover DA function Late (6 weeks): TH mRNA	Late (6 weeks): TH protein TH activity DA	Rosenblad et al. (2003) Georgievska et al. (2004a) Georgievska et al. (2004b) Sajadi et al. (2005)

Table 1.2: Summary of studies showing effects of ectopic GDNF delivery to intact rodent brain. Adapted from doctoral thesis by Planken (2012).

Some phenotypic effects of exogenous GDNF varied based on method (Procedure section, table 1.2) of GDNF delivery emphasizing that biological outcome of a trophic factor like GDNF is critically dependent upon the injected dose as well as on the method of GDNF delivery. The main challenge with transgenic overexpression and ectopic GDNF delivery is the inability to control the spread of GDNF and other NTF molecules to the correct biological sites of action, which likely leads to undesired biological effects. Apart from this, GDNF is a particularly poorly diffusing molecule, since it strongly interacts with heparin sulfates in the extracellular matrix (Bespalov et al., 2011). An alternative measure enabling elevation of GDNF and possibly BDNF at correct sites would be of interest to uncover but such a tool has been missing. In search for such a tool, we turned to study the biology of 3′UTRs of GDNF and BDNF.

1.6 Gene regulation by 3'UTR

Mature mRNAs in eukaryotes contain a protein coding sequence (CDS) with a 5′ and 3′ UTRs. The last decade of research has revealed that 3′UTRs are crucial regulators of gene expression through interaction with various factors (Barrett et al., 2012) . 3′UTRs are used to control mRNA stability, translation, polyadenylation and cellular localization (Siepel et al., 2005, Mercer et al., 2011). At least about 60% of 3′UTRs of protein coding genes are predicted to contain various cis- acting regulatory elements like miR response elements, AU rich elements (AREs), iron responsive elements(IREs), alternate polyadenylation signals or they fold into various secondary structures with functional significance, all of which can affect transcripts' stability, translation or localization (Barrett et al., 2012).

1.6.1 MicroRNAs

miRs were discovered already in the early 1990s , but were recognized as a distinct class of biological regulators almost a decade later (Lee et al., 1993, Ambros, 2001). miRs are a family of small ~22 nucleotide long non coding RNAs which regulate gene expression by binding to complementary nucleotide sequences in their target mRNAs (Ambros, 2004). miR binding domains are mostly present in the 3′UTR region of target mRNA in mammals with few exceptions (Gu et al., 2009, Forman and Coller, 2010). miRs are evolutionarily ancient regulatory molecules and conserved during the course of evolution (Wheeler et al., 2009).The majority of miRs are derived from long pri-miR transcripts that are synthesized from specific miR genes or from the introns of protein coding genes (host gene) by RNA polymerase II (Lee et al., 2004, Rodriguez et al., 2004). The processing of primary miR (pri-miR) transcripts to precursor miRs (pre-miRs) is mediated by ribonuclease complex (Dorsha) and specialized double stranded RNA binding proteins (DGCR8) in the nucleus, followed by transport to the cytoplasm for final cleavage by another ribonuclease called Dicer resulting in double stranded mature miRs (Figure 1.5) (Lee et al., 2002, Lee et al., 2003, Landthaler et al., 2004). After sequential processing to mature miR duplexes, a ribonucleoprotein complex called RNA induced silencing complex (RISC) is formed in the cytosol by interaction of argonaute (AGO) proteins with mature duplex miRs (Yoda et al., 2010). One of two strands of miRs (guiding strand) guides the RISC complex to complementary target mRNAs sequences while the other strand known as the passenger strand is degraded (Matranga et al., 2005, Rand et al., 2005, Kawamata et al., 2009). Whether miRs repress the expression of the target gene by inhibiting translation or by promoting target mRNA decay is, at least in part, determined by seed matching of the guiding strand to target mRNA sequences (Figure 1.6) (Yekta et al., 2004). Interestingly, in animals just 6-8 complementary nucleotides matching at the 5' end of the guiding strand of miRs can be enough to inhibit the expression of target mRNAs (Lai, 2002, Lewis et al., 2003). This is in stark contrast to plants where almost 100% complementarity is required to promote repression of target mRNA expression (Rhoades et al., 2002)

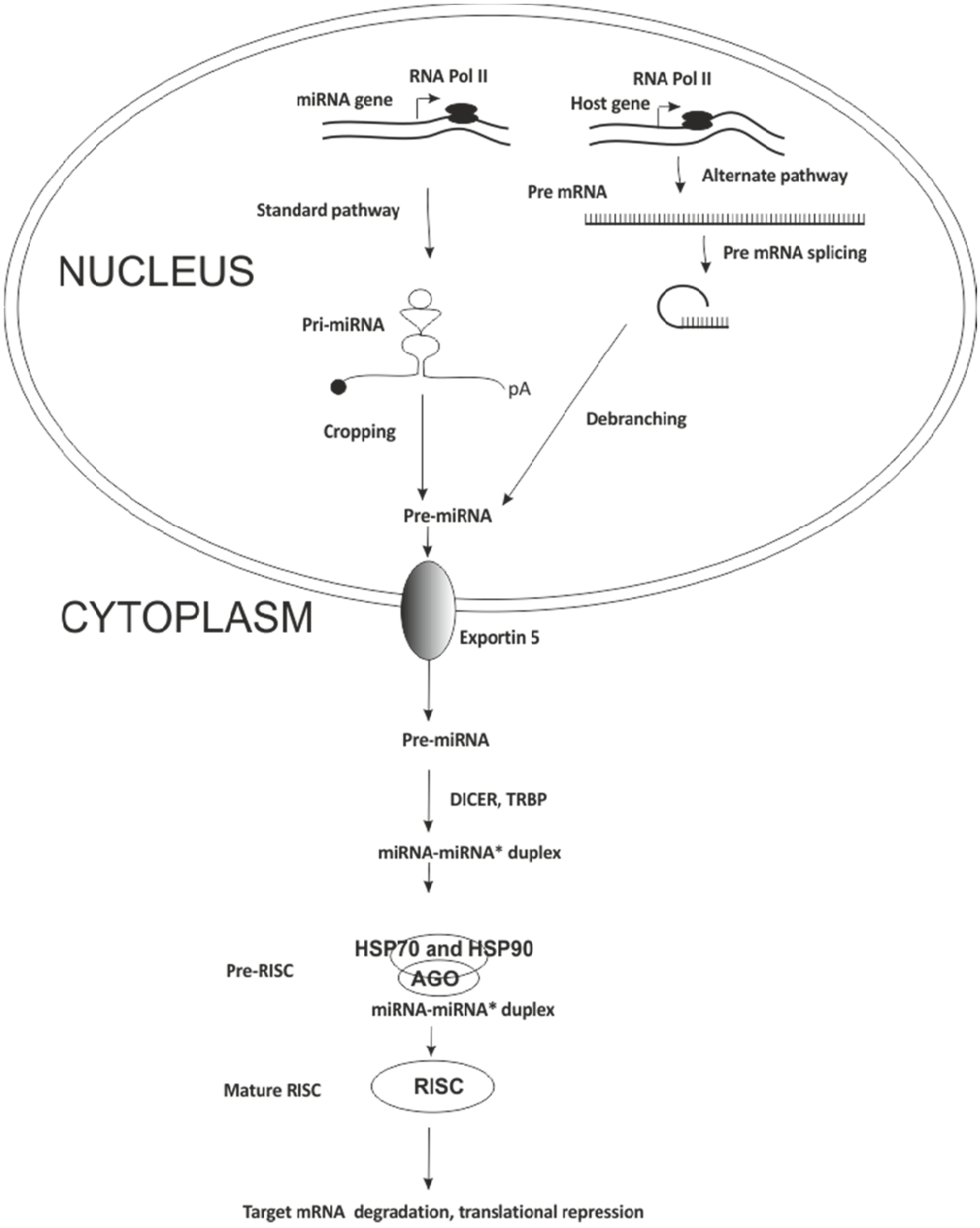


Figure 1.5: In the standard biogenesis of miRs primary microRNA (Pri-miR) is transcribed in the nucleus from specific miR gene by RNA polymerase II (RNA pol II) and is processed by Drosha and DGCR8 microprocessor complex subunit to form precursor miR (pre-miR).Alternatively pre-miR can be derived from splicing and debranching of transcripts from introns of a host gene. Processed pre-miR is exported to the cytoplasm by exportin 5. In the cytoplasm, Dicer cleaves and processes pre-miR to mature ~22bp duplex miR .One of the two strands of miRs (guiding strand) guides the RISC complex to complementary target mRNA sequences while other strand (passenger strand) is usually degraded. Adapted from review by Ameres and Zamore (2013).

During recent years, various freely available *in silico* web-tools able to predict animal miR binding sequences have been developed (Saito and Saetrom, 2010).

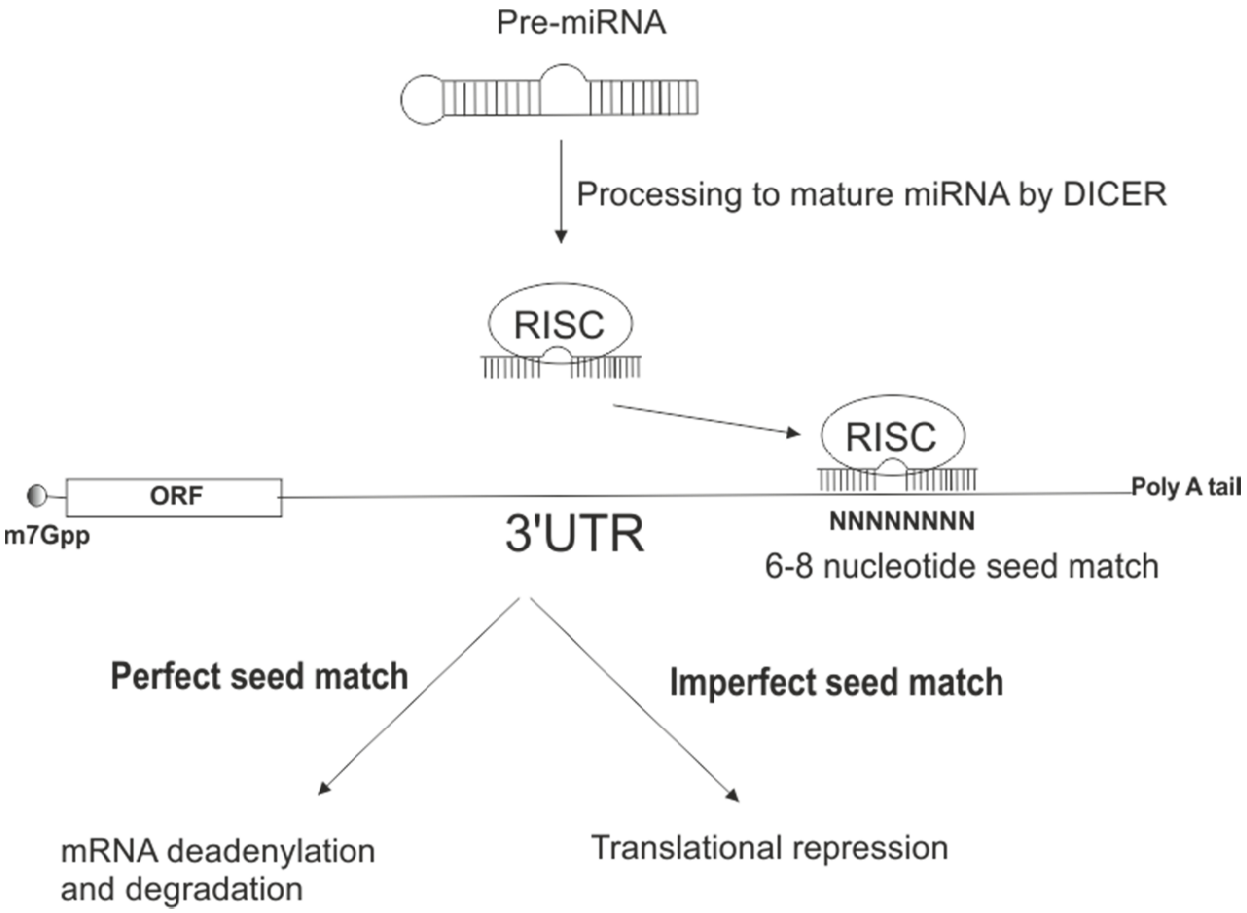


Figure 1.6: Perfect seed match of miR to target mRNA promote deadenylation and degradation of target mRNA whereas imperfect seed matching leads to translational repression of target (Krol et al., 2010).

miRs regulate multiple biological processes ranging from embryonic development to a wide range of physiological and pathological events (Chang and Mendell, 2007, Pauli et al., 2011, Suh and Blelloch, 2011). Each miR can influence multiple biological processes as a single miR has potentially hundreds of mRNA targets (John et al., 2004). For example, miR-1 plays a critical role in early embryonic development of the heart by facilitating a balance between differentiation and proliferation of cardiomyocytes (Zhao et al., 2005). Using human embryonic stem cell-derived neural progenitors (NPs), Delaloy et al. (2010) have shown a role for miR-9 in NP proliferation and migration. In zebra fish, mutating maternal-zygotic Dicer leads to abnormal brain formation suggesting a role for miRs in brain morphogenesis (Giraldez et al., 2005). The importance of miRs in brain has also been addressed in mouse models. The late embryonic deletion of Dicer in mice CNS leads to defective neurogenesis and gliogenesis (Kawase-Koga et al., 2009). In another study, knockdown of Dicer in the neurons of adult mouse forebrain results in abnormal tau hyperphosphorylation contributing to loss of neurons in the forebrain and hippocampus (Hebert et al., 2010).
miRs are also implicated in various human diseases ranging from cancer to neurological disorders (Sun et al., 2014, Li et al., 2014, Feng et al., 2009, Chatterjee et al., 2014). For example, a single point mutation in the seed region of human miR-96 leads to progressive hearing loss (Mencia et al., 2009). Parkinson's disease patients have decreased levels of miR-29c, miR-29a, and miR-19b in serum (Botta-Orfila et al., 2014). Thus miRs are proposed as diagnostic markers and potential future therapeutic targets for a wide range of pathological conditions in humans (Saito et al., 2013, Hermansen and Kristensen, 2013, Briasoulis et al., 2013).

1.6.2 AREs and ARE-BPs

AREs are 50-150 nucleotide long regions in the 3'UTRs which serve as binding sites for ARE binding proteins (ARE-BPs) that can either stabilize or de-stabilize their target mRNAs (Barreau et al., 2005). Roughly about 8% of mRNAs are estimated to contain functional AREs but their relevance in biological functions is largely unexplored (Bakheet et al., 2003, Bakheet et al., 2001). They are classified into three groups (Class I-III AREs), based on the presence of a core pentameric AUUUA sequence. Class I AREs have a core AUUUA sequence flanked by either A or U, Class II AREs have more than one overlapping AUUUA sequences, whereas Class III AREs are not well defined and do not have a typical AUUUA sequence (Chen and Shyu, 1995, Xu et al., 1997). Table 1.3 summarizes the known effects of ARE-BPs on the target mRNAs.

ARE-BPs	Stabilizing effect on mRNA	De-stabilizing effect on mRNA
Tristetraprolin(TTP)		TNF-α,c-Fos, GM-CSF,Cox -2,IL-2,IL-3, IL-6, IL-8, IL-10, IL-17, IL-1a, IL-23a, Csf2, Ccl2, Ccl3, Vegfa, Cxcl1, Nos2, Pitx2, Pim1, MYC, MMP1, TLR4, Ptgs2, HIV-1, Approx. 250 transcripts upregulated in TTP KO fibroblasts
Butyrate response factor 1 (BRF1)		TNF-α, GM-CSF, IL-3, Vegf, c-IAP2, STAR
Butyrate response factor 2 (BRF2)		TNF-α, , GM-CSF, IL-3
AU rich element binding protein 1 (AUF1/HNRPD)	c-myc, c-fos, PTH, GM-CSF, TNF-α	c-myc, c-fos, p21, Cyclin D1, GM-CSF, IL-3
ELAV like RNA binding protein 1 (ELAVL1/HuR)	c-fos, MyoD, p21, Cyclin A, Cyclin B1, CyclinD1, NOS II/iNOS, GM-CSF, TNF-α, COX-2, IL-3, Vegf, Myogenin	
ELAV like neuron-specific RNA binding protein (ELAVL4/HuD)	GAP-43, BDNF	
KH-type splicing regulatory protein (KSRP)		c-fos, TNF-α, NOS II/iNOS, IL-2, c-jun

Table 1.3: ARE-BPS and their effects on known target mRNAs. Broadly they can also be classified in two major groups based on their stabilizing or destabilizing effects on target mRNAs. Notably, some ARE-BPs like AUF1 can have stabilizing and de-stabilizing effect on same mRNA target in different biological contexts. Based on information from publications by Barreau et al. (2005), Baou et al. (2009), Allen et al. (2013) and Brooks and Blackshear (2013).

1.6.2.1 Tristetraprolin (TTP)

Tristetraprolin/ Zinc finger protein 36 (TTP/Zfp36) is one of the most widely studied ARE-BPs and the founding member of the TTP/family. A characteristic feature of the TTP ARE-BP family is the presence of a CCCH zinc finger motif (Lai et al., 1990, DuBois et al., 1990). Butyrate response factor 1 (BRF1 /ZFP36L1) and Butyrate response factor 2 (BRF2 /ZFP36L2) are the other two members of the TTP/TIS11 family. TTP, BRF1 and BRF2 are expressed in most mammals including humans, while rodents have an additional fourth member called ZFP36L3 (Brooks and Blackshear, 2013).

TTP and its family members BRF1 and BRF2 have been shown to bind to ARE containing mRNAs resulting in mRNA decay by mainly promoting deadenylation (Carballo et al., 1998, Stoecklin et al., 2002, Ciais et al., 2004). Interestingly, some reports also suggest a role of TTP in regulation of gene expression at the translational level (Qi et al., 2012, Pfeiffer and Brooks, 2012).

TTP KO mice are healthy at birth but develop progressive inflammatory disease with symptoms like patchy alopecia, dermatitis, erosive arthritis, cachexia, conjunctivitis, myeloid hyperplasia, glomerular mesangial thickening, and develop antinuclear antibodies 1-8 weeks postnatally. The phenotype of TTP KO mice is largely rescued by injecting monoclonal antibodies against TNF-α (Taylor et al., 1996). Later, TTP was demonstrated to bind directly to ARE present in the 3′UTR of TNF-α, validating it as the first physiological target of TTP *in vivo* (Lai et al., 1999). Since then, TTP has been found to regulate a plethora of cytokines and growth related transcripts (Table 1.3). Notably, NTFs such as BDNF are also involved in regulating inflammation in certain pathological conditions (Amoureux et al., 2008, Lin et al., 2011, Uchida et al., 2013, Luhder et al., 2013). However, to date the potential of TTP to regulate NTF levels have remained unexplored and is one of the objectives of this thesis.

1.6.3 Other mediators of 3'UTR regulation

Apart from the above mentioned miR response elements and AREs, other regulatory elements exist in the 3′UTRs. 3′UTR contains signal sequences which lead to addition of roughly 200 adenosine bases (polyA tail) at the 3′ end of a mRNA that serve as a binding site for polyA binding proteins (PABPs) known to play a role in processes like mRNA export, decay, stability and translation (Mangus et al., 2003). So far, five different PABPs have been identified (Gorgoni et al., 2011). Furthermore, alternate polyadenylation signals in the 3′UTRs facilitate generation of mRNAs containing 3′UTRs of various lengths. Roughly 50% of human genes are estimated to contain alternate polyadenylation signals (Dickson and Wilusz, 2010). Iron response elements (IREs) are another regulatory sequences present in 3′UTRs of various mRNAs encoding for proteins involved in iron metabolism and serve as binding sites for IRE binding proteins (IRE-BPs) which stabilize the target mRNAs (Guo et al., 1994). Finally, 3′UTR-s of several mRNAs may contain localization signals which determine their active transport to different subcellular compartments particularly in such highly polarized and differentiated cells as neurons. The disruption of these localization signal sequences can cause a reduction in the corresponding mRNA and respective encoded protein levels in those sub-cellular locations (Andreassi and Riccio, 2009). Last but not least are the secondary structure motifs of the 3′UTRs of mRNAs which can regulate RNA turnover (Sarnowska et al., 2007). For example, the 3′UTR of BDNF has a secondary loop structure that stabilizes BDNF mRNA in neurons in the presence of Ca2+ signal (Fukuchi and Tsuda, 2010). BDNF and GDNF transcripts contain long and conserved 3′UTRs (Results and discussions, Figure 4.1). The current understanding of 3′UTR mediated regulation of BDNF and GDNF is discussed in following section.

1.7 3' UTR mediated regulation of BDNF and GDNF

Apart from multiple promoter-mediated control, BDNF is additionally regulated by the 3′ UTR . A single study suggested that the 3′UTR of BDNF mRNA plays a role in the subcellular localization of BDNF mRNA in neurons leading to distinct cellular functions, although this conclusion needs further validation (An et al., 2008). According to this study BDNF mRNAs containing long 3′UTRs are localized in dendrites whereas short 3′UTR containing transcripts generated due to presence of alternate polyA signal (See Figure 1.3A) are restricted to the soma *in vivo*. An et al. (2008) made a mouse model generating truncated long form of BDNF 3′UTR (BDNF-L) and presented data suggesting impaired BDNF mRNA localization to dendrites leading to defects in pruning and enlargement of dendritic spines resulting in selective impairment of long-term potentiation in dendrites of hippocampal neurons . Despite the authors claim of no change in BDNF levels in the 3′UTR truncated animal model the presented data suggests derepression of endogenous BDNF levels which may alone or in addition to the localization defect explain the outcome. Future studies should resolve this important issue.

In another study, Lau et al. (2010) has shown that the BDNF mRNA containing long 3′ UTR specifically undergoes robust translational activation in the hippocampus upon seizure-induced neuronal activation. Furthermore, regions of 3′UTR stabilize BDNF mRNA in response to Ca^{2+} signaling (Fukuchi and Tsuda, 2010). These studies highlight the importance of the 3′UTR in the regulation of BDNF expression and function, stressing the need for further in depth analysis of 3′UTR mediated regulators like miRs and RBPs. Later studies indeed reported several miRs targeting the BDNF 3′UTR. miR-206 was shown to regulate BDNF in the mouse model of Alzheimer disease and in *vitro* model of skeletal muscle differentiation (Lee et al., 2012, Miura et al., 2012). Caputo et al. (2011) showed in another study that miR-26a and miR-26b down regulates BDNF expression in a reporter assay and that two single nucleotide polymorphisms (SNPs) in BDNF 3′UTR specifically abolish their binding to 3′UTR in the same reporter assay. Table 1.4 shows an overview of previous studies addressing regulation of BDNF by miRs.

Target miR	Model	Reference
miR-206	*In vitro*, *In vivo*	Lee et al. (2012) Miura et al. (2012) Yang et al. (2014)
miR-26a, miR-26b	*In vitro*	Caputo et al. (2011)
miR-16	*In vitro, In vivo*	Sun et al. (2013)
miR-22	*In vitro*	Muinos-Gimeno et al. (2011)
miR-30a-5p, miR-195	*In vitro*	Mellios et al. (2008)

Table 1.4: Overview of BDNF targeting miR studies.

Importantly, the *in vitro* reporter based studies mentioned above used only a small fragment of the 3′UTR of BDNF for validation of miR targets. This leaves an open question about the validity and availability of these target sequences in the context of full-length BDNF 3′UTR.

Apart from microRNAs, RBP HuD is shown to stabilize selectively BDNF mRNAs containing long 3′UTR *in vitro* and *in vivo* but in this study as well, only a small fragment of BDNF 3′UTR, containing ARE was used for *in vitro* assay (Allen et al., 2013). Interestingly, microarray studies on TTP KO cell line identified BDNF among the 250 upregulated mRNAs, suggesting that TTP may, directly or indirectly, regulate BDNF levels (Lai et al., 2006).

The structure of the mouse GDNF gene is far simpler in comparison to the BDNF gene (Figure 1.3B), it contains three exons coding for transcripts including 1 kb long 5'- and 2.8 kb long 3′-untranslated regions

(UTRs) (Matsushita et al., 1997). Regions in the 3′UTR of GDNF are quite evolutionarily conserved (Results and discussion section, Figure 4.1) indicating functional significance but surprisingly, there is just one single study by Oh-hashi et al. (2012) which analyzed fragments from mouse GDNF 3′ UTR with varying lengths with luciferase reporter assay and concluded presence of inhibitory elements in GDNF 3′UTR region. However, analysis of GDNF 3′UTR for various regulators including miRs is still lacking and is one of the primary objectives of the current thesis.

2 Aims of the study

The aim of this study was to investigate 3′UTR mediated regulation of the neurotrophic factors BDNF and GDNF by miRs and RBPs. Along these lines, an alternative approach to study gene function was explored by 3′UTR replacement. The hypothesis is that when a 3′UTR is a subject of negative regulation, its replacement with a 3′UTR devoid of negative regulation would lead to overexpression limited to natively expressing cells, i.e. derepression of endogenous gene expression.

Specific aims:

- Characterize the 3′UTRs of BDNF for miR binding sites and identify BDNF regulating miRs *in vitro.*

- Analyze BDNF 3′UTRs for AREs and screen for BDNF regulating ARE-BPs *in vitro.*

-Characterize the 3′UTR of GDNF for miR binding sites and identify GDNF regulating miRs *in vitro.*

- Study mice where the GDNF 3′UTR is replaced with a 3′UTR less responsive to negative regulation by miRs.

3 Materials and methods

List of material and methods used in this thesis work. See original publications/manuscripts for detailed information about material and methods.

METHODOLOGY	PUBLICATION/MANUSCRIPT
RNA and DNA methods	
Isolation of RNA and DNA	I,II,III
Reverse transcription(RT), cDNA synthesis	I,II,III
Quantitative RT-PCR	I,II,III
Northern Blot	III
In situ hybridization	III
Genotyping	III
Dual-Glo® Luciferase Assay System and CellTiter-Glo® Luminescent Cell Viability Assay	I,II,III
Molecular cloning	I,II,III
Electrophoretic mobility shift assay (EMSA)	II
RNA immunoprecipitation assay (RNA-IP)	II
Cell culture methods	
In vitro cell culture	I,II,III
Primary cell culture	III
Immunological methods	
ELISA (GDNF/BDNF)	I,II,III
Western blotting	II,III
Immunohistochemistry	III
Other methods	III
Estimation of monoamines and their metabolites, HPLC	III

Corridor test	III
Amperometry	III
Generation of knockin animals and tissue dissection	III
Web tools for *in silico* analysis	Web page (Publication/manuscript)
Targetscan	http://www.targetscan.org (I,III)
AREsite	http://rna.tbi.univie.ac.at/cgi-bin/AREsite.cgi (II,III)
Genecards	http://www.genecards.org/ (II)

4 Results and discussion

4.1 The 3' UTRs of BDNF and GDNF contain regulatory sequences (I, II, III)

in silico analysis of BDNF and GDNF 3′ UTR was performed using publically available web-tools (Material and methods). Targetscan predicted multiple evolutionary conserved miR binding sequences in the both isoforms of 3′UTRs of BDNF (BDNF-S and BDNF-L) and GDNF 3′UTR (Figure 4.1). AREsite predicted multiple evolutionally conserved class I AREs (AUUUA) in both isoforms of BDNF 3′UTRs and GDNF 3′UTR. Notably, regions closer to 5' and 3′ end of both BDNF and GDNF 3′ UTR sequences were highly (81-90%) conserved between M. musculus and H. sapiens (Figure 4.1) . Importantly, all miR binding sequences present were shared between M. musculus and H. sapiens 3′UTRs except miR-190/190ab site for BDNF and miR-216/216a site for GDNF (Targetscan). Additionally, Human BDNF 3′UTR had one extra ARE site (AUUUA) and GDNF 3′ UTR had 3 extra ARE sites (AREsite). Furthermore, BDNF-S contained 7 miR sites out of total 13 predicted conserved miR sites and 2 AREs out of total 8 predicted conserved ARE sites in mouse BDNF 3′UTR (Figure 4.1).

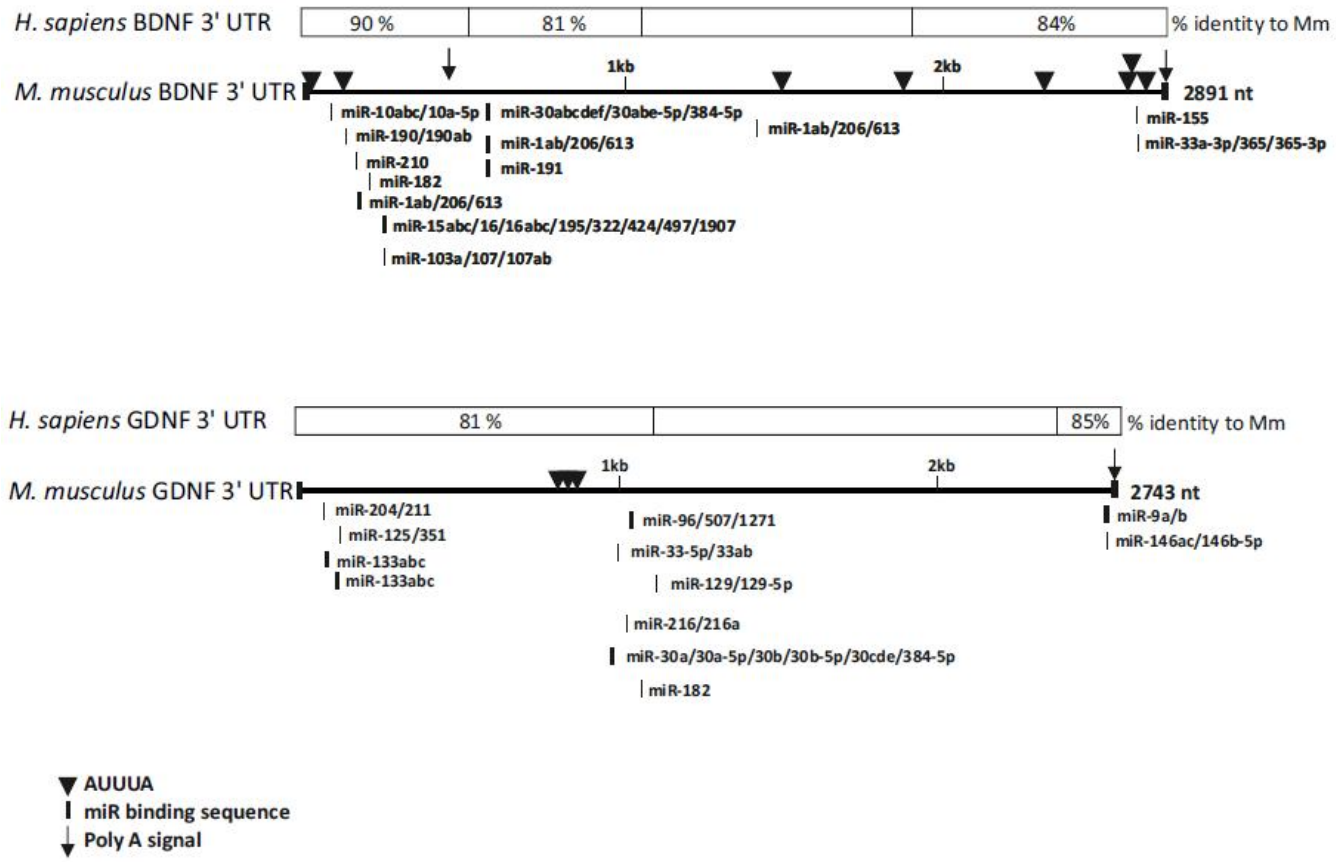


Figure 4.1: Schematic diagram showing percentage of sequence identities between mouse (M. musculus) and human (H. sapiens) 3′UTR of BDNF and GDNF. Vertical bars show predicted miR binding sequence (Thicker bars indicates highly conserved miR binding sequences among vertebrates), black triangles show predicted conserved AREs (AUUUA) and arrows show PolyA (polyadenylation) signals.

4.2 BDNF and GDNF level is regulated by miRs *in vitro* (I, III)

4.2.1 BDNF regulation by miRs *in vitro* (I)

The objective of this study was to analyze the regulation of both isoforms of BDNF 3′UTR : BDNF-L and BDNF-S, by ten miRs (miR-1, miR-10b, miR-15a, miR-16, miR-30a,miR-30b, miR-155, miR-182, miR-191 and miR-195) selected based on evolutionary conservation of their binding sequences within 3′UTRs of BDNF and their co-expression with BDNF in the tissues or cell lines with known BDNF functions using *in vitro* tools.

miR-1, miR-10b, miR-155 and miR-191 as novel regulators of BDNF were identified using luciferase reporter assay, miR binding site mutational analysis, assessment of pre-miR overexpression effects on endogenous BDNF levels and measurements of endogenous BDNF derepression upon application of anti-miRs against identified endogenously expressed miRs. In addition, data suggest direct interaction between BDNF long 3′UTR and endogenous miR-1/206 family miRs in C2C12 cell model of muscle differentiation, where regulation of BDNF level is known to be critical. The findings were confirmed by showing that the binding sites for miR-1 and mIR-10 in BDNF 3′UTR are used synergistically by endogenous miRs in several cell lines.

This study is the first functional analysis of BDNF 3′UTR which addresses the effects of the ten most conserved miRs on full-length BDNF 3′UTR isoforms. All the previous studies focused on one or two miRs at a time and studied their effects on various fragments of BDNF 3′UTR making direct comparisons of different studies difficult. Due to the co-expression of investigated miRs with BDNF in various tissues and conservation of these miR sites among most vertebrates, it is speculated that BDNF/miR interaction plays an important role *in vivo*.

4.2.2 GDNF regulation by miRs *in vitro* (III)

The first objective of the study was to analyze the regulation GDNF 3′UTR by nine miRs (miR-9, miR-133a, miR-133b, miR-125a-5p, miR-125b-5p, miR-30a, miR-30b, miR-96, miR-146a) selected based on conservation of their binding sites within the GDNF 3′UTR and their co-expression with GDNF in various tissues where GDNF has a known or presumed biological function. All the nine miRs negatively regulated expression of a reporter construct containing the GDNF 3′UTR compared to controls. Furthermore, mutating the evolutionary conserved miR-146a and miR-96 target sequence abolished the inhibitory effects of respective miRs on the GDNF 3′UTR containing reporter, suggesting their direct interaction with the 3′UTR of GDNF. Mutational analysis of the presumed miR-9 binding site and miR-133 family binding sites in the GDNF 3′UTR did not abolish inhibition, indicating that these two miRs bind to different sites or their effect are indirect. Endogenous GDNF protein and mRNA levels were suppressed when precursors of miR-9, miR-96, miR-146a and mir-133b (selected based on their most potent inhibitory effects in the luciferase based reporter assay) were transfected to U-87 cells which express detectable amounts of endogenous GDNF protein and mRNA(III, Figure 3B-E).

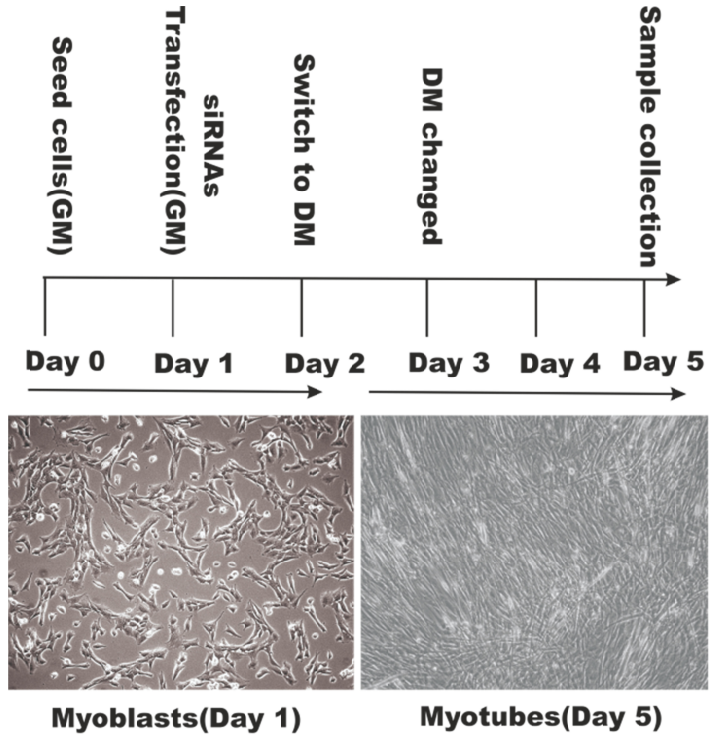
4.3 BDNF but not GDNF is regulated by TTP ARE-BP via the 3'UTR *in vitro* (II, III)

4.3.1 BDNF is regulated by TTP (II)

AREs in both isoforms of BDNF 3′UTRs are well conserved (AREsite, Figure 4.1). Selection of six ARE-BPs (TTP, BRF1, BRF2, ELAVL1, ELAVL2 and AUF1) were based on their co-expression with BDNF in various tissues (Genecard, Material and methods). ARE-BPs TTP, BRF1 and BRF2, but not ELAVL1 or ELAVL2,

inhibited luciferase reporter expression containing either BDNF-L or BDNF-S. Furthermore, a direct interaction between the proximal 5' fraction of BDNF-S and recombinant TTP protein were demonstrated using EMSA. Additionally, endogenous BDNF mRNA co-immunoprecipitated with endogenous TTP in cell lysate from differentiated C2C12 cells (myotubes). Finally, siRNA-mediated knockdown of TTP during myogenic differentiation of mouse myoblast C2C12 cells led to increased BDNF protein expression. Taken together, this data suggests TTP as a novel regulator of BDNF expression. Additionally, upon TTP knock-down, morphological changes such as deformed myotubes were observed. However, since the TTP regulates levels of at least 250 mRNAs (Lai et al., 2006), this deformed myotube phenotype cannot solely be attributed to the elevation of BDNF levels (Figure 4.2). BDNF and TTP family members are co-expressed in many cells and tissues (Genecard, see material and methods section), suggesting a broader scope of observed regulation in the context of a wide range of biological processes ranging from CNS function to inflammation and muscle differentiation.

A.



B.

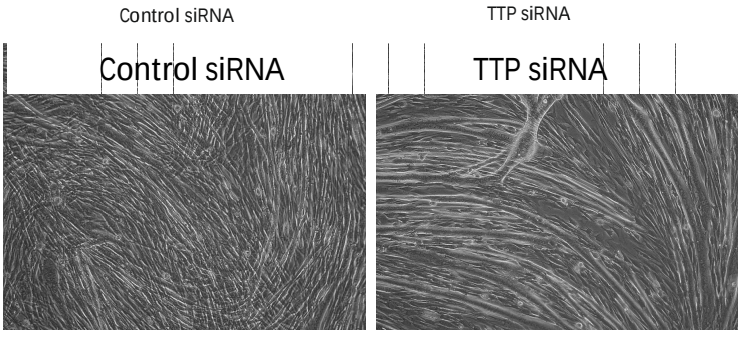


Figure 4.2: A. C2C12 cell differentiation model B. Phenotypic change in differentiated myotubes at day 5 after knockdown of TTP by siRNA. Representative image, N= 4

Since we found that miRs (miR-1, miR-10b, miR-155 and miR-191) and TTP were direct regulators of BDNF levels, it was tempting to speculate that they might act together. We explored the possibility of additive

effect of miRs and TTP. Using the same reporter containing long 3'UTR (BDNF-L), we studied miR-1 and TTP alone and together and found an additive effect in this system (Figure 4.3). This is an indication that TTP and miR-1 can co-operate but this finding needs further validation in different systems including studies on endogenous TTP and miR-1 co-inhibition on endogenous BDNF mRNA and protein levels.

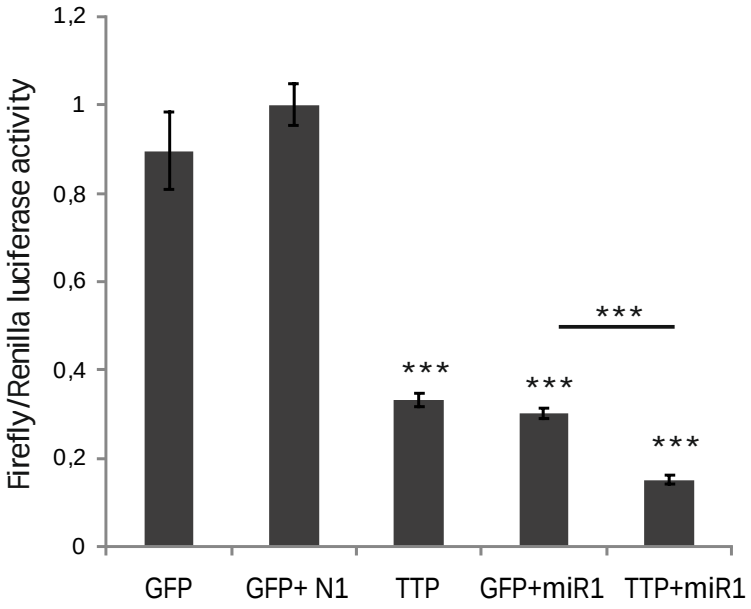


Figure 4.3: Additive effects of TTP and miR-1on the inhibition of luciferase reporter construct expression containing BDNF-L. In the graph, Firefly/Renilla luciferase ratio for GFP+N1 control sample was set 1 and fold changes compared to GFP is presented for other samples. Data were analyzed with 2-tail student's t-test assuming unequal variance. The level of significance was set at p= 0.01 . N1= Non-targeting miR control, *** P< 0.001, N=3

4.3.2 GDNF is not regulated by tested RBPs *in vitro* reporter assay (III)

Reporter construct containing GDNF3'UTR co-transfected with plasmid encoding for the selected ARE-BPs (TTP, ELAVL1, AUF1, TIA1, ELAVL2, BRF1 and CELF1) revealed that the above RBP-s had no or mild effect (In case of, BRF1) on reporter expression (Figure 4.4 and III, Supplementary Figure 3A). Mild binding effects in luciferase reporter assay with RBPs is subject to further validation by alternate direct methods such as EMSA or RIP as indirect luciferase reporter based method is known to have nonspecific effect (Auld et al., 2008). An independent study by Oh-hashi et al. (2012) tested several ARE-BPs in a luciferase reporter assay (ELAV family members: HuR, HuD, HuB and HuC) with similar conclusions.

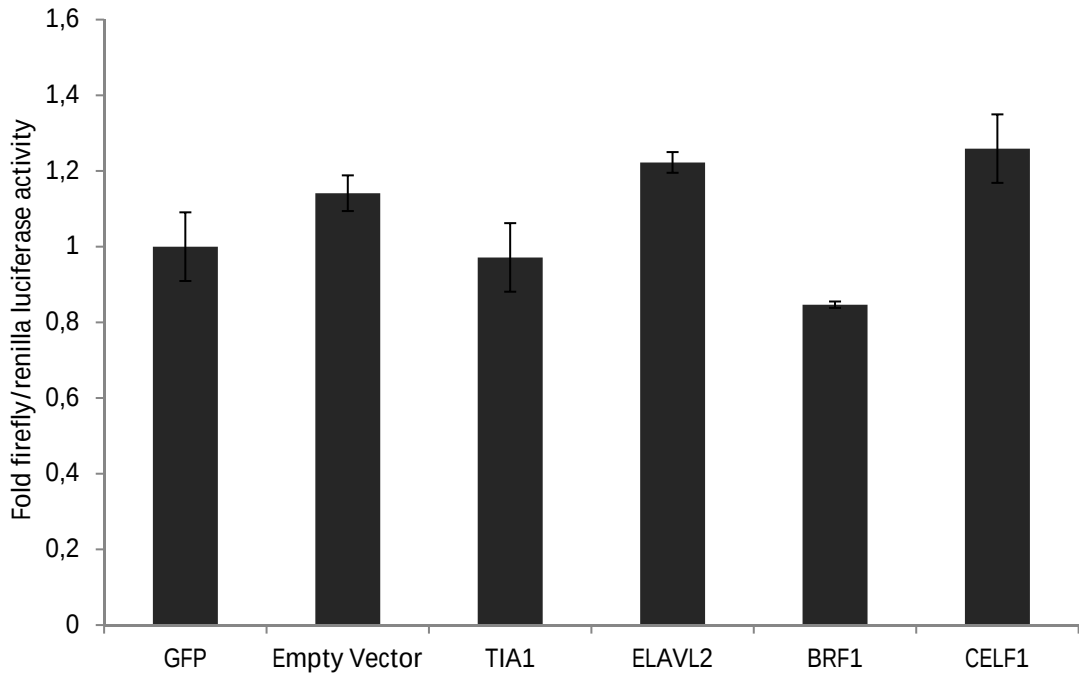


Figure 4.4: No effect of RBPs on luciferase reporter expression containing GDNF 3′UTR. In the graph, Firefly/Renilla luciferase ratio for GFP control sample was set 1 and fold changes compared to GFP is presented for other samples. Data were analyzed with 2-tail student's t-test assuming unequal variance. The level of significance was set at p= 0.01.

Importantly, luciferase reporter containing full length GDNF 3′UTR was used in this thesis work whereas Oh-hashi et al. (2012) used almost 13 different luciferase reporter constructs containing various lengths of GDNF 3′UTR (ranging from full length GDNF 3′UTR to shorter fragments (~80 nucleotides) with predicted 3 AREs, Figure 4.1). This indicates that these predicted AREs are not interacting with or unavailable to tested RBPs and independent of the length of GDNF 3′UTR used with the luciferase reporter.

4.4 Generation of GDNF 3'UTR-crR mice (III)

A mouse model with conditionally reversible replacement of GDNF 3′UTR (GDNF 3′UTR-crR mice) was generated, as the GDNF 3′UTR possesses inhibitory properties (III, Figure 1). The effect of GDNF specific miRs on the GDNF 3′UTR replacement cassette (designated as "GDNF 3′UTR-crR") was assessed in luciferase reporter assay and none of the tested miRs retained any inhibitory effect on the replacement sequence (III, Figure 1). Compared to a reporter containing GDNF 3′UTR a reporter containing the GDNF 3′UTR-crR yielded two-fold elevated expression in brain-derived U87 cells and eight-fold elevated expression in kidney-derived HEK293 cells (III, Figure 1). The replacement cassette was flanked with FLP recombinase recognition sites enabling conditional removal of the replacement cassette to restore transcription to the GDNF native 3′UTR (GDNF 3′UTR-rest -/-, III).

4.5 *In vivo* replacement of 3'UTR elevates GDNF level (III)

GDNF 3′UTR replacement in mice resulted in elevation of GDNF mRNA and protein levels in the different parts of the brain and in the developing kidneys (III, Figure 2 and Supplementary Figure 2) in both homozygous and heterozygous animals in a gene dose-dependent manner. Analysis of GDNF mRNA expression by *in situ* hybridization (ISH) in various organs revealed that GDNF mRNA expression in those mice is limited to cells which natively express GDNF (III, Figure 2). This elevation in the GDNF levels in brain and kidney was restored to wild type levels by conditional removal of the replacement cassette by crossing

with Deleter-Flp mouse line (III, Figure 2C). This shows an advantage of the 3′UTR replacement method over conventional transgene overexpression mouse models where variations in integration site and recombinant construct most often lead to spatiotemporal misexpression confounding conclusions on endogenous gene functions. Moreover, elevation in the GDNF levels in brain and kidney was restored to wild type levels by conditional removal of the replacement cassette by crossing with Deleter-Flp mouse line (III, 2C). This property of GDNF 3′UTR-crR mice allows future studies on the biology of GDNF levels in various organ systems where GDNF is known to play important role.

4.6 Phenotypic effect of 3'UTR replacement of GDNF in mice (III)

Homozygous GDNF 3′UTR-crR mice die before post-natal day (P) 18 because of defective kidneys and urogenital tract (Andressoo group, unpublished) but heterozygous GDNF 3′UTR-crR mice are healthy with normal life-span. Bodyweight measurements and studies with a physiological cage revealed no difference between heterozygous and wild type littermates. Dopamine levels in the rostral brain of homo- (-/-)and heterozygous (+/-) GDNF 3′UTR-crR mice at the age of P7.5 were elevated compared to wild type (III, Figure 4B). Similarly, dopamine levels in the striatum of adult (2.5-3 months age) GDNF 3′UTR-crR (+/-) mice were elevated (III, Figure 4F). Restoration of wild type GDNF level by removing 3′UTR replacement cassette leads to restoration of wild type dopamine level (III, 4B and 4F).

Furthermore, dopamine transporter (DAT) activity is increased up to 5-fold in a dopamine concentration dependent manner and amphetamine-induced locomotor activity (but not basal) is significantly increased in the GDNF3′UTR-crR (+/-) mice (III, Figure 5A-B). Additionally, GDNF 3′UTR-crR (+/-) mice were protected in a unilateral supranigral lactacystin injection based rodent model of Parkinson's disease (III, Figure 5C-G). Thus, about 2-fold elevation in endogenous GDNF levels in the striatum (III, Figure 2C) results in significant boost in brain dopamine system function and is sufficient to protect animals from chemically induced PD.

Since classical GDNF knockout mice die at birth due to a lack of kidneys with an intact midbrain dopamine system little in vivo data on the postnatal function of endogenous GDNF exists. Since GDNF 3′UTR-crR mice (+/-) are viable, we were able to analyze the postnatal role of endogenous GDNF in the development and function of the postnatal dopamine system. GDNF 3′UTR-crR mice can also be useful for study of endogenous GDNF in other organ systems with known GDNF functions such as the urogenital tracts and enteric nervous system (Moore et al., 1996, Sainio et al., 1997).

We demonstrate a qualitative difference between ectopic and endogenous GDNF action on the dopamine system (III, Table 1). Ectopic GDNF transiently increases dopamine turnover without lifting total dopamine levels (Hudson et al., 1995, Georgievska et al., 2004b, Hebert et al., 1996), while elevation of endogenous GDNF by 3′UTR replacement permanently elevates total dopamine levels but not its turnover. In addition, results with GDNF3′UTR-crR mice show dopamine concentration-dependent increase in DAT activity which has not been reported in studies with ectopic GDNF. We also show that, unlike ectopic GDNF which reduces TH levels, endogenous GDNF has no effect on TH levels in SNpc and striatum.

5 Conclusion and future perspectives

The physiological consequence of 3′UTR mediated levels regulation of important regulatory genes such as neurotrophic factors in the brain has largely remained unexplored. In this thesis work we present analysis of full length 3′UTRs of the neurotrophic factors BDNF and GDNF *in vitro*. We show role of miRs (miR-1, miR-10b, miR-191 and miR-155) and ARE-BP TTP in the negative regulation of BDNF. We conclude that GDNF is regulated by multiple miRs in cell lines and identify binding sites for miR-146 and miR-96 in the GDNF 3′UTR. Finally, the knowledge gained from *in vitro* experiments about 3′UTR mediated negative regulation of GDNF was applied to generate a new mouse model based on 3′UTR replacement of GDNF which led to *in vivo* upregulation of GDNF levels. The latter mouse model enables studies on postnatal GDNF function in midbrain and other organ systems that are largely inaccessible with previous genetic tools.

The specific conclusions for each study:

I. miR-1, miR-10b, miR-155 and miR-191 are novel direct regulators of BDNF. Co-expression of BDNF and miR-1/miR-10b/miR-155/miR-191 in various cell types and tissues suggests potential relevance of miR mediated post-transcriptional regulations of BDNF in various physiological and pathological processes.

II. TTP is a direct post-transcriptional regulator of BDNF. BDNF and TTP are co-expressed in many cell types and tissues.
Together, I and II report two different classes of negative regulators of BDNF expression via 3'UTR. These findings open doors to future studies addressing the *in vivo* relevance of the identified miRs and TTP mediated regulation of BDNF function in health and disease.

III. GDNF is regulated by multiple miRs in cell lines and at least miR-146 and miR-96 directly bind to the GDNF 3′UTR. *In vivo* replacement of 3′UTR uncovers novel functions of GDNF and provides a potent tool for future studies on GDNF.

The results presented in this thesis also indicate that the 3′UTR replacement approach could be of broad interest to the scientific community. Currently, gene function *in vivo* is mostly studied by the gene knock-out approach or by transgenic overexpression. However, the knock-out approach fails to reveal the extent to which compensatory effects by functional homologues and, in the case of the brain, the use of alternative neuronal networks, masks the effects of the lack of a gene. On the other hand, the transgenic overexpression of a gene has its own limitations because of frequent spatiotemporal mis-expression resulting from recombinant construct and integration site variations. Our results suggest that *in vivo* studies by targeting 3′UTRs with regulatory properties may provide information on gene function previously inaccessible with current methods, and in a highly specific manner in natively expressing cells.

Of all organs, it is the brain where precision of spatial regulation of regulatory factors expression is expected to play the most prominent role in building and maintaining the organ. We hypothesize that systematic species and organ system-spanning genetic studies of 3′UTRs would provide important new information on gene function and regulation. Based on our findings, we propose an improved approach enabling studies on 3′UTR function and changes in endogenous gene product levels both spatially and temporally. We compared sequences of important regulatory genes involved in development and plasticity of nervous system.

Our analysis revealed long 3′UTRs with the majority of miR binding sequences conserved between mouse and human for several important regulatory genes involved in brain development and function (Table 5.1).

Gene	Gdnf	Bdnf	EfnA3	EfnB2	Ntn1	En2	Robo1	Sema3A	Ncam1
3′ UTR bp	2743	2891	954	3141	3632	1764	1730	2954	3275
No. of conserved miR sites	12	13	6	15	5	15	6	11	7
Identical miR sites Mm/Hs	11	12	6	14	5	13	6	11	7

Table 5.1: Representative genes with known regulatory function in brain (III, Figure 5L)

As mentioned above, these results with the 3′UTR replacement model suggest that this method can provide information beyond that accessible with previously available genetic manipulation tools. Along these lines, Andressoo group has already generated a "Flex-cassette" (Schnutgen et al., 2005) based system allowing conditionally replace GDNF 3′UTR *in vivo*. This model will provide additional opportunities to use Cre-based mouse lines to conditionally derepress GDNF in a specific tissue and/or time.

Whether presented findings in this thesis can be transferred into clinically meaningful applications to treat human diseases or age-related decline in brain function awaits the development and demonstration of brain-deliverable and sufficiently gene specific 3′UTR regulators. Those may include virally encoded shRNAs targeting specific miRs, miR sponges and miR target site protectors (Choi et al., 2007, Gentner et al., 2009, Li et al., 2009). It is tempting to speculate that these as well as future results from 3′UTR replacement studies will motivate development of new gene-specific means to manipulate 3′UTR regulation.

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RESEARCH ARTICLE

miR-1, miR-10b, miR-155, and miR-191 are novel regulators of BDNF

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Abstract Brain-derived neurotrophic factor (BDNF) is a secreted protein of the neurotrophin family that regulates brain development, synaptogenesis, memory and learning, as well as development of peripheral organs, such as angiogenesis in the heart and postnatal growth and repair of skeletal muscle. However, while precise regulation of BDNF levels is an important determinant in defining the biological outcome, the role of microRNAs (miRs) in modulating BDNF expression has not been extensively analyzed. Using in silico approaches, reporter systems, and analysis of endogenous BDNF, we show that miR-1, miR-10b, miR-155, and miR-191 directly repress BDNF through binding to their predicted sites in BDNF 3′UTR. We find that the overexpression of miR-1 and miR-10b suppresses endogenous BDNF protein levels and that silencing endogenous miR-10b increases BDNF mRNA and protein levels. Furthermore, we show that miR-1/206 binding sites within BDNF 3′UTR are used in differentiated myotubes but not in undifferentiated myoblasts. Finally, our data from two cell lines suggest that endogenous miR-1/206 and miR-10 family miRs act cooperatively in suppressing BDNF through their predicted sites in BDNF 3′UTR. In conclusion, our results highlight miR-1, miR-10b, miR-155, and miR-191 as novel regulators of BDNF long and short 3′UTR isoforms, supporting future research in different physiological and pathological contexts.

Keywords Brain-derived neurotrophic factor · MicroRNAs · 3′ untranslated region · Differentiation

Introduction

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family and has important functions in brain development, synaptogenesis, and memory and learning [19, 32, 37, 44, 50]. Disrupted BDNF function has been associated with several nervous system disorders, such as Huntington's disease [23, 69], depression [8, 11], and anxiety [55], in both humans and rodent models. In addition, BDNF is involved in several physiological processes outside the brain, such as angiogenesis in the heart and skeletal muscles [16], myogenic differentiation [49], and skeletal muscle regeneration [13]. As evident from studies with mice heterozygous for the BDNF gene, 50 % reduction in BDNF protein levels is sufficient to cause notable phenotypic changes [1, 2, 15, 45]. Thus, precise regulation of BDNF levels is important in several developmental and pathological processes, highlighting the importance of understanding the mechanisms that control BDNF expression.

The 3′ untranslated regions (3′UTRs) of mammalian genes contain highly conserved sequences [60] important in the regulation of translational efficiency, polyadenylation, and stability of the mRNA. These functions are mediated by binding to RNA-interacting factors, such as microRNAs (miRs) [6, 48]. miRs are short, non-coding RNA molecules predicted to interact with the transcripts of about 60 % of all mammalian protein-coding genes [7]. miRs bind their target mRNAs through a fully complementary seed sequence of 7–8 nucleotides in their 5′ end and less complementary area in the 3′ end, inducing translational repression and/or mRNA degradation [7, 24].

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One way to modulate miR-mediated regulation of gene expression is through the generation of alternative mRNA transcripts that differ in the length of the 3′UTR [58]. Brain-derived neurotrophic factor transcripts contain either short (BDNF-SH, 350 nt) or long 3′UTR (BDNF-L, 2891 nt [27]). In addition to length, secondary structure of the 3′UTR is also known to influence mRNA stability, at least in part by modifying accessibility to miR target sites in the mRNA [29, 67, 68]. In general, miR sites near the ends of the 3′UTR are more effective than sites in the center of the 3′UTR, partly because regions in the middle of the 3′UTR are more likely to be incorporated into hairpin structures, hindering access to miRs [29]. BDNF-L contains a stem-loop structure necessary for the regulation of transcript stability [26], raising the possibility that a secondary structure could determine access to factors like miRs that influence mRNA stability.

Despite the presence of more than ten in silico predicted highly conserved binding sites for different miRs in BDNF 3′UTR (www.targetscan.org, see below) and the physiological and pathological relevance of BDNF levels, to date only a few miRs have been studied in relation to BDNF. Although BDNF regulation by miR-206 has been well established in several studies [42, 47, 54], it has remained controversial which of the three miR-206 sites is functional [42, 47]. The potential effect of another miR-1/206 family member, miR-1, which differs from miR-206 by four nucleotides outside the seed region and has a distinct expression pattern from miR-206, has not been investigated to date. Other studies suggesting miR-BDNF 3′UTR interaction lack evidence that the effect of the putative BDNF-regulating miR on BDNF 3′UTR is direct [25, 46]. Most importantly, the effects of endogenous miRs on putative miR binding sites within BDNF 3′UTR have remained unaddressed altogether. Since 3′UTR length and secondary structure are likely to play an important role in miR-mediated target suppression [29, 58], the fact that all previous studies have exclusively analyzed BDNF 3′UTR fragments [25, 42, 46, 47] further stresses a need for analysis of BDNF 3′UTR-miR interaction in the context of native, full-length 3′UTR.

The objective of the current study was to analyze the regulation of both BDNF-L and BDNF-SH full-length 3′UTR isoforms by ten miRs selected based on evolutionary conservation of their seed sequences within BDNF and their expression within tissues or cell lines with known BDNF function [21, 40, 49, 51, 61, 63]. We assessed the effects of miR-1, miR-10b, miR-15a, miR-16, miR-30a, miR-30b, miR-155, miR-182, miR-191 and miR-195 on full-length BDNF-L and BDNF-SH isoforms and identified miR-1, miR-10b, miR-155, and miR-191 as novel direct regulators of BDNF 3′UTR. In addition, we provide data suggesting direct interaction between BDNF long 3′UTR and endogenous miR-1/206 family miRs in a model system of muscle differentiation, where the regulation of BDNF levels is known to be critical. Finally, our results indicate that the binding sites for miR-1 and miR-10 in BDNF 3′UTR are used synergistically by endogenous miRs in several cell lines.

Materials and methods

Plasmids and constructs

Full-length and short BDNF 3′UTR sequences were obtained from BAC clone RP24-149F11 (RPCI-24: Mouse (C57BL/6 J Male) (*Mus musculus*) BAC library; BACPAC Resources, Oakland, CA, USA) using primers in Online resource 1 and inserted downstream of Renilla luciferase gene in pGL4.73[*hRluc*/SV40] vector (E6911, Promega, Madison, WI, USA) using restriction with XbaI. Luciferase constructs containing BDNF 3′UTR with mutations in miR binding sites were generated with inverse PCR from the pGL4.73-BDNF3′UTR using primer pairs indicated in Online resource 1 (mutated nucleotides shown in bold). All constructs were verified by sequencing. Cotransfection with pGL4.13[*luc2*/SV40], encoding for firefly luciferase (E668A, Promega, Madison, WI, USA), was used for normalization in the dual luciferase assay.

Cell culture

Human embryonic kidney 293 (HEK-293) cells were cultured at 37 °C with 5 % CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (FBS; SV30160, Thermo Fisher Scientific, Waltham, MA, USA) and 1× Normocin (ant-nr-2, InvivoGen, San Diego, CA, USA).

Human retinal pigment epithelium 19 (ARPE-19, [17]) cells were cultured at 37 °C with 5 % CO_2 in DMEM/F-12 (1:1) medium containing L-glutamine and 15 mM HEPES (31330, Invitrogen/Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10 % FBS and 1× Normocin.

Human primary glioblastoma U-87 MG cells were cultured at 37 °C with 5 % CO_2 in DMEM supplemented with 10 % FBS and 1× Normocin. In all experiments, HEK-293, ARPE-19 and U-87 MG cells were split 1:2 on two consecutive days prior to seeding.

C2C12 mouse skeletal myoblast cells [65] were maintained at subconfluent densities at 37 °C with 5 % CO_2 in DMEM supplemented with 10 % FBS and 1× Normocin (growth medium, GM). Cells were kept at low density to prevent differentiation. Myogenic differentiation was

induced by replacing the growth medium with DMEM supplemented with 2 % horse serum (HS; B15-021, PAA/GE Healthcare Life Sciences, Helsinki, Finland) and 1× Normocin (differentiation medium, DM).

Luciferase assay

For the luciferase assay, cells were seeded to a 96-well plate at a density of 15,000–20,000 cells/well in a final volume of 100 µl. After 24 h of incubation, 80–90 % confluent cells were transfected with 50 µl Opti-MEM I Reduced Serum Medium, GlutaMAX (51985, Gibco/Thermo Fisher Scientific, Waltham, MA, USA) containing 0.3 µl Lipofectamine 2000 Transfection Reagent (11668, Invitrogen/Thermo Fisher Scientific, Waltham, MA, USA), 100 ng pGL4.73[*hRluc*/SV40] encoding for Renilla luciferase with BDNF 3′UTR (BDNF-L), BDNF short 3′UTR (BDNF-SH) or mutated BDNF 3′UTR cloned downstream of the luciferase coding sequence, 10 ng pGL4.13 encoding for firefly luciferase and 10 nM pre-miRs (where indicated, Applied Biosystems/Thermo Fisher Scientific, Waltham, MA, USA) per well. In experiments where miR binding site mutants were compared to each other, equimolar plasmid quantities were used. Cells were incubated for 3 h and transfection medium was replaced with 100 µl of fresh growth medium. Luciferase activity was measured after 24 h with Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) as recommended by the manufacturer. Briefly, cells were lysed with 35 µl of Passive Lysis Buffer per well. Culture plate was incubated for 15 min with shaking at 400 rpm at room temperature. To record luminescence, 100 µl of Luciferase Assay Reagent II was added to 30 µl of lysate for first measurement (firefly luciferase) and 100 µl of Stop & Glo Reagent was added for the second measurement (Renilla luciferase). Renilla/firefly luciferase ratio was used for statistical analysis. Three to four replicate wells were used in each experiment and experiments were repeated 3–5 times.

To assess the utilization of miR binding sites in the BDNF 3′UTR during myogenic differentiation, C2C12 cells were seeded to a 96-well plate at a density of 10,000–15,000 cells/well in a final volume of 100 µl. After 24 h of incubation, 40–50 % confluent cells were transfected with 50 µl OptiMEM containing 0.3 µl Lipofectamine, 100 ng pGL4.73[*hRluc*/SV40] encoding for Renilla luciferase with BDNF 3′UTR or mutated BDNF 3′UTR cloned after the luciferase coding sequence and 10 ng pGL4.13 encoding for firefly luciferase per well. Cells were incubated for 3 h and transfection medium was replaced with 100 µl of fresh medium, either GM (to keep cells growing as undifferentiated myoblasts) or DM (to induce differentiation to myotubes). Luciferase activity in myoblasts was measured after 8 h. DM was replaced every day and luciferase activity in myotubes was measured after 4 days, as described above. Renilla/firefly luciferase ratio was used for statistical analysis. Three to four replicate wells were used in each experiment.

Transfection with pre-miRs

To quantify endogenous BDNF protein levels after treatment with pre-miRs, ARPE-19 cells and U-87 MG cells were seeded to 6- or 12-well plates and incubated for 24 h to reach 90–100 % confluency. The medium was changed to GM and cells were transfected using OptiMEM containing 5 µl (2.5 µl for 12-well plate) Lipofectamine and 100 nM pre-miRs per well in a final volume of 1 ml (500 µl) for 4 h, then replaced with GM and cultured for 48 h. All experiments were performed with at least two biological repeats.

Silencing endogenous miRs with anti-miRs

To assess endogenous BDNF expression after silencing endogenous miRs, ARPE-19 and U-87 MG cells were seeded to 12-well plates at 90–100 % confluency. Medium was changed to 250 µl OptiMEM containing 6 µM Endoporter transfection agent, (GeneTools, LLC, Philomath, OR, USA) and 10 nM anti-miRs (Exicon, Vedbaek, Denmark) and cultured for 24 h before substituting with 500 µl GM. Cells were cultured for 48 h before mRNA and protein isolation. All experiments were performed with two biological repeats.

BDNF enzyme-linked immunosorbent assay (ELISA)

Culture medium from cells treated with pre- or anti-miRs was centrifuged at 1,000 rpm to remove debris and used immediately for ELISA or stored at −80 °C. Cells were lysed with lysis buffer containing 137 mM NaCl, 20 mM Tris–HCl (pH 8.0), 1 % NP40, 10 % glycerol, 1 mM PMSF, 10 µg/ml aprotinin, 1 µg/ml leupeptin and 0.5 mM sodium vanadate. Lysate was centrifuged for 20 min at 13,000 rpm at 4 °C and supernatant was used immediately or stored at −80 °C.

To assess BDNF expression in C2C12 myoblasts and myotubes during differentiation, C2C12 cells were seeded to a six-well plate in a density of 500,000 cells/well in 1 ml of GM. After 24 h, culture medium was isolated from undifferentiated myoblasts and replaced with DM to induce differentiation. DM was changed daily and culture medium from myotubes was isolated on day four. Culture medium was centrifuged at 1,000 rpm and used immediately for BDNF ELISA.

Brain-derived neurotrophic factor ELISA was performed using BDNF E_{max} ImmunoAssay System (Promega, Madison, WI, USA) as recommended by the manufacturer. Two replicates from each biological repeat were included in

ELISA. Brain-derived neurotrophic factor levels were normalized to total protein concentration using DC Protein Assay (500-0116, Bio-Rad, Helsinki, Finland), as recommended by the manufacturer.

RNA isolation

ARPE-19 and U-87 MG cells and hippocampi of C57BL/6 J mice were homogenized with TRI Reagent (TR 118, Molecular Research Center, Inc., Cincinnati, OH, USA) and RNA was isolated as recommended by the manufacturer. Briefly, after incubation of the homogenate at room temperature for 5 min, 1-bromo-3-chloropropane (BP151, Molecular Research Center, Inc., Cincinnati, OH, USA) was added and the tubes were shaken vigorously for 15 s. The mix was incubated at room temperature for 5 min and centrifuged at 12,000 × *g* for 15 min at 4 °C. The aqueous phase was transferred to a fresh tube containing isopropanol (59300, Sigma-Aldrich, St. Louis, MO, USA), mixed by vortexing and incubated at room temperature for 10 min. Samples were centrifuged at 12,000 × *g* for 8 min at 4 °C and RNA pellet was washed with 70 % ethanol (Altia Oyi, Helsinki, Finland), followed by centrifugation at 7,500 × *g* for 5 min. Ethanol was then removed and the RNA pellet was allowed to briefly air dry and then dissolved in 30–50 µl H_2O. The RNA samples were frozen immediately and stored at −80 °C until further processing. RNA quantity was measured with NanoDrop (Thermo Scientific, Waltham, MA, USA). The A260/A280 ratio was 1.78–2.01 and RNA yield was 3.5–9.5 µg (18–40 µg for hippocampus RNA).

Reverse transcription

RNA samples were treated with Turbo DNA-free DNase treatment and removal reagents, as recommended by the manufacturer (AM1907, Invitrogen/Thermo Fisher Scientific, Waltham, MA, USA), to prevent contamination with genomic DNA. cDNA was synthesized from 150–500 ng of RNA (equal amount of RNA was used within a single experiment) with random hexamer primers in a final volume of 20 µl using Transcriptor First Strand cDNA synthesis kit as recommended by the manufacturer (04896866001, Roche, Basel, Switzerland). Briefly, 2 µl of random hexamer primers was mixed with 11 µl of RNA sample diluted with nuclease-free water, and incubated at 65 °C for 10 min. Then 7 µl of mix containing 4 µl of 5× RT buffer, 2 µl of 100 mM dNTP, 0.5 µl of RNase inhibitor and 0.5 µl of Transcriptor reverse transcriptase was added, mixed gently, and incubated at 25 °C for 10 min, 55 °C for 30 min, and 85 °C for 5 min. No reverse transcriptase control was included in each experiment. cDNA was cooled on ice, diluted 1:10, and stored at −20 °C or used immediately for qPCR.

Quantitative real-time PCR

Quantitative PCR reaction was performed with the LightCycler 480 real-time PCR system (Roche Diagnostics, Basel, Switzerland) using LightCycler 480 SYBR Green I Master, complemented with 2.5 pmol of primers in the final volume of 10 µl on white 384-well plates sealed with adhesive plate sealer (04729749001, Roche, Basel, Switzerland). An amount of 2.5 µl of the diluted cDNA product was used in each reaction. Oligonucleotide primers (Oligomer Oy, Helsinki, Finland) used for the qPCR reactions are indicated in Online resource 1. No-reverse transcription control and no-template control were included for each experiment. Two or three replicates of each reaction were included in the qPCR runs. The following qPCR program was used: [1] pre-incubation 10 min at 95 °C, [2] amplification 10 s at 95 °C, 15 s at 60 °C, 15 s at 72 °C for 45 cycles, [3] melting curve 5 s at 95 °C, 30 s at 55 °C, continuous acquisition mode at 95 °C with two acquisitions per degree Celsius, and [4] cooling 10 s at 40 °C. The results were analyzed with LightCycler 480 Software Release 1.5.0 SP1 using the Absolute Quantification/2nd Derivative Max calculation. The quantification cycle (C_q) for the no-template control was 40 (or 0) in all experiments. Beta-actin was used as a reference gene. Results for a biological repeat were discarded when the C_q value for one or more of the replicates was 40 (or 0) or when the C_q difference between replicates was >1. For each primer pair, primer efficiencies (the ratio of amplified products if average C_q difference is one; the ideal efficiency would be two) were determined (Online resource 1). Fold difference to the reference gene was calculated according to the following formulation: $\mathrm{FD} = (E_{(\mathrm{GOI})}^{-Cq1(\mathrm{GOI})} + E_{(\mathrm{GOI})}^{-Cq2(\mathrm{GOI})})/(E^{-Cq1(\mathrm{ref})}_{(\mathrm{ref})} + E^{-Cq2(\mathrm{ref})}_{(\mathrm{ref})})$, where E_{GOI} and E_{ref} are the primer efficiencies of the gene of interest (GOI) and reference gene (ref), respectively, and C_{q1} and C_{q2} are the C_q values of individual replicates.

microRNA expression

MicroRNA expression was assessed using TaqMan MicroRNA Assay reactions (Applied Biosystems/Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's recommendations with minor modifications. Briefly, RNA was isolated as described above. cDNA from 0.3–1 µg RNA was synthesized with TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems/Thermo Fisher Scientific, Waltham, MA, USA) using Megaplex RT Primers, Rodent Pool A or B (Applied Biosystems/Thermo Fisher Scientific, Waltham, MA, USA) without preamplification in a final volume of 7.5 µl. With the exception of miR-155 and miR-182, all the primers in the MegaPlex Rodent Pool were suitable for the amplification of human microRNAs. For miR-155

and miR-182, 5x RT primers provided by the TaqMan MicroRNA Assay were used for reverse transcription reaction. The cDNA product was diluted 1:30 and 2.5 µl of the diluted cDNA was used for each real-time PCR reaction in a final volume of 10 µl in 384-well plates. Each sample was run in duplicate. microRNA expression from mouse-derived cells was normalized to sno202 and microRNA expression from human-derived cells was normalized to miR-191 [52].

Statistical analysis

All values are presented as mean ± SEM. Statistical significance level was set at $p < 0.05$. Quantitative PCR data was calculated based on primer efficiencies and analyzed using fold difference compared to reference gene. Statistical analysis was performed using paired or unpaired Student's *t* test, Mann–Whitney *U* test, or one-way ANOVA followed by Tukey's HSD (honestly significant difference) or Games–Howell post hoc analysis.

Results

Both BDNF 3′UTR isoforms are predicted to contain conserved binding sites for multiple miRs

We performed in silico analysis of BDNF 3′UTR-miR interactions using publicly available bioinformatics tool TargetScan (www.targetscan.org) and found that the 3′UTR of BDNF contains evolutionarily conserved seed sequences for multiple miRs (Fig. 1). In addition to miR sites, the overall nucleotide sequence of BDNF 3′UTR is highly conserved across species, especially near both ends of the 3′UTR (Fig. 1), suggesting important biological function of these regions. Most of the predicted miR binding sites fall into the 5′ end of the 3′UTR and the nucleotide sequences near miR sites are highly conserved across vertebrates (Online resource 2). Brain-derived neurotrophic factor transcripts have either a long (BDNF-L, 2891 nt) or short (BDNF-SH, 350 nt) 3′UTR ([27], Fig. 1). Although in silico analysis with different target prediction tools, including TargetScan ([43], http://www.targetscan.org), PITA ([34], http://genie.weizmann.ac.il/pubs/mir07/index.html), miRanda ([33], http://www.microrna.org), PicTar ([38], http://pictar.mdc-berlin.de/) implies that BDNF 3′UTR can be regulated by a multitude of miRs (Online resource 3), to date only a few BDNF 3′UTR-miR interactions have been investigated, and in these, relatively short BDNF 3′UTR fragments have been used [25, 42, 46, 47]. Since there is potential for several miRs to bind BDNF 3′UTR, we set out to systematically investigate the role of ten miRs with conserved putative binding sites in BDNF 3′UTR isoforms in the regulation of BDNF expression. miRs of interest were selected based on the conservation of their binding site within BDNF 3′UTR, co-expression with BDNF in cells and tissues where BDNF is known to have important roles, and/or their overall expression level (Online resource 2, microRNA.org, [20, 22, 31, 40]). We used full-length BDNF-L and BDNF-SH sequences to better preserve the potential effects that secondary structure and nucleotide context may have on miR binding.

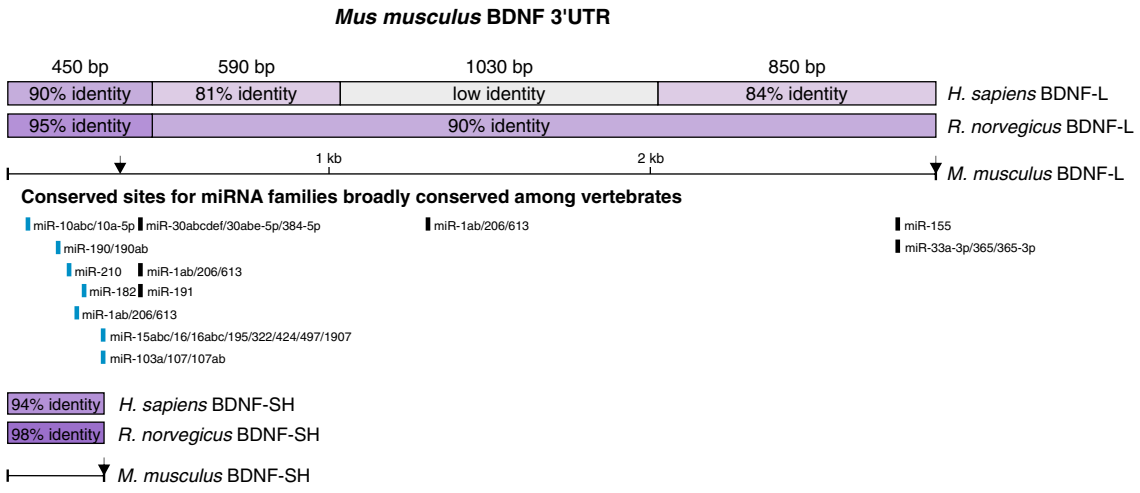
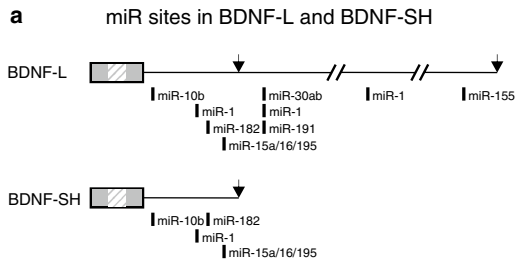


Fig. 1 Scheme of long (BDNF-L) and short (BDNF-SH) BDNF 3′UTR isoforms with evolutionarily conserved miR sites predicted by TargetScan. Sites within BDNF-SH are shown in *light blue*. Conservation between mouse, rat, and human BDNF 3′UTR is shown in *purple bars*. *Arrows* denote polyadenylation sites



c Number of miR sites

microRNA	BDNF-L	BDNF-SH
miR-9	0	0
miR-1	**3**	**1**
miR-10b	1	1
miR-15a	1	1
miR-16	1	1
miR-30a	**1**	**0**
miR-30b	**1**	**0**
miR-155	**1**	**0**
miR-182	1	1
miR-191	**1**	**0**
miR-195	1	1

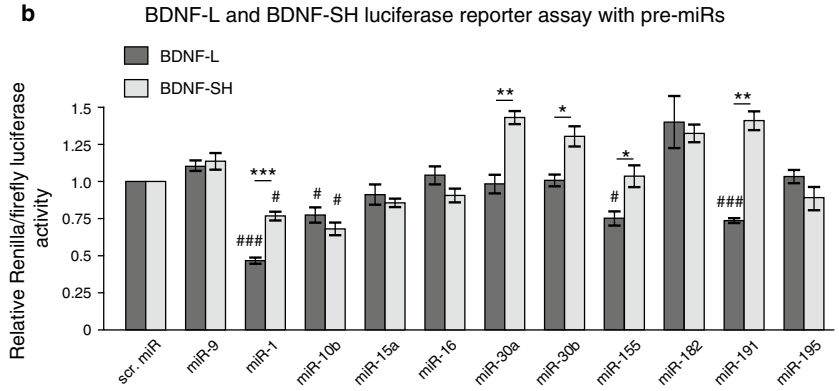


Fig. 2 Several of the predicted miRs regulate BDNF 3′UTR. **a** Scheme of miR sites within BDNF-L and BDNF-SH. Only miRs included in the current study are shown. *Arrows* denote polyadenylation sites. **b** Luciferase activity of reporter constructs containing either BDNF-L or BDNF-SH, co-transfected with pre-miRs. *Dark grey bars* show inhibition of BDNF-L by miRs and *light grey bars* show inhibition of BDNF-SH by miRs. # indicates difference from treatment with negative control and * indicates differences between the extent of miR inhibition between BDNF-L and BDNF-SH. $n = 3–8$, #$p < 0.05$, ###$p < 0.001$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. **c** Number of miR sites in BDNF-L and BDNF-SH predicted by TargetScan. miRs that have a different number of predicted binding sites within BDNF-L and BDNF-SH are shown in *bold*

Analysis of BDNF 3′UTR isoforms for miR regulation

We asked whether the in silico predicted miRs (Fig. 1) regulate protein synthesis from a transcript containing full-length BDNF-L. For that, we transfected human embryonic kidney 293 (HEK-293) cells with precursors for ten miRs predicted by four different target prediction tools (Online resource 3) to bind BDNF-L (miR-1, miR-10b, miR-15a, miR-16, miR-30a, miR-30b, miR-155, miR-182, miR-191, and miR-195, Fig. 2a). Scrambled pre-miR (scr. miR) and precursor for miR-9 that do not have a strongly conserved predicted binding site in BDNF-L were used as negative controls. We found that miR-1, which has three binding sites in BDNF-L (Fig. 2a), reduces luciferase signal most efficiently (Fig. 2b). Of the other miRs, miR-10b, miR-155, and miR-191 significantly inhibited luciferase signal compared to the scrambled miR control. Unlike in previous studies, which utilized fragments of BDNF 3′UTR in the same reporter assay, we found no significant effect for miR-30a or miR-195 (Fig. 2b).

In parallel, we tested the ability of the same miRs to inhibit protein synthesis from transcript containing BDNF-SH, which contains binding sites for six miRs out of the ten assessed in BDNF-L (Fig. 2a, c). We found that miR-1 and miR-10b significantly reduced luciferase activity. Of the three miR-1 sites present in BDNF-L, only the first is located within BDNF-SH (Fig. 2a, c). If miR-1 sites act additively to regulate BDNF levels, miR-1 is expected to have a milder effect on a reporter construct carrying BDNF-SH compared to BDNF-L. Matching the prediction, we found a statistically significant difference in the extent of luciferase signal reduction by miR-1 on BDNF-SH compared to BDNF-L (Fig. 2b). Similarly, unlike BDNF-L, BDNF-SH lacks binding sites for miR-155 and miR-191.

As expected, miR-155 and miR-191 had no effect on BDNF-SH, while they had a significant effect on BDNF-L (Fig. 2b). Thus, we found a statistically significant difference in the extent of luciferase signal reduction between BDNF-L and BDNF-SH by miR-1, miR-155 and miR-191 that have a different number of predicted binding sites in the long and short BDNF 3′UTR isoforms. In contrast, there was no difference in luciferase activity after treatment with miR-10b that has a single binding site predicted to regulate both BDNF-L and BDNF-SH (Fig. 2a–c). These data suggest that miR-1, miR-10b, miR-155, and miR-191 are novel regulators of BDNF 3′UTR and that a subset of them are able to differentially regulate expression from transcripts containing either short or long 3′UTR isoform.

Regulation of endogenous BDNF levels by miRs

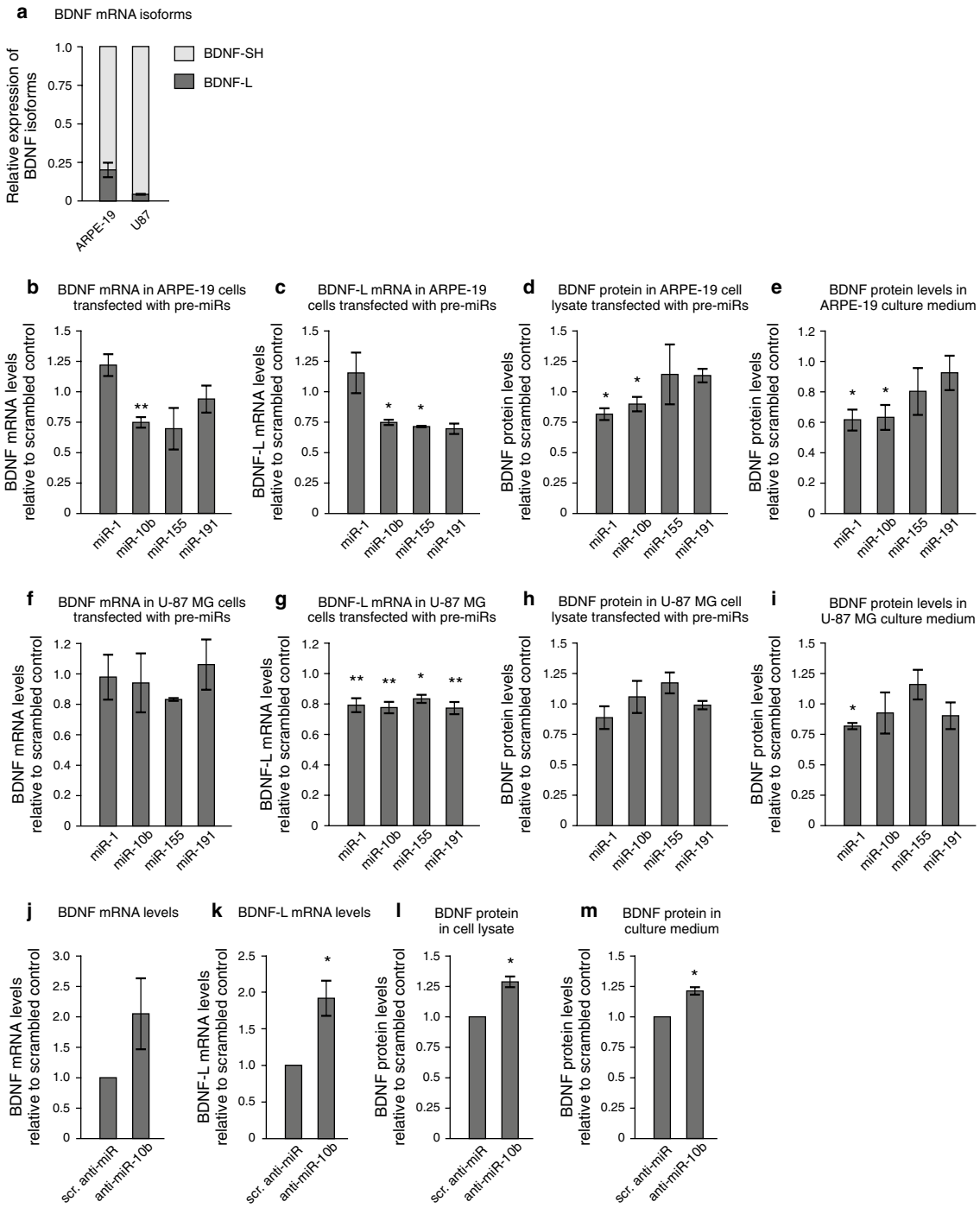
Next, we asked if the miRs that regulate BDNF 3′UTR in a reporter assay can also regulate endogenous BDNF. Towards that end, we made use of human retinal pigment epithelial (ARPE-19) cells and human glioblastoma cell-line U-87 MG that secrete a detectable amount of endogenous BDNF. Relative expression of BDNF and BDNF-regulated miRs in different cell lines used in this study is shown in Online resource 4a and 5. We found that approximately 80 % of BDNF mRNA transcripts in ARPE-19 cells and 95 % of BDNF transcripts in U-87 MG cells are expressed as short 3′UTR isoforms (Fig. 3a), suggesting that miR-155 and miR-191, which only have a binding site within BDNF-L, should be unable to regulate the majority of BDNF transcripts in these cells (Fig. 2a). We transfected ARPE-19 cells with precursors for miR-1, miR-10b, miR-155, and miR-191, and measured BDNF mRNA levels with primers recognizing total BDNF and BDNF transcript carrying the long 3′UTR (BDNF-L). miR-10b significantly decreased both total BDNF and BDNF-L levels, while miR-155 and miR-191 only reduced BDNF-L expression, as expected (Fig. 3b–c). We then determined BDNF protein levels in the cell lysate and culture medium by ELISA. We found that miR-1 and miR-10b effectively suppressed endogenous BDNF synthesis, while miR-155 and miR-191 did not significantly reduce BDNF levels (Fig. 3d–e). Brain-derived neurotrophic factor levels in the culture medium were decreased more compared to the whole cell lysate, indicating that in addition to BDNF, miR-1 and miR-10b could regulate the expression of proteins involved in BDNF secretion. Surprisingly, although BDNF mRNA levels were increased by transfection with miR-1, protein levels were decreased (Fig. 3b–e), suggesting that the mechanism by which miR-1 suppresses BDNF may be different from that of other miRs and may involve other factors that regulate BDNF expression. Taken together, these data suggest that miR-1 and miR-10b inhibit endogenous BDNF levels but do not exclude the ability of miR-155 and miR-191 to do the same in cells expressing BDNF transcript with the long 3′UTR isoform.

Since BDNF has multiple functions in the central nervous system, we also tested pre-miRs in U-87 MG glioblastoma cells that originate from the CNS. However, only 5 % of BDNF transcripts in these cells carry the long 3′UTR isoform (Fig. 3a). We found that while total BDNF levels were not reduced after treatment with any of the pre-miRs, the expression of BDNF transcripts carrying the long 3′UTR was significantly decreased by about 20 % by all the pre-miRs tested (Fig. 3f–g). Interestingly, although miR-1 and miR-10b have a binding site within BDNF-SH and should therefore be able to regulate the expression of all BDNF transcripts, they seem to be effective only in suppressing BDNF-L in U-87 MG cells (Fig. 3f–g). Consistent with our finding that miRs only regulate a marginal subpopulation of BDNF transcripts, resulting in about 20 % decrease in BDNF-L levels, BDNF protein levels in U-87 MG cells were not suppressed by transfection with pre-miRs (Fig. 3h–i).

Finally, we transfected ARPE-19 cells with anti-miR-10 and found that silencing endogenous miR-10b significantly increased BDNF mRNA and protein levels (Fig. 3j–m). We were unable to address the effect of miR-1 in ARPE-19 cells, since it is not expressed in this cell line (Online resource 5). In summary, our results suggest that miR-1, miR-10b, miR-155, and miR-191 are able to regulate BDNF expression, although the effect may vary depending on the cell line.

miR-1, miR-10b, miR-155, and miR-191 regulate BDNF 3′UTR directly through their predicted sites

Quantifying endogenous BDNF expression after treatment with pre- or anti-miRs does not allow to distinguish whether miRs regulate BDNF levels directly or indirectly. To investigate the interaction between the miRs identified to suppress BDNF-L (Fig. 2b) and their concomitant binding sites within the BDNF 3′UTR, we created constructs harboring mutations in one or several miR binding sites in BDNF-L (Fig. 4a–b). Since miR-1 has three predicted sites, we generated mutant constructs for each of the sites individually (miR1-1m, miR1-2/191m, miR1-3m) and a triple mutant with all three binding sites mutated (miR1m). It should be noted that the second binding site of miR-1 (miR-1-2) partly overlaps with miR-191 binding site and therefore both sites were mutated in miR-1-2 mutant (Fig. 4a–b). We co-transfected mutant constructs with the corresponding pre-miRs into HEK-293 cells as above and assessed the relative extent of inhibition. In accordance with analysis of BDNF-L vs. BDNF-SH (Fig. 2b), mutating the first site (miR1-1m), which is also present within BDNF-SH, reduced



suppression by miR-1 most effectively compared to mutating the other miR-1 sites (Fig. 4c), suggesting that miR-1-1 site is more important in regulating BDNF 3′UTR than miR-1-2 and miR-1-3 sites. Furthermore, mutating the third miR-1 site (miR1-3m) did not change 3′UTR inhibition by exogenous miR-1 (Fig. 4c), suggesting that this site may not

◀**Fig. 3** Regulation of endogenous BDNF levels by pre- and anti-miRs. **a** Expression of BDNF-L and BDNF-SH mRNA isoforms in ARPE-19 and U-87 MG cells. Endogenous BDNF levels in ARPE-19 (**b–e**) and U-87 MG (**f–i**) cells transfected with pre-miRs, relative to scrambled control. **j–m** Endogenous BDNF levels in ARPE-19 cells transfected with anti-miRs, relative to scrambled control. **b, f, j** Total BDNF mRNA levels, normalized to β-actin. **c, g, k** mRNA levels of BDNF transcripts containing the long 3′UTR (BDNF-L), normalized to β-actin. **d, h, l** BDNF protein levels in whole cell lysate, normalized to total protein content. **e, i, m** BDNF protein levels in culture medium, normalized to total protein content. $n = 3$. *Asterisks* show difference from negative control, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. *Error bars* denote mean ± SEM

be used by miR-1 for suppression of BDNF 3′UTR. Mutating miR-10b (miR10m), miR-155 (miR155m), and miR-191 (miR1-2/191m) sites effectively abolished the inhibitory effect of the corresponding exogenous miRs (Fig. 4d), suggesting that these miRs also directly inhibit protein synthesis by binding to the predicted site in the BDNF 3′UTR.

miR-1/206 sites in BDNF 3′UTR are used by endogenous miRs in differentiated but not in undifferentiated muscle cells

Regulation of BDNF expression has been shown to be required for postnatal growth and repair of skeletal muscle in vivo [13]. To gain further insight into the physiological relevance of miR sites identified to regulate BDNF 3′UTR, we extended our analysis to a model of muscle differentiation, the mouse myoblast C2C12 cells, where reduction in BDNF levels is believed to be required to allow myotube generation from undifferentiated myoblasts [47]. miR-206, a miR-1/206 microRNA family member that has identical seed sequence to miR-1 but differs from miR-1 by four nucleotides (Online resource 4b), is believed to be involved in BDNF downregulation in C2C12 cells [47]. However, it is unknown whether the predicted miR-1/206 sites are the sites used by endogenous miR-1 and/or miR-206 during muscle differentiation. Furthermore, it has remained unclear if BDNF suppression by miR-1/206 is used in myoblasts to induce differentiation, in myotubes to maintain differentiation, or both. To gain further insight into these questions, we first confirmed earlier findings that BDNF is downregulated with concurrent upregulation of miR-1 and miR-206 upon myogenic differentiation of muscle cells (Online resource 4c–e; [12, 35, 47, 49]). Then we analyzed the effect of miR-1/206 site mutations on reporter construct expression in myoblasts and myotubes. We found that BDNF-L was repressed in differentiated myotubes by about 50 %, while miR1m mutant construct was not (Fig. 5a). Furthermore, although both miR-1 and miR-206 are expressed in myoblasts (Online resource 4e, 5), there was no difference in the expression of BDNF-L and miR1m containing reporters in those cells (Fig. 5a). This suggests that interaction between BDNF 3′UTR and miR-1/206 family miRs is utilized in C2C12 myotubes to maintain differentiation by BDNF repression but not to induce myogenic differentiation.

Endogenous miRs regulate BDNF 3′UTR in different cell lines through the predicted sites

Next we turned to cell lines where interaction between BDNF 3′UTR and endogenous miRs has not been addressed before. We determined the endogenous miR expression levels in human embryonic kidney cell line HEK-293 and human retinal pigment epithelial cell line ARPE-19. We found that miR-1 was not expressed in ARPE-19 cells and miR-155 was not expressed in HEK-293 cells, while the other miRs were expressed but their relative levels were different between HEK-293 and ARPE-19 cells (Online resource 5). To assess the interaction between BDNF 3′UTR and endogenous miRs, we transfected HEK-293 and ARPE-19 cells with luciferase assay reporter containing native BDNF-L or constructs harboring various miR site mutants: mutations for each individual miR-1 site (miR1-1m, miR1-2/191m, miR1-3m), all three miR-1 binding sites (miR1m), miR-10b site (miR10m), miR-155 site (miR155m) and a quadruple mutant for three miR-1 sites and miR-10b site (miR1,10m). As indicated above, miR-191 site was mutated in miR1-2/191m mutant construct (Fig. 4b). We found that mutating miR-10b site significantly derepressed luciferase construct in both cell lines (Fig. 5b–c). Consistent with our findings with miR-1 transfection (Fig. 4c), we found that mutating the miR-1-1 site, but not the other miR-1 sites increased luciferase signal in HEK-293 cells (Fig. 5c). In ARPE-19 cells, luciferase signal obtained with the quadruple mutant miR1,10m was increased by approximately two-fold compared to wild-type BDNF-L. However, in HEK-293 cells, mutating all three sites for miR-1 and the single miR-10b site (miR1,10m) derepressed luciferase activity to the level comparable to the empty luciferase vector carrying SV40 late 3′UTR (Fig. 5c), which is in essence devoid of binding sites for strongly conserved miRs (Online resource 6). These results indicate that miR-1 and miR-10 sites within BDNF 3′UTR are used synergistically by endogenous miR-1/206 and miR-10 family miRs to repress BDNF 3′UTR, and at least in HEK-293 cells miR-1/206 and miR-10 sites are responsible for the majority of BDNF 3′UTR repression.

Discussion

Here we present the first systematic functional analysis of nine evolutionarily conserved miR recognition sites in BDNF 3′UTR. Unlike previous studies, we have analyzed full-length sequences of both BDNF 3′UTR isoforms, BDNF-L (2891 nt) and BDNF-SH (350 nt). We identify

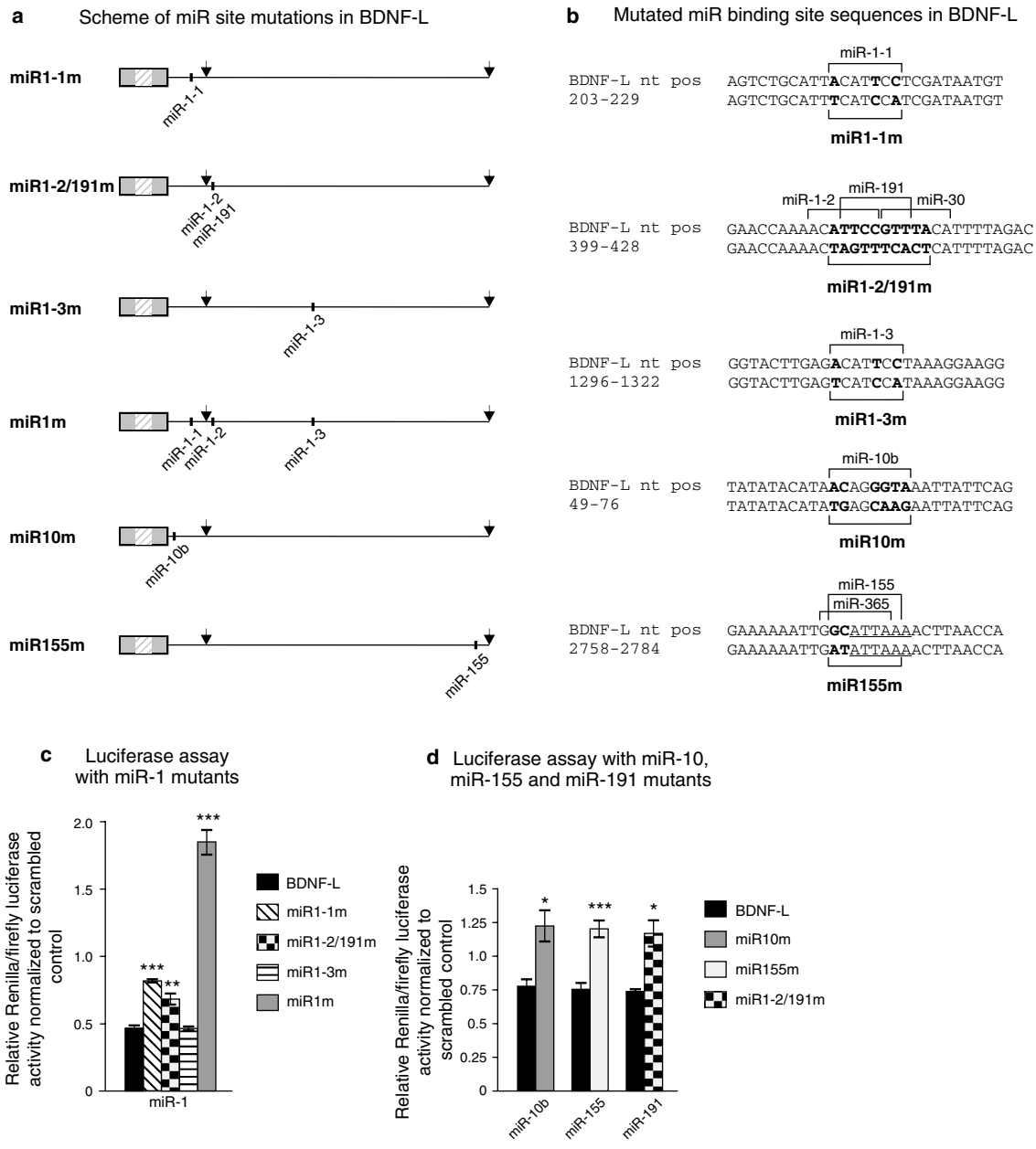


Fig. 4 Regulation of luciferase signal by miRs is specific to the predicted miR binding sites. **a** Schemes of miR site mutations in BDNF-L. Mutated sites are shown for each BDNF-L mutant. *Arrows* denote polyadenylation sites. **b** miR binding site mutations in BDNF-L. BDNF 3′UTR sequences complementary to miR seed sites are indicated with *brackets*. Mutated nucleotides are shown in *bold*. **c** Mutating miR-1 predicted binding sites in BDNF-L prevents repression by miR-1 in a luciferase reporter assay in HEK-293 cells. $n = 3$. **d** Mutating miR-10b, miR-155, and miR-191 predicted binding sites prevents repression by the respective miRs in a luciferase reporter assay in HEK-293 cells. $n = 3$. **c, d** Reporter expression is shown relative to scrambled pre-miR. *Error bars* denote mean ± SEM. *Asterisks* show difference from BDNF-L; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

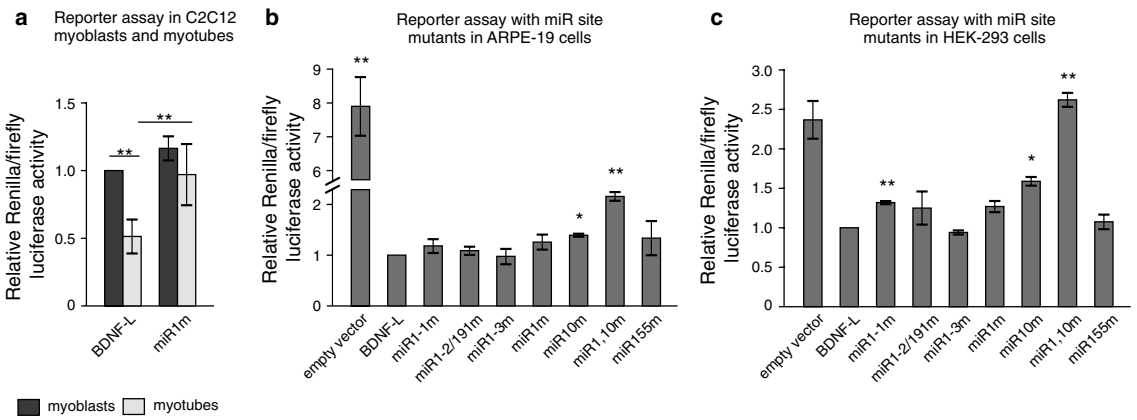


Fig. 5 Mutating miR-1 and miR-10 sites abolishes suppression of BDNF-L by endogenous miRs in a reporter assay. **a** Reporter activity in C2C12 cells before and after differentiation with constructs containing either wild-type BDNF-L or BDNF-L miR site mutants. $n = 7$. **b, c** Luciferase activity of reporter constructs containing wild-type BDNF-L or BDNF-L miR site mutants. *Asterisks* show difference from BDNF-L. $n = 3$–4. **b** Luciferase activity in ARPE-19 cells. **c** Luciferase activity in HEK-293 cells. $n = 3$–5. *Error bars* denote mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

miR-1, miR-10b, miR-155, and miR-191 as novel regulators of BDNF 3′UTR and show that BDNF-L and BDNF-SH can be differentially regulated by a subset of miRs. Using mutated constructs we show that the interaction between the identified miRs and BDNF 3′UTR is direct. Our results suggest that miR-1/206 sites within BDNF 3′UTR are used in C2C12 myotubes to maintain differentiation rather than in myoblasts to induce differentiation. Finally, we show that the predicted binding sites for miR-1/206 and miR-10 family miRs are used in a synergistic manner to repress BDNF 3′UTR in ARPE-19 and HEK-293 cells.

The potential physiological importance of BDNF regulation by miRs has been suggested by a study where Dicer, an enzyme required for miR maturation, was conditionally deleted from the forebrain. Prior to the onset of neurodegeneration, BDNF levels in such animals were increased with concomitant functional changes in the CNS [36]. Recently, the conclusion was confirmed by Lee et al., who demonstrated BDNF regulation by miR-206 in the brain [42]. In addition, other studies have addressed the potential interaction between BDNF 3′UTR and specific miRs. Interestingly, miR-30a and miR-195 did not have an effect in our study (Fig. 2b) while previous results have suggested that these miRs regulate BDNF 3′UTR in a reporter assay [46]. These different results can at least partly be explained by the use of 3′UTR fragments in the reporter assay of the previous study, which may affect the accessibility of miRs to their binding site in the target mRNA. It has been shown that miR sites residing near the two ends of long 3′UTRs are generally more effective than those near the center [29]. Thus, a site normally unavailable in the native 3′UTR may become more accessible if only a small region of the 3′UTR is analyzed in reporter assay and in some cases, the situation may be the other way around. miR-191 has been shown not to inhibit BDNF 3′UTR [46], whereas our results clearly show that miR-191 does in fact directly inhibit BDNF 3′UTR via the predicted site (Fig. 2b, 4d).

Further illustrating the potential problems stemming from analysis of fragments rather than full-length 3′UTRs are the conflicting results of studies by Miura et al. and Lee et al. [42, 47], where the first study showed that of three miR-206 sites within BDNF 3′UTR, the first two and not the third are functional, while the other study reached the opposite conclusion. Although both studies analyzed fragments of BDNF 3′UTR, the size of fragments used in [47] was longer than those studied in [42] and thus resembled the full-length BDNF 3′UTR better. Our analysis of full-length BDNF 3′UTR interaction with miR-1, another family member of miR-1/206 family, supports the conclusion of (Fig. 2b, 4c, [47]). Our data on miR-1 also confirms the in silico prediction that miR-1 and miR-206 share the binding sites on BDNF 3′UTR (Fig. 1). However, it is important to keep in mind that miR-1 and miR-206 differ by four nucleotides outside the seed sequence (Online resource 4b). Therefore, it would be interesting to analyze miR-206 and BDNF 3′UTR interaction in the context of full-length 3′UTR.

We also found that endogenous miR-1/206 and miR-10 family miRs act cooperatively to repress BDNF expression through its 3′UTR in two cell lines of different origin (Fig. 5b–c). This is consistent with the notion from previous reports that multiple miR sites within a 3′UTR can act in a synergistic manner, especially if the sites are located

close together [29, 56, 57]. In line with this, recent experimental evidence shows that noncoding pseudogenes can bind to and compete for the same combination of miRs as their ancestral gene [53], suggesting that there has been evolutionary pressure on pseudogenes to preserve the same miR binding site pattern or "miR code" that is used to regulate protein synthesis from the ancestral transcript. Even though the expression levels of pseudogenes are low, they seem to be sufficient to derepress endogenous parent gene expression in case their 3′UTR contains binding sites for multiple miRs that act in synergy. Our data imply that regulation of BDNF by its 3′UTR is important in tissues where miR-1/206 and miR-10 family miRs are co-expressed. In addition, although mutating miR-155 and miR-191 sites had no effect on reporter activity in HEK-293 and ARPE-19 cells, it cannot be excluded that BDNF 3′UTR interacts with miR-155 or miR-191 in other tissues or cell types.

Although some studies have suggested that inhibition by miRs depends mostly on miR expression levels [18, 28], others suggest that additional factors such as specific nucleotide composition [53, 57], or the number of target genes [4] may be more important in defining the repressive activity of miRs. Our results indicate that endogenous miR expression levels do not solely determine the inhibition efficiency. In assays utilizing reporter constructs and pre-miR transient transfection, miR-1 through its sites 1 and 2, miR-10b and miR-191 all suppress BDNF 3′UTR (Fig. 2b, 4c–d). However, despite the presence of relatively high levels of endogenous miR-191 compared to endogenous miR-1 and miR-10b in both HEK-293 and ARPE-19 cells (Online resource 5), mutating miR-191 site did not lead to an increase in luciferase signal, whereas mutating miR-10b site or miR-1 sites together with miR-10b site had a clear effect (Fig. 5b–c). Thus, results suggesting direct interaction between miR and 3′UTR in transient transfection assays do not allow conclusions on the interaction between endogenous miR and the corresponding site within the 3′UTR, even if the endogenous miR expression levels are high, underlining the need to test each case separately.

While the role of miR-1/206 family member miR-206 in the regulation of BDNF levels is well established [42, 47, 54], the interaction between BDNF 3′UTR and miR-1 has not been investigated. Although miR-1 and miR-206 have identical seed sequences, the mature miR-1 differs from miR-206 by four nucleotides (Online resource 4b). miR-1 is encoded by two distinct genomic locations in both mouse and human genomes and displays partially different expression pattern from miR-206. For example, both miR-1 and miR-206 are expressed in high levels in the skeletal muscle [12, 35, 39, 59, 68], but unlike miR-206, miR-1 levels are high in the heart [40]. Since BDNF is known to be required for heart angiogenesis [16], it would be interesting to assess the role of miR-1 and BDNF interaction in heart development. Moreover, appropriate BDNF levels are important for adjusting the number of dopaminergic neurons in the substantia nigra midbrain [5] and regulation of BDNF expression in the hippocampus is important in learning and memory (see [64]). miR-1 is expressed within the midbrain [40], hippocampus (Online resource 5), and in several other neuron populations within the central nervous system [30]. Taken together, there is potential for miR-1-mediated regulation of BDNF levels during development, warranting further investigation.

While miR-191 is widely expressed in different tissues and cell types, miR-10b and miR-155 have a more restricted expression pattern [40]. For example, one of the few expression sites of miR-10b is the skin [40], whereas expression of BDNF in the skin is required for its proper sensory innervation [21]. miR-155, on the other hand, is robustly upregulated after the activation of immune cells, including microglia [9], whereas BDNF expression by microglia has been implicated in the induction of neuropathic pain [14, 66].

Our results also suggest that selected miRs are able to differentially regulate BDNF transcripts with either long or short 3′UTR isoform. Indeed, the considerably shorter half-life of BDNF mRNAs containing the long 3′UTR compared to the short 3′UTR [10] may at least partly be a consequence of miR regulation. Furthermore, the relative abundance of BDNF 3′UTR isoforms varies during developmental processes, for example, in muscle differentiation [47], and in different brain areas, such as the cortex, hippocampus and cerebellum [3, 62], suggesting that the long and short 3′UTR isoforms have different biological functions. In line with this, it was recently shown that in response to neuronal activation, BDNF protein synthesis in hippocampal neurons is rapidly initiated from transcripts containing the long 3′UTR, while expression from transcripts containing the short 3′UTR maintains basal BDNF levels [41].

Overall, co-expression of BDNF and miR-1/miR-10b/miR-155/miR-191 in various cell types and tissues suggests potential relevance of the interaction between miRs and BDNF 3′UTR isoforms in different physiological and pathological processes, which remain a target of future studies addressing post-transcriptional BDNF regulation.

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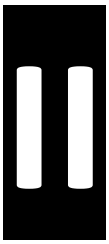

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Tristetraprolin is a novel regulator of BDNF

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Abstract

Brain-derived neurotrophic factor (BDNF) regulates multiple biological processes ranging from central nervous system development and function to neuroinflammation and myogenic differentiation and repair. While coordination of BDNF levels is central in determining the biological outcome, mechanisms involved in controlling BDNF levels are not fully understood. Here we find that both short (BDNF-S) and long (BDNF-L) BDNF 3'UTR isoforms contain conserved adenylate- and uridylate rich elements (AREs) that may serve as binding sites for RNA-binding proteins (ARE-BPs). We demonstrate that ARE-BPs tristetraprolin (TTP) and its family members butyrate response factor 1 (BRF1) and 2 (BRF2) negatively regulate expression from both BDNF-S and BDNF-L containing transcripts in several cell-lines and that interaction between TTP and AU-rich region in proximal 5' end of BDNF 3'UTR is direct. In line with the above, endogenous BDNF mRNA co-immunoprecipitates with endogenous TTP in differentiated mouse myoblast C2C12 cells and TTP overexpression destabilizes BDNF-S containing transcript. Finally, RNAi-mediated knock-down of TTP increases the levels of endogenous BDNF protein in C2C12 cells. Our findings uncover TTP as a novel regulator of BDNF assisting future studies in different physiological and pathological contexts.

Keywords: Brain-derived neurotrophic factor; 3' untranslated region; Tristetraprolin; C2C12 cells

Introduction

BDNF is involved in a wide range of developmental, functional and pathological processes in the central nervous system (CNS) (Nagahara and Tuszynski 2011; Kirschenbaum and Goldman 1995; Cohen-Cory et al. 2010; Bamji et al. 2006; Nieto et al. 2013; Mu et al. 1999). Outside the CNS, processes regulated by BDNF include inflammation (Uchida et al. 2013; Lin et al. 2011; Gomes et al. 2013; Amoureux et al. 2008; Luhder et al. 2013), development of neuromuscular junctions (Je et al. 2012), muscle regeneration after injury (Clow and Jasmin 2010) and myogenic differentiation (Mousavi and Jasmin 2006).

Precise regulation of BDNF levels is critical in determining the biological outcome. Reduction of BDNF levels by 50% in BDNF knock-out heterozygous mice is associated with a range of phenotypes in the CNS (Lyons et al. 1999; Dluzen et al. 2002; Abidin et al. 2006; Abidin et al. 2008). On the other hand, a 2-fold elevation in endogenous BDNF by the suppression of miR-206, a direct negative regulator of BDNF levels, alleviates disease phenotype in a mouse model of Alzheimer's disease (Lee et al. 2012). In muscle tissue, about 50% reduction in BDNF levels is believed to be required to allow myogenic differentiation (Mousavi and Jasmin 2006). However, despite the biological and potential clinical relevance, mechanisms controlling BDNF levels are not fully understood.

As a result of alternative polyadenylation, BDNF transcripts have either a long (BDNF-L, 2891 nt) or short (BDNF-S, 350 nt) 3'UTR (Timmusk et al. 1993). The ratio between transcripts containing BDNF-L and BDNF-S varies with BDNF-L levels ranging between 20-50% in different tissues and cells-lines (Timmusk et al. 1994; Miura et al. 2012). Alternative 3'UTR isoforms allow cells to differentially regulate expression from transcripts containing long or short 3'UTR.

Elements within the 3'UTR that control mRNA stability enable to adjust the expression of important regulatory proteins, including neurotrophic factors. Adenylate- and uridylate (AU)-rich elements (AREs) are typically 50–150 bp areas in the 3'UTR that serve as binding sites for *trans*-acting ARE binding proteins (ARE-BPs) which either stabilize or destabilize transcripts (Xu et al. 1997; Barreau et al. 2005). Although the exact consensus sequence of AREs is not in depth understood, AREs are often highlighted by high AU content and concomitant presence of AUUUA pentamers (Chen and Shyu 1995;

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Barreau et al. 2005). According to current estimations, approximately 8% of the human transcriptome contains AREs. However, relatively few AREs are experimentally verified as functional targets of ARE-BPs (Barreau et al. 2005; Bakheet et al. 2006; Barrett et al. 2012; Apponi et al. 2011; Gruber et al. 2011; Pascale and Govoni 2012). ARE-BPs tristetraprolin (TTP), butyrate response factor 1 (BRF1) and 2 (BRF2) form the TIS11/TTP family of ARE-BPs that target mRNAs for rapid degradation by binding to AREs (Hudson et al. 2004; Brooks and Blackshear 2013; Sanduja et al. 2011). TIS11/TTP family proteins are central regulators of the expression of inflammatory cytokines and several oncogenes (Hudson et al. 2004; Brooks and Blackshear 2013; Sanduja et al. 2011). Whether TIS11/TTP family could control the expression levels of neurotrophic factors, such as BDNF, has remained unknown.

In the current study, we used publicly available *in silico* tools to search for conserved AREs in BDNF-L and BDNF-S, and look for ARE-BPs co-expressed with BDNF in sites with known BDNF function. We find that ARE-BPs TTP, BRF1 and BRF2, but not ELAVL1 or ELAVL2, inhibit expression from luciferase reporters containing BDNF-L and BDNF-S, and that AUF1 has a mild inhibitory effect in the same assay. Using electrophoretic mobility shift assay (EMSA), we demonstrate a direct interaction between the 5' region of BDNF-S and recombinant TTP protein and find that endogenous BDNF mRNA co-immunoprecipitates with TTP. In line with the above, over-expression of TTP destabilizes transcript containing BDNF-S. Finally, we show that siRNA-mediated down-regulation of TTP during myogenic differentiation of mouse myoblast C2C12 cells leads to increased BDNF protein expression. Altogether, our findings suggest that TTP is a new post-transcriptional regulator of BDNF expression.

Materials and methods

Cell culture

Human Embryonic Kidney 293 (HEK-293), Chinese Hamster Ovary (CHO), HeLa and C2C12 cells were cultured at 5% CO_2 and 37°C in growth medium (GM) containing Dulbecco's Modified Eagle Medium (DMEM, Invitrogen/Gibco) supplemented with 10% fetal bovine serum (FBS; SV30160, Thermo Fisher Scientific) and 100 μg/ml Normocin (InvivoGen). Cells were kept at sub-confluent density and split one day before plating for an experiment. C2C12 wells were seeded to 6-well plates pre-coated with 0.1% gelatin. Myogenic differentiation of C2C12 mouse skeletal myoblast cells was induced by replacing growth medium with differentiation medium (DM) containing DMEM supplemented with 2% horse serum (HS; B15-021, PAA) (Miura et al. 2012) and 100 μg/ml Normocin.

Constructs and cloning

Gateway® pcDNA™-DEST40 vectors encoding for ARE-binding proteins for expression in mammalian cells were obtained from Genome Biology Unit (Institute of Biotechnology, University of Helsinki, Finland). Long and short BDNF 3'UTR-s (BDNF-L and BDNF-S) were amplified from BAC clone RP24-149 F11 (RPCI-24: *Mus musculus* (C57BL/6 J male) BAC library; BACPAC Resources) using primers with *XbaI* sites (Additional file 1: Table S1), and cloned into *XbaI* site in pGL4.13 vector downstream Firefly luciferase gene (E6681,Promega) and in *XbaI* site in pGL4.73 vector downstream Renilla luciferase gene (E6911,Promega). Similarly, U1 and U2 fragments were amplified with primers containing *XbaI* sites (Additional file 1: Table S1) from BDNF long 3' UTR sequence and cloned into pGL4.73 vector (E6911,Promega) and Bluescript KS + (Stratagene). All constructs were verified by sequencing. In addition, ARE-BP protein expression from various ARE-BP encoding Gateway® pcDNA™-DEST40 vectors in HEK-293 cells was verified using Western blotting (Additional file 1: Figure S1a). We also assessed whether the C-terminal tag (V5 or His) encoded by pcDNA™-DEST40 vectors has an impact on RBP activity in luciferase assay and found no difference between the tested tagged and non-tagged ARE-BP activity (Additional file 1: Figure S1b and see below).

Luciferase reporter assay

For luciferase reporter assay, cells were seeded to 96-well plates (pre-coated with 0.1% gelatin for HEK-293 cells), plating density per well was 15,000 (HEK-293 and CHO cells) and 10,000 (HeLa cells) in a volume of 100 μl one day before transfection. Reporter plasmids in 10:1 ratio (100 ng Luc-BDNF-L, Luc-BDNF-S, Luc-U1 and Luc-U2, if not indicated otherwise) and 10 ng either pGL4.73 [hRluc/SV40] or pGL4.13 [luc2/SV40]) as internal controls were co-transfected per well on 96 well plate to normalize the luciferase signal in dual luciferase assay. BDNF-L/BDNF-S transcript ratio in HEK-293 cells transfected with BDNF-L is 60/40, as assessed by QPCR analysis. Transfections were done according to standard protocol recommended for Lipofectamine 2000 (11668–019, Invitrogen). Growth medium was replaced with fresh cell culture medium after 3–4 hours after transfection. Luciferase assay was performed with Dual-Luciferase® Reporter Assay System (E1960, Promega) as recommended by the manufacturer. Briefly, cells were lysed 24 hours after the transfection with Passive Lysis Buffer (E1960, Promega). Plates were either stored at −80°C or analyzed immediately with Dual-Luciferase Reporter Assay reagents. Results from each experiment were normalized to controls from the same experiment. Data for each figure panel was collected from a set of several experiments where constructs indicated on the figure were present in each experiment.

We find that TTP suppressive effect on BDNF 3'UTR containing reporter gene expression is observed in all experiments, but the strength of inhibition on reporter gene expression varies up to about 1.7 fold between different sets of experiments. The reason for variance between different experimental sets is not known, but likely reflects normal variance in cell-culture experiments. Each experiment contained 3–4 replicates per construct/treatment and was repeated 2–8 times as specified in the figure legends.

Recombinant TTP protein production

Human TTP open reading frame was cloned into a T7lac based vector containing a His-tag (Peranen et al. 1996). The vector was transformed into BL21 (DE3) cells (Novagen), and the protein was expressed in the presence of IPTG (isopropyl β-D-thiogalactoside) for 4 h at 24°C. The cells were lysed in buffer A (20 mM Tris HCl, pH 8.0, 0.5% Triton X-100, 10 mM b-mercaptoethanol, 0.4 mM PMSF) by sonication. Then, NaCl and imidazole were added to a final concentration of 0.5 and 0.02 M, respectively. After centrifugation (15,000 × *g* for 15 min at 4°C) the supernatant was passed through a 0.45 μm filter. The His-TTP protein was purified by the HisTrap kit according to the manufacturer (GE Healthcare). Buffer B (50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.5 mM EDTA, 1 mM DTT) exchange was done by using a PD-10 column (GE Healthcare). His-tag was cleaved from His-TTP by AcTEV (Invitrogen). The His-tag and AcTEV were removed by application to a HiTrap Chelating column and TTP was collected from the flow-through. TTP was concentrated using an Amicon Ultra-4 filter device (Millipore). Aliquots of TTP were snap frozen in liquid nitrogen and stored at −80°C.

Electrophoretic Mobility Shift Assay (EMSA)

Depending on the orientation of U1 and U2 fragments, T7 or T3 RNA polymerase was used to synthesize RNA probes *in vitro*. RNA 3' End Biotinylation Kit (20160, Thermo Scientific Pierce) was used to label RNA probe at the 3' end according to manufacturer's protocol. Biotin labeling efficiency was assessed by dot plot as recommended by the manufacturer. Biotin-labeled RNA probes were diluted (5-10x) based on labeling efficiency obtained by dot plot assay. Diluted RNA probes were incubated with different concentrations of purified TTP protein at room temperature for 30 minutes in 20 μl reaction mixture containing 10 mM HEPES (pH 7.3), 40 mM KCl, 3 mM $MgCl_2$, 2 mM DTT, 5% glycerol and 2 mg/ml tRNA. The reaction was mixed with 1X REMSA loading buffer (Thermo Scientific Pierce) and run for 2 hours in 6% native polyacrylamide gel at 130 V. The bands were transferred to positively charged nylon membrane (Roche) and UV cross-linked at 120 mJ/cm^2 for 1 min. The detection of biotin-labeled RNA probes was done using LightShift Chemiluminescent RNA EMSA Kit (20158, Thermo Scientific Pierce) according to manufacturer's protocol.

RNA isolations and quantitative real-time PCR

RNA from C2C12 cells was isolated using either TRI reagent (TR 118, Molecular Research Center Inc.) or Trizol (15596–018, Ambion) according to protocol provided by the manufacturers. RNA samples were treated with Turbo DNA-free DNase (AM1907, Invitrogen) to remove DNA. cDNA synthesis using random hexamer primers in a final volume of 20 μl was performed using Transcriptor First Stand cDNA synthesis kit (04896866001, Roche) as recommended by manufacturer. Quantitative real-time PCR (RT-PCR) was done with Lightcycler 480 real-time PCR system (Roche Diagnostics) using Lightcycler 480 SYBR Green I Master Mix. Three replicate wells were run for each sample. Primers used for RT-PCR are indicated in Additional file 1: Table S1.

RNA interference

SMART pool siGENOME mouse Zfp36 (TTP) siRNA (M-041045-01-0005,Thermo Scientific) and siGENOME Non-Targeting siRNA Pools (D-001206-13, Thermo Scientific) were transfected to C2C12 cells 50–75 pmol each using Lipofectamine® RNAiMAX Transfection Reagent (13778030, Invitrogen) as recommended by the manufacturer.

Western blot

For detection of RBP expression in cell-lines, samples were run on 12% acrylamide gel for 1 hour and blotted to Hybond-ECL membrane (G1492720, GE Healthcare). The membrane was incubated in blocking solution (5% Non-fat milk, TBS, 0.1% Tween) for 30 minutes and incubated in anti-V5 Mouse Monoclonal Antibody (R960-25, Invitrogen) at 1:6000 dilution in the blocking solution for 50 minutes. The membrane was washed and incubated in polyclonal goat-anti-mouse HRP-conjugated secondary antibody (P0447, Dako) at 1:2000 dilution in the blocking solution for 50 minutes. Pierce ECL Western Blotting Substrate (32106, Thermo Scientific Pierce) was used for signal detection according to manufacturer's instructions.

Enzyme-linked immunosorbent assay (ELISA)

Differentiation medium (DM) from C2C12 cells was collected at day 5 as described in the experimental plan in Figure 1a and centrifuged at 2000 rpm at +4°C for 2 minutes to remove cell debris. BDNF protein levels in the DM were measured using BDNF Emax® ImmunoAssay System (G7611, Promega) according to manufacturer's protocol. BDNF levels were normalized to total protein

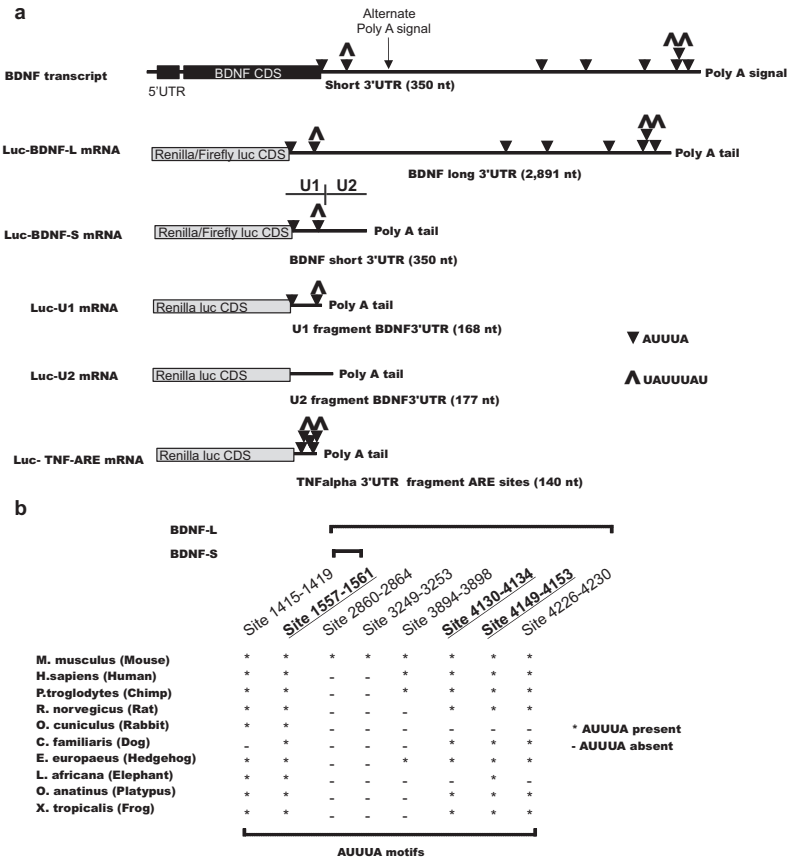

Figure 1 AUUUA- motifs in mouse BDNF 3′ UTR and their conservation among vertebrates. a. Schematic diagram of BDNF mRNA products with long (BDNF-L) and short (BDNF-S) 3′UTRs. AUUUA sequences, often serving as ARE-BP binding motifs within AU-rich elements (AREs) are indicated with black triangles, AREs UAUUUAU are indicated with up arrowheads, black box denotes BDNF coding sequence (Source: AREsite and AREscore). mRNA-s transcribed from luciferase reporter constructs (grey boxes) containing BDNF-L, BDNF-S, U1, U2 and ARE-rich fragment from TNFα 3′UTR. The positions of U1 and U2 (see below) are indicated above Luc-BDNF-S. **b**. Scheme depicting conservation of AUUUA sequences among vertebrates. UAUUUAU motifs are underlined and in bold.

concentration using DC Protein Assay (500–0114, Bio-Rad) according to manufacturer's recommendations.

RNA immunoprecipitation (RNA-IP) assay

RNA-IP was performed as described previously (Ishmael et al. 2008). Briefly, differentiated C2C12 cells in six-well plate (CellStar) were lysed in 250 μl of polysome lysis buffer (100 mM KCL, 5 mM $MgCl_2$, 10 mM HEPES (pH 7.0), 0.5% Nonidet P-40, 1 mM DTT, 100 U/ml RNaseOUT (Invitrogen), 0.2% vanadyl-ribonucleoside complex (New England Biolabs), Protease Inhibitor Cocktail (Roche)) and centrifuged at 12000 rpm for 15 minutes at 4°C. A/G agarose beads (Santa Cruz) were precoated with 10 μg of antibody against TTP or normal rabbit IgG (Santa Cruz). 100 μl of cell lysate was mixed with precoated A/G agarose beads in 900 μl of NT-2 buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM $MgCl_2$, 0.5% Nonidet P-40) supplemented with 1 mM DTT, 100 U/ml RNaseOUT, 0.2% vanadyl-ribonucleoside complex and 20 mM EDTA and incubated for 2 hours at RT. Washed beads were incubated with NT-2 buffer supplemented with 0.1% SDS and 0.5 mg/ml Proteinase K at 55°C for 30 minutes. RNA was isolated and real time quantitative PCR was performed as described above.

RNA turnover assay

HEK-293 cells were transfected as described above with Luc-BDNF-S, empty vector (pDEST 40) or TTP encoding plasmids. After 24 hours 1 μg/ml of Actinomycin D (A 1410, Sigma Aldrich) was added to the medium. Cells

were lysed at indicated time points, RNA was extracted and semi-quantitative PCR was performed using primers indicated in Additional file 1: Table S1.

Statistical analysis

For luciferase assay, data points from experiments within one experimental set (experimental set is defined as repeated experiments containing the same set of constructs) were averaged and used for statistical calculations. For other experiments, data was averaged from repeated experiments and used for statistical calculations. Error bars of graphs represent standard deviations (±SD). Data was analyzed with 2-tailed Student's t-test assuming unequal variance. The level of significance was set at $p < 0.01$ unless otherwise indicated. Experiments were repeated 2–8 times with 2–4 replicates per experiment as indicated in specific figure legends.

Results

In silico prediction of ARE sites in the BDNF 3'UTR

Using publically available web tools ARESite (Gruber et al. 2011) (http://rna.tbi.univie.ac.at/cgi-bin/ARESite.cgi) and AREscore (Spasic et al. 2012) (http://arescore.dkfz.de/arescore.pl), we identified five conserved AUUUA motifs in the BDNF 3'UTR flanked by AU-rich sequences (Figure 1, Additional file 1: Figure S2). Two of the five AUUUA motifs were present in BDNF-S, while three AUUUA motifs were only present in BDNF-L (Figure 1, Additional file 1: Figure S2). Interestingly, three of the predicted AUUUA motifs are UAUUUAU (7-mer) sites, which may serve as high-affinity binding sites for TTP family ARE-BPs (Hudson et al. 2004; Brooks and Blackshear 2013) and one of the core 7-mer motifs is located in BDNF-S (Figure 1 Additional file 1: Figure S2).

TTP family ARE binding proteins inhibit expression of a luciferase reporter containing BDNF-L and BDNF-S

To analyze the biological importance of the *in silico* identified ARE motifs in BDNF 3'UTR, we cloned mouse BDNF-L and BDNF-S into luciferase reporter constructs (Figure 1a, see MM for details). Co-transfection of luciferase reporter constructs along with cDNAs encoding for ARE-BPs TTP, BRF1, BRF2, ELAVL1, ELAVL2 and AUF1 selected based on co-expression with BDNF in various tissues, (Additional file 1: Table S2) revealed that ARE binding proteins forming the TTP family - TTP, BRF1 and BRF2, but not ELAVL1 or ELAVL2, significantly inhibit expression of luciferase reporter containing BDNF-L in the human embryonic kidney 293 (HEK-293) cells, while AUF1 had a marginal effect (Figure 2a). Next we compared the effects of ARE-BP-s on BDNF-S and BDNF-L in the same reporter assay and found that the ability of TTP family members to suppress expression does not differ between luciferase reporter constructs

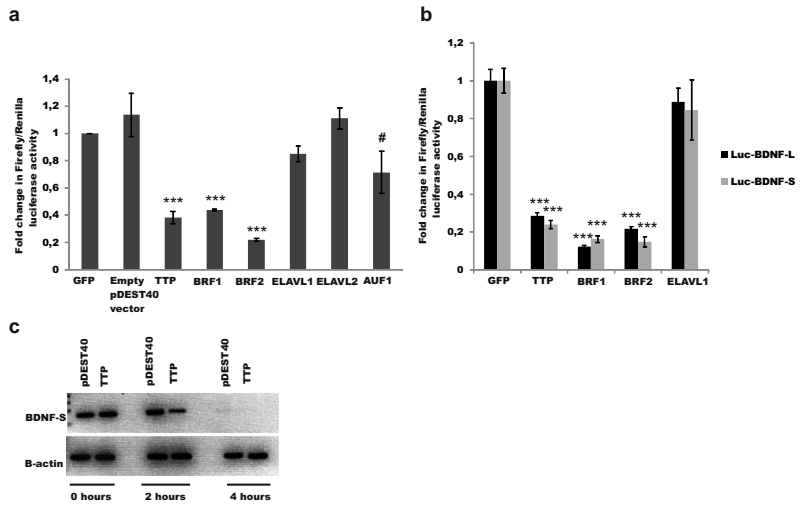

Figure 2 Effects of ARE-BPs on expression from luciferase reporter containing BDNF-L or BDNF-S. a. Effects of TTP, BRF1, BRF2, ELAVL1, ELAVL2 and AUF1 on the expression of luciferase reporter containing BDNF-L (Luc-BDNF-L) compared to Green Fluorescent Protein (GFP) and empty vector (pDEST40) as negative controls in HEK-293 cells, N = 2-8 experiments with 3–4 repeats per experiment, ***$p < 0.001$, #$p < 0.035$, Error bars indicate SD. **b**. Comparison of effects of TTP, BRF1 and BRF2 on Luc-BDNF-L and Luc-BDNF-S expression in HEK-293 cells revealed no statistically significant difference between BDNF-L and BDNF-S, N = 3-4, experiments with 4 repeats per experiment, ***$p < 0.001$. Error bars indicate SD. **c**. Effect of TTP expression on Luc-BDNF-S mRNA turn over in the HEK-293 cells upon Actinomycin D (1 μg/ml) treatment at the indicated time points, N = 2 experiments, a representative experiment is shown. BDNF-S and β-actin mRNA was detected with semi-quantitative PCR.

containing BDNF-S and BDNF-L (Figure 2b). Then we studied the effect of TTP on the stability of a transcript containing BDNF-S upon transcriptional inhibition using actinomycin D. In line with results from luciferase assay, TTP overexpression destabilized BDNF-S containing transcript (Figure 2c). To test whether the observed inhibition by TTP family members is also observed in other cell-lines from different species we performed the luciferase assay in Chinese hamster ovary (CHO) cells and in Hela cells. TTP and BRF1 suppressed reporter gene expression in both cell-lines (Additional file 1: Figure S3a).

TTP interacts with 5′ proximal end of BDNF-S without the requirement for AUUUA motif

BDNF-S contains a proximal AUUUA site and a distal UAUUUAU site (Figure 3a). Both sites are conserved among vertebrates (Figure 1b) and may serve as binding site for ARE-BPs (Hudson et al. 2004; Brooks and Blackshear 2013). However, within the AU-rich region, (U)AUUUA(U) sequence may also be dispensable for RBP, including for TTP binding (Chen et al. 1994; Lopez de Silanes et al. 2004; Lopez de Silanes et al. 2005). To further characterize the suppressive effect of TTP on BDNF-S, we cloned the 5′ proximal end of the BDNF 3′UTR containing both conserved AUUUA site-s (designated as U1, Figure 3a) and the adjacent 3′UTR fragment (designated as U2, Figure 3a) into luciferase reporter construct as above. The overall AU-content of U1 and U2 fragments is comparable (Additional file 1: Figure S4). As a positive control for TTP inhibition in the luciferase reporter system we used a well-characterized target of inhibition by TTP, a 140 bp AU-rich 3′UTR fragment from TNF-alpha gene (TNF-ARE), which contains five overlapping UUAUUUAUU motifs identified as high-affinity binding sites for TTP (Hudson et al. 2004; Barreau et al. 2005). The overall AU-content of TNF-ARE was comparable to AU-rich regions of U1 and U2 of BDNF 3′UTR (Additional file 1: Figure S4). We found that luciferase reporter containing BDNF-S, 5′ proximal BDNF-S fragment U1 and TNF-ARE are significantly inhibited at lower TTP concentrations than U2, the distal BDNF-S fragment, on which TTP had moderate suppressive effect only at the highest concentration (Figure 3b). Repression by TTP was comparable between Luc-TNF-ARE, Luc-BDNF-S and Luc-U1 at the highest TTP concentration (Figure 3b). To assess whether similar repression is observed in cells derived from other species we repeated the experiment in CHO cells and observed a similar outcome (Additional file 1: Figure S3b). To study whether inhibition of U1 containing reporter by TTP can be attributed to a direct interaction between TTP and U1, we synthesized RNA probes corresponding to U1 and U2, produced recombinant TTP protein and performed electrophoretic mobility shift assay (EMSA). EMSA analysis showed a shift in the band representing U1 probe but no shift in the band representing U2 probe after addition of recombinant TTP protein (Figure 3c), suggesting a stronger interaction between TTP protein and U1 fragment of BDNF 3′ UTR. (U)AUUUA(U) elements within the AU-rich region may either be important or dispensable for binding by RBP-s (Chen et al. 1994; Lopez de Silanes et al. 2004; Lopez de Silanes et al. 2005).

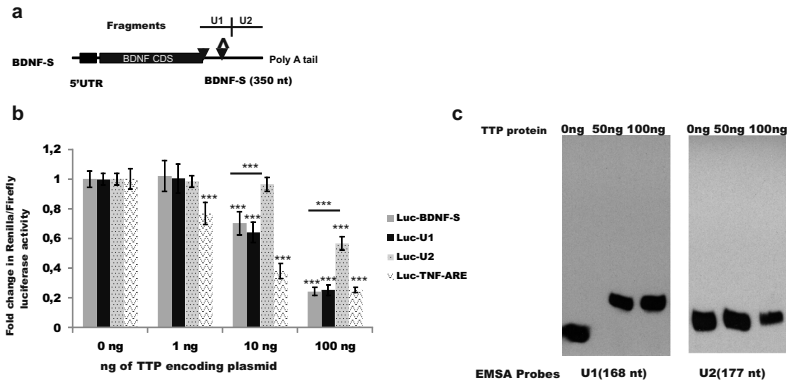

Figure 3 TTP binds to the 5′ proximal fragment of BDNF 3′UTR. a. Illustration showing the position of analyzed 3′UTR fragments U1 and U2 in BDNF-S, AREs are marked as on Figure 1a. **b**. Effects of 1–100 ng of plasmid/96-well encoding for TTP on reporter construct expression carrying BDNF-S, U1 and U2 in HEK-293 cells. TTP encoding plasmid has a moderate effect on U2 containing reporter construct expression only at the highest concentration (100 ng), while U1 is inhibited already at a 10 fold lower TTP encoding plasmid concentration (10 ng), suggesting different affinity of U1 and U2 towards TTP. A well-established target of inhibition by TTP, a 140 bp ARE-rich fragment from TNF-alpha 3′UTR (Luc-TNF-ARE) was included as a positive control, N = 2-3 experiments with 4 repeats per experiment. Error bars indicate SD, ***$p < 0.001$. **c**. Electrophoretic mobility shift assay showing band shift of RNA probe U1 with recombinant TTP protein (50 ng and 100 ng) and no band shift of RNA probe U2 under the same conditions. Experiment was repeated twice.

To discriminate between these two options, we studied the requirement of proximal AUUUA site, the distal UAUUUAU site and the necessity of both sites together in U1 (Figure 3a) for suppression by TTP. We generated constructs where the proximal AUUUA site (Luc-BDNFSmut1), distal UAUUUAU site (Luc-BDNFSmut2) and both (Luc-BDNFSmut1-2) AUUUA sequences were replaced with UAUAU. Changing either one or both AUUUA sequences to UAUAU did not affect the ability of TTP to inhibit BDNF-S (Additional file 1: Figure S3c), suggesting that BDNF-S falls into the class of 3'UTR-s where AUUUA elements are dispensable for RBP function (Chen et al. 1994; Lopez de Silanes et al. 2004; Lopez de Silanes et al. 2005).

TTP interacts with BDNF mRNA and regulates endogenous BDNF levels in myogenic C2C12 cells

TTP is acutely and transiently induced within 30 minutes following skeletal muscle injury in satellite cells, which give rise to myoblasts (Sachidanandan et al. 2002; Apponi et al. 2011). However, targets of TTP regulation upon muscle differentiation are unknown. On the other hand, BDNF is expressed in skeletal muscle satellite cells and down-regulation of BDNF levels is believed to be required to allow myogenic differentiation of myoblasts into myotubes (Mousavi and Jasmin 2006; Miura et al. 2012;). Our results suggest that TTP may suppress BDNF levels by directly interacting with its 3'UTR. To gain further insight into this, we first studied the expression levels of TTP, BRF1 and BRF2 in differentiating C2C12 cells, an established model of muscle differentiation (Burattini et al. 2004). Confirming and extending the earlier findings, we found that TTP, but not BRF1 or BRF2, is upregulated upon differentiation in C2C12 cells (Figure 4a-b). Next, we performed RNA-immunoprecipitation in differentiating C2C12 cells using antibodies against TTP and found that endogenous TTP co-immunoprecipitates with endogenous BDNF mRNA (Figure 4c). Finally, we tested the effect of RNAi knock-down of endogenous TTP (Figure 4d) on endogenous BDNF levels in C2C12 cells, and found concomitant upregulation of BDNF protein levels (Figure 4e).

Discussion

Recently, 3'UTRs have emerged as an important site of gene expression regulation by binding of micro-RNAs (miRs) and RBPs. Long and conserved 3'UTRs that provide a binding platform for miRs and RBPs are especially

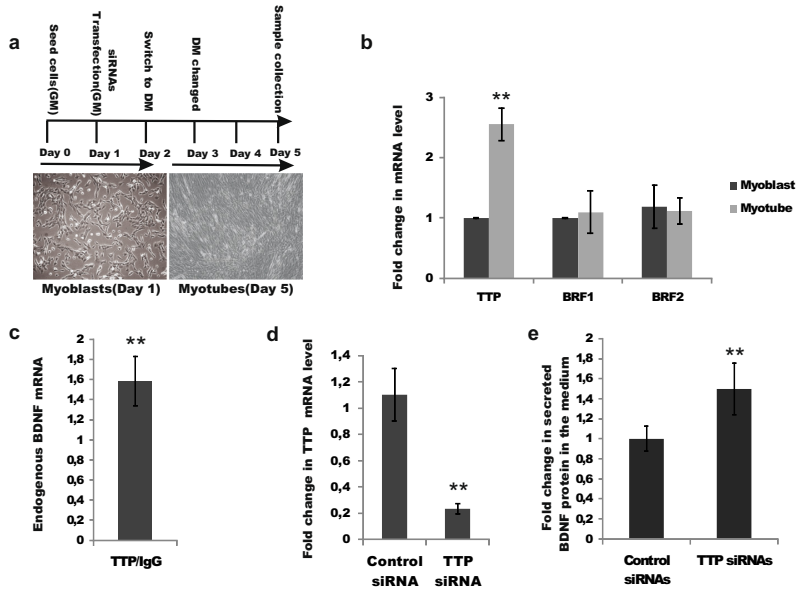

Figure 4 TTP interacts with BDNF mRNA and regulates endogenous BDNF levels in myogenic C2C12 cells. a. Outline of an experiment with C2C12 cells. Left: illustrative image of undifferentiated cells (myoblasts) at day 1; Right: illustrative image of differentiated cells (myotubes) at day 5. GM-growth medium, DM-differentiation medium. **b**. TTP but not BRF1 and BRF2 mRNA levels are upregulated in differentiating myotubes at day 5. **p < 0.01, N = 2-4, with 2–3 repeats per experiment. **c**. Endogenous BDNF mRNA co-immunoprecipitates with endogenous TTP protein in differentiating C2C12 cells, N = 2 experiments. **p = 0.01. **d**. Reduction in TTP mRNA levels after siRNA-mediated TTP knock-down at day 5, N = 4 experiments with 2–3 repeats per experiment, **p < 0.01. **e**. Increase in BDNF protein levels after siRNA-mediated TTP knock-down (c) in the cell-culture medium at day 5. N = 3 experiments with two repeats per experiment, **p < 0.01. Error bars indicate SD on all graphs.

common for regulatory genes, such as neurotrophic factors (Barrett et al. 2012). During the last two years, several miRs have been shown to directly interact with BDNF 3'UTR and regulate its expression(Miura et al. 2012; Lee et al. 2012; Varendi et al. 2014). However, how RNA binding proteins impact BDNF expression has largely remained unexplored. Microarray analysis of primary fibroblasts derived from TTP deficient mice revealed BDNF mRNA among ca 250 elevated mRNAs (Lai et al. 2006), suggesting that BDNF is either directly or indirectly regulated by TTP. Our results suggest that the suppressive effect of TTP on BDNF mRNA level is direct.

BDNF and TTP family members are co-expressed in many tissues, suggesting that the observed regulation may be involved in controlling a range of biological processes from CNS function to inflammation and muscle differentiation. That fine-tuning of BDNF levels via its 3'UTR has physiological and clinical importance is illustrated by a recent study by Lee *et al.*, where anti-miR based knockdown of miR-206, a negative regulator of BDNF, increased endogenous BDNF levels and alleviated Alzheimer's disease in a mouse model (Lee et al. 2012).

So far only one ARE-BP, ELAVL4, is reported to impact BDNF mRNA stability by specifically stabilizing the BDNF-L but not the BDNF-S isoform (Allen et al. 2013). BDNF-L makes up 20-50% of BDNF transcripts in the CNS (Timmusk et al. 1994). We find that TTP binds to the 5' proximal end (U1) of the BDNF 3'UTR and suppresses expression from both BDNF-S and BDNF-L to a comparable extent. This suggests that in BDNF 3'UTR, either U1 is the only binding platform for TTP, that binding of TTP distal to U1 has no additive effect on suppression, or that in BDNF-L only the distal binding site is accessible for TTP. Future studies will help to discriminate between these options.

TTP was discovered and has been characterized as an important regulator of inflammation (Sanduja et al. 2011; Taylor et al. 1996; Carballo et al. 1998; Brooks and Blackshear 2013). BDNF is the first neurotrophic factor identified as a target of TTP regulation. Interestingly, similar to TTP, BDNF regulates inflammatory processes including microglial activation and neuropathic pain (Uchida et al. 2013; Lin et al. 2011; Gomes et al. 2013; Amoureux et al. 2008), as well as neuroimmunological disease (Luhder et al. 2013). How TTP-mediated regulation of BDNF impacts the inflammatory processes remains to be explored.

Interestingly, as a rule, ARE-BP proteins share target mRNA-s. For an example GM-CSF, TNF-alpha and c-fos are shared targets of AUF1, ELAVL1 and TTP, see (Barreau et al. 2005) for review. We find that BDNF 3'UTR is directly regulated by TTP but the effect of AUF1 and ELAVL1 is either minor or absent. Therefore, further analysis of BDNF 3'UTR involving bioinformatics and laboratory techniques may enable to disclose which sequence/structure components in the BDNF 3'UTR underlie its specificity for TTP.

Taken together, co-expression of BDNF and TTP family members in various cell types and tissues suggests potential relevance of the interaction between TTP family and BDNF 3'UTR isoforms in different physiological and pathological processes. We hope our work facilitates future studies addressing the physiological and therapeutic potential of TTP/BDNF interaction in various organ systems.

Additional file

Additional file 1: Table S1. List of cloning and Q-PCR primers. **Table S2.** BDNF is co-expressed with ARE-BPs in various tissues with known BDNF function. **Figure S1. a**. Western blotting using antibodies against V5-tag showing that ARE-BP encoding constructs used in the study result in proteins with expected sizes. In addition, constructs used in this study were sequenced (see also MM). **b**. C-terminal tag (V5 or His) has no effect on TTP, ELAVL1 and ELVAL2 ability to modulate expression from reporter construct containing Luc-BDNF-L. N = 2 experiments with 4 repeats per experiment, ***p < 0.001. Error bars indicate SD. **Figure S2.** Mouse Bdnf 3'UTR sequence. Location of AUUUA motifs is indicated by red, UAUUUAU motifs are red in italics; the sequence of BDNF-S is bold and underlined. **Figure S3. a**. Similar to HEK293 cells (Figure 3), inhibitory effect of TTP and BRF1 on BDNF-L expression is observed in Hela cells and in Chinese hamster ovary cells (CHO). N = 1-2 experiments with 4 repeats per experiment, error bars indicate SD, ***p < 0.001, **p < 0.01. **b**. Similar to HEK293 cells (Figure 4b), low concentration of TTP encoding plasmid (0.5 ng/96-well plate well) has inhibitory effect on TNF-ARE and U1 but not on U2 in CHO cells. N = 3 experiments with 3–4 repeats per experiment, ***p < 0.001. **c**. Mutation in 5', 3' or both AUUUA sites present in BDNF-S has no effect on TTP-mediated inhibition on expression from Luc-BDNF-S reporter construct. N = 2 experiments with 3–4 repeats per experiment, ***p < 0.001. Error bars indicate SD on all graphs. **Figure S4.** AU percent in BDNF 3' UTR fragments U1, U2 and in TNFα 3'UTR ARE fragment.

Competing interests
All authors declare that they have no competing interests.

Authors' contributions
KV cloned the mutation constructs, performed luciferase assay for mutation analysis, analyzed data and contributed to the writing of the manuscript. JP produced recombinant TTP protein for EMSA. AK conceived the idea, planned and executed experiments, analyzed data and wrote the manuscript. JOA planned the experiments, analyzed data, provided funding and wrote the manuscript. All authors read and approved the final manuscript.

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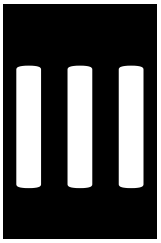

In vivo replacement of 3'UTR as a versatile method to study gene function – the case of GDNF

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ABSTRACT

Genetic studies on the effect of 3'UTR on gene expression levels in mammalian nervous system are currently missing. GDNF has been tested in clinical trials to treat Parkinson's disease with variable outcome, but mechanisms of GDNF regulation and its post-natal function have remained obscure. Here we show that GDNF expression is negatively regulated via its 3'UTR *in vitro* and *in vivo.* Our data suggest suppression of GDNF expression via direct interaction between *Gdnf* 3'UTR and miR-96 and miR-146a, and identify other candidate miR-s for GDNF regulation. Further, we show that elevation of endogenous GDNF by 3'UTR replacement in mice augments striatal extracellular dopamine (DA) clearance rate in DA concentration-dependent manner, increases striatal DA levels and elevates the number of DA neurons in adult substantia nigra. Adverse effects associated with ectopic GDNF applications are not observed, while striatal DA levels and function in proteasome inhibitor lactacystin induced Parkinson's disease model are preserved. Our results reveal novel post-natal functions of endogenous GDNF and highlight 3'UTR replacement as a potent genetic tool to study mammalian genes *in vivo.*

SIGNIFICANCE

Currently, gene function *in vivo* is mostly concluded based on effects associated with its deletion. To what extent compensation occurs remains unknown. Transgenic overexpression on the other hand, is limited due to spatiotemporal misexpression. Recently, 3'UTR-s have emerged as important site of gene levels regulation but genetic studies have remained scarce. We find that the 3'UTR of GDNF, a dopaminergic growth factor currently in phase 2 clinical trials for Parkinson's disease, contains strong inhibitory property and we identify candidate microRNA-s involved in this process. Replacement of GDNF's 3'UTR in mice derepressed endogenous GDNF levels and allowed studies inaccessible with alternative methods. Our data pinpoint that 3'UTR-s are an important target for genetic studies.

INTRODUCTION

Currently, gene function *in vivo* is mostly studied by transgenic overexpression or deletion within endogenous locus (gene knock-out) methods. However, transgenic overexpression of a gene is limited due to spatiotemporal misexpression resulting from recombinant construct and integration site variations. The gene knock-out approach, on the other hand, fails to reveal the extent to which compensatory effects by functional homologues and, in the case of the brain, the use of alternative neuronal networks, masks the effects of the lack of a gene.

3'UTR replacement strategy to modulate endogenous gene product levels to complement the limitations of existing methods has been proposed almost ten years ago(1) but follow up studies have remained scarce(2, 3). Genetic studies on the role of 3'UTR in the regulation of gene expression levels in mammalian nervous system are currently lacking.

Neurotropic factors (NTFs) such as Glial cell line-derived neurotrophic factor (GDNF) carry great therapeutic potential. However, whether *in vivo* upregulation (derepression) of endogenous NTFs by 3'UTR replacement provides scientifically and therapeutically valuable information has remained unexplored.

GDNF has been identified as a potent NTF particularly for the midbrain dopamine (DA) neurons in substantia nigra (SN) both *in vitro* and *in vivo*(4, 5). DA neurons in SN innervate mainly the dorsal striatum(6) and primarily regulate initiation of voluntary movements and basal motor activity(7). Degeneration and loss of these neurons is specifically accelerated in

Parkinson's disease (PD), which is characterized by reduction in striatal dopamine levels and concomitant slowness of movement, resting tremor and rigidity (8).

Because of its strong dopaminotrophic effects, GDNF is extensively studied in pre-clinical and clinical trials of PD by intracranial delivery(8). However, intracranial ectopic GDNF application has undesired side-effects and benefits have remained inconclusive, underpinning a need for basic studies on GDNF biology (9-16). To what extent results on ectopic GDNF applications reflect endogenous GDNF function has remained unclear. This is at least in part because knock-out mice of *Gdnf* and its receptors, *Gfrα1* and *Ret* die at birth due to the lack of kidneys, while the DA system, which largely matures postnatally, is intact at that age (17).

Here, we show that GDNF levels are negatively regulated via its 3'UTR and generate mice in which *Gdnf* 3'UTR is conditionally reversibly replaced resulting in enhanced, but spatially unchanged expression of endogenous GDNF. While homozygous *Gdnf* 3'UTR-crR mice die by P18 due to defects in kidney development, heterozygous animals are healthy. Analysis of the new mouse model revealed that moderate, 30-50% elevation in endogenous GDNF levels augments the number of adult DA neurons in SNpc and striatal DA levels, boosts extracellular striatal DA clearance rate in a DA concentration dependent manner and increases amphetamine-induced but not spontaneous motor activity in mice. Further, we find that endogenous GDNF protects DA neuron function in proteasome inhibitor lactacystin induced mouse PD model. Side effects associated with ectopic GDNF applications such as spontaneous hyperactivity, reduction in key enzyme in DA synthesis tyrosine hydroxylase (TH) levels and loss of appetite were not observed.

Our findings suggest that next to the classical gene deletion or transgenic over-expression methods, 3'UTR replacement is a potent tool to study gene function and regulation *in vivo*.

RESULTS

The 3'UTR of *Gdnf* contains an inhibitory property

We found that *Gdnf* 3'UTR contains an inhibitory property that suppresses Firefly luciferase expression in human embryonic kidney (HEK293) cells. We also found that suppression by *Gdnf* 3'UTR is similar when different reporter gene is used, suggesting that it is independent of the preceding coding sequence (Fig. 1*A*), extending the findings of an earlier study(18). Human brain-derived U87 glioblastoma cells are one of the few cell-lines known to produce and secrete detectable amounts of endogenous GDNF protein(19). We found that inhibition by *Gdnf* 3'UTR occurs, although less prominently than in HEK293 cells, also in U87 cells (Fig. 1*A*).

Conditionally reversible replacement of *Gdnf* 3'UTR

Our goal was to study the *in vivo* relevance of the inhibitory property in *Gdnf* 3'UTR with the focus on midbrain DA system development and function. We set to generate mice with conditionally reversible replacement of *Gdnf* 3'UTR (*Gdnf* 3'UTR-crR) as shown on Fig 1*B*. To test the principle, we preceded 2.8kb *Gdnf* 3'UTR in the reporter construct with FLP recombinase recognition site flanked 2.5 kb marker gene PR/TK (20), where transcription termination is induced by bovine Growth Hormone polyadenylation signal (bGHpA), (Fig S1*A*). bGHpA is a strong initiator of transcription termination broadly used for gene-trap experiments in mice(21, 22). We confirmed that bGHpA prevents transcription to *Gdnf*

3'UTR in our construct (Fig. S1*B-C*). Inhibition of transcription using actinomycin D revealed that *Gdnf* 3'UTR-crR renders the preceding gene product more stable than *Gdnf* 3'UTR, suggesting that negative regulation occurs at post-transcriptional level (Fig. S1*D*). Comparison of *Gdnf* 3'UTR and *Gdnf* 3'UTR-crR on reporter gene expression revealed 8-fold derepression relative to *Gdnf* 3'UTR in HEK293 cells and about 2-fold derepression in U87 cells (Fig. 1*C*). Next, *Gdnf* 3'UTR-crR cassette was flanked with homologous arms to generate *Gdnf* 3'UTR-crR mice carrying an allele depicted on (Fig. 1*D*). Because we also aimed to compare phenotypes of heterozygous *Gdnf* 3'UTR-crR mice to heterozygous *Gdnf* coding sequence knock-out (KO) mice, *Gdnf* exon 3 encoding for GDNF protein was "floxed" (Fig. 1*D*).

Gdnf levels and site of expression in the *Gdnf* 3'UTR-crR mice

We found that homozygous *Gdnf* 3'UTR-crR animals died before post-natal day (P) 18 due to kidney defects while heterozygous (+/-) *Gdnf* 3'UTR-crR mice were healthy (see below).

Similar to HEK293 and U87 cells, GDNF mRNA and protein in *Gdnf* 3'UTR-crR homozygous mice were about 6-fold derepressed in the kidney (Fig. S2*A*) and up to about 2-2.5 fold both in the rostral brain at P7.5 (Fig. 2*A-B*) and in various brain regions in adult mice (Fig 2*C-D*) as measured using qPCR and ELISA methods, respectively. We noted that *Gdnf* mRNA was more efficiently derepressed in the brain areas where *Gdnf* mRNA levels were relatively higher (Fig. 2*C*). Removal of the 3'UTR replacement cassette with crosses to Deleter-Flp line resulting in a *Gdnf* 3'UTR-restored allele, normalized GDNF levels in brain (Fig. 2*C*) and kidney (Fig S2*A*).

Next, we studied the site of *Gdnf* expression. Unfortunately, antibodies reliably recognizing endogenous GDNF protein in histological sections are currently not available. To further address this, we tested over ten commercially available anti-GDNF antibodies and all recognized a 37kD band of a "wrong "size in western blotting analysis of brain lysates derived from GDNF gene knock-out mice (MW of GDNF monomer is ca 15kD). We therefore used *in situ* hybridization (ISH) method to detect *Gdnf* mRNA. *Gdnf* mRNA levels in mouse brain are the highest at around P12.5 facilitating the analysis (23, 24). In the striatum (Fig. 2*E*) and in other brain areas (Fig. S2*B*) *Gdnf* expression site in *Gdnf* 3'UTR-crR+/- mice matched the wt *Gdnf* expression pattern and the previously published results (24, 25). In line with enhanced *Gdnf* expression, ISH signal was stronger in *Gdnf* 3'UTR-crR+/- mice striatum (Fig. 2*E*). Because there are always cells with borderline ISH signal (Fig. 2*E*) it is impossible to directly assess whether the seemingly increased number of *Gdnf*+ cells in the striata of *Gdnf* 3'UTR-crR+/- mice reflects an increase in *Gdnf*-positive cell number or ca 2-fold increase in *Gdnf* mRNA expression (Fig 2*C,E*). However, *Gdnf* and *parvalbumin* (PV) are almost exclusively co-expressed in the same striatal neurons (24). Quantification of striatal PV+ cells did not reveal difference between the genotypes (Fig. 2*F*). ISH analysis of *Gdnf* mRNA in other organs with well-defined *Gdnf* mRNA expression sites, including developing kidney, testis and hind-limb revealed stronger signal in *Gdnf* 3'UTR-crR+/- mice but no difference in the site of expression between wt and *Gdnf* 3'UTR-crR mice (Fig S2*C-F*). Northern blot analyses of *Gdnf* mRNA (Fig. S2*G*) and *Gdnf* transcript sequencing (Fig. S2*H-I*) further confirmed that *Gdnf* 3'UTR replacement was successful *in vivo*.

Identification of *Gdnf* 3'UTR regulating micro-RNAs

Gdnf 3'UTR contains inhibitory property but the inhibitors involved remained unknown (18) (Fig 1*A*). miRs and RNA-binding proteins (RBPs) are the most important mRNA stability and translation regulating factors that can bind to 3'UTR-s (2, 26). Oh-Hashi *et al.* tested several RBP-s but found no impact on *Gdnf* 3'UTR containing reporter gene expression (18), a finding which we independently confirmed (Fig S3*A*).

Next we turned our attention to micro-RNAs (miRs). Analysis of *Gdnf* and its family ligands (GFL-s: *neurturin, artemin, persephin*) and their receptors (*Gfrα1-4, Ret*)(17) revealed multiple conserved miR sites in the 3'UTR's of *Gdnf* (Fig. 3*A*, Table S1*A*) and GDNF family ligands (GFL) signaling receptor *Ret* mRNAs, a single conserved miR site in the 3'UTR of neurturin and *Gfrα2* but none in other GFL-s and their receptors (Fig. S3*B*).

We studied whether the 3'UTR of *Gdnf* can be specifically regulated by the predicted miRs using the Dual-Glo reporter system. Currently there are no reports on miR regulation of GDNF. We selected miR-133a, miR-133b, miR-125a-5p, miR-125b-5p, miR-30a, miR-30b, miR-96, miR-9 and miR-146a for further analysis based on their co-expression with *Gdnf* in various brain areas (24, 25, 28) and (30), www.microrna.org. We also confirmed that miR-9, miR-133a, miR-125a-5p, miR-125b-5p, miR-30a, miR-30b and miR-146a are expressed in adult dorsal striatum (Fig. S3*C*). HEK-293 cells were transfected with RNA-s encoding for different control pre-miRs (Negative control-N) and with putative GDNF-regulating pre-miRs: miR-133a, miR-133b, miR-125a-5p, miR-125b-5p, miR-30a, miR-30b, miR-9, miR-96 and miR-146a. All of the above miRs, relative to the controls, negatively regulated the expression of a reporter construct containing the *Gdnf* 3'UTR (Fig. 3*B*). Mutating the

predicted miR binding sites (seed sequence, Table S1) in the *Gdnf* 3'UTR abolished the inhibitory capability of the tested miR-146a and miR-96 (Fig. 3*C*). We also found that miR-s lack effect on the *Gdnf* 3'UTR-crR cassette (Fig. 3*F*) in the same assay. This is in line with *in silico* prediction that *Gdnf* 3'UTR-crR cassette contains about 2-fold less total potential miR sites and 9-fold less conserved sites present in *Gdnf* 3'UTR (Fig. S3*E-F*). Transient expression of a combination of multiple miRs (miR-9, miR-96, miR-146a and miR-133b), which were selected based on their ability to interact with *Gdnf* 3'UTR and suppress the *Gdnf* 3'UTR-containing reporter construct (Fig. 3*B*), additively suppressed both endogenous GDNF protein and mRNA levels (Fig. *3D-E*), without affecting U87 cell survival (Fig. *S3D*).

Kidney development disturbed but enteric nervous system function maintained

Heterozygous *Gdnf* knock-out (KO) animals with about 50% reduced GDNF levels frequently have one kidney (17). Both homo- and heterozygous *Gdnf* 3'UTR-crR animals always had two kidneys, but excess GDNF negatively regulated kidney size in a GDNF concentration-dependent manner (Table S2*A*, Fig. S4*A-B*). However, while homozygous *Gdnf* 3'UTR-crR-/- mice died most likely due to miniature and poorly functioning kidneys by P18, kidney defect in heterozygous mice was mild or absent and animals were found at the Mendelian frequency and healthy at all ages (Table S2*B*, Fig. S4*C-F,* and see below). Analysis of the GI tract revealed that the number of enteric neurons in *Gdnf* 3'UTR-crR animals was increased, while the GI tract in both homo- and heterozygous mice appeared anatomically normal. Bodyweight measurements and studies with physiological cage revealed no difference between heterozygous 3'UTR-crR and wt littermates (see below), supporting the idea of normally functioning GI tract. Detailed role of the increased GDNF levels in the kidney and ENS will be described elsewhere.

Effect of GDNF derepression on postnatal nigrostriatal DA system development

Since homozygous *Gdnf* KO mice die at birth due to lack of kidneys, the post-natal role of GDNF in DA system function has remained poorly understood (17, 31). We found that in *Gdnf* 3'UTR-crR mice striatal phosphorylated extracellular signal-regulated kinase 2 (Erk2) levels, a known target of GDNF signaling(17), are increased at P7.5 (Fig. 4*A*). DA levels in the rostral brain at P7.5 were elevated to a comparable level in both homo- and heterozygous *Gdnf* 3'UTR-crR animals and normalization of GDNF levels restored wt DA levels (Fig. 4*B*). Unbiased stereological cell counts of TH immunoreactive DA neurons in the SNpc revealed analogous increase in both homo- and heterozygous *Gdnf* 3'UTR-crR animals at P7.5 (Fig. *4C*). Similarly, quantification of the number of all monoaminergic neurons using immunostaining against vesicular monoamine transporter 2 (VMAT2), revealed a tendency towards increase in both hetero- and homozygous mutants, which did not reach statistical significance (Fig. 4*D*). DA metabolite levels in the rostral brain at P7.5 remained unaltered in Gdnf 3'UTR+/- and -/- mice (Fig. S4*G-H*). DA system and kidney development are not linked, since kidney function was severely compromised only in *Gdnf* 3'UTR-crR homozygous animals, which was further confirmed by correlation analysis of kidney and DA system parameters in individual heterozygous animals (Table S3).

Nigrostriatal DA system in adult Gdnf 3'UTR-crR+/- mice

Next we addressed the effects of GDNF derepression in adult mice. At 2.5-3 months of age tissue DA levels in *Gdnf* 3'UTR-crR+/- mice striatum were elevated (Fig 4*E*). To control for the effects of one mutant allele, we measured DA levels in *Gdnf* KO heterozygous mice. In

line with previous studies (32, 33), DA levels in *Gdnf* KO heterozygous mice were at the wt level (Fig. 4*E*). Restoration of wt *Gdnf* levels restored wt DA levels (Fig. 4*F*). Similar to P7.5, we found no change in striatal DA metabolite levels and turn-over in striata of adult *Gdnf* 3'UTR-crR+/- mice (Fig. S4*I-K*). Unbiased stereological cell counts of TH immunoreactive DA neurons in the SNpc revealed a tendency towards increase (13%) in *Gdnf* 3'UTR-crR+/- animals which did not reach statistical significance (Fig. *4G*). However, unbiased stereological cell counts of VMAT2 immunoreactive neurons revealed a significant increase in the SNpc *Gdnf* 3'UTR-crR+/- animals (Fig. 4*H*). Microscopy analysis of TH immunostaining of striatum and midbrain revealed no gross anatomical or other changes (Fig. S4*L-M*).

Dopamine transporter activity in adult *Gdnf* 3'UTR-crR+/- mice

To assess dopamine transporter activity in adult Gdnf 3'UTR-crR+/- mice, we measured extracellular DA clearance rate in the striatum using *in vivo* amperometry and found that DAT activity is increased up to five fold in a DA concentration-dependent manner in *Gdnf* 3'UTR-crR +/- mice (Fig. 5*A*). While striatal DA uptake rate in wt littermate controls reached a plateau, DAT activity in the striata of *Gdnf* 3'UTR-crR +/- animals increased at all DA concentrations tested (Fig. 5*A*). Measurements of total DAT protein levels in the nigrostriatal system revealed no difference between genotypes (Fig. S5*A*). Changes in DAT activity in amperometry measurements occur within seconds suggesting that DAT activity in *Gdnf* 3'UTR-crR +/- mice is regulated by fast post-translational modifications and/or protein-protein interactions. Amphetamine releases DA from synaptic vesicles into the cytoplasm and reverses its flow across the synaptic DAT, increasing extracellular DA and concomitantly inducing an increase in locomotor activity (34). To gain insight whether the observed augmentation in DAT function can be related to changes in locomotor function, mice were injected i.p. with

1mg/kg amphetamine. Compared to wt littermate controls, amphetamine-induced locomotor activity was significantly enhanced in *Gdnf* 3'UTR-crR +/- mice (Fig. 5*B*), suggesting that DAT activity is enhanced also in awake *Gdnf* 3'UTR-crR +/- animals.

Effects of GDNF derepression on DA system in lactacystin model of Parkinson's disease

Mouse models phenocopying the slow progression of human PD are not available(35). Currently, the most widely used animal models for PD are based on toxins MPTP and 6-OHDA, which are delivered specifically into the DA neurons via DAT (35). Since DAT activity in *Gdnf* 3'UTR-crR+/- mice is five-fold increased (Fig. 5*A*) the use of DAT-based toxins would confound the outcome. Therefore, we looked for alternative PD models. Abnormal protein aggregation is generally accepted as a pathological process common in most neurodegenerative disorders including PD. In line with this, intracranial application of proteasome inhibitors such as lactacystin (LC) has recently been shown to induce PD in rodent and fish models (36-38). We found that unilateral supranigral LC injection induced significant side bias in corridor test in wt, but not in *Gdnf* 3'UTR-crR heterozygous mice (Fig. 5*C*). DA and its metabolite levels were better preserved in the *Gdnf* 3'UTR-crR +/- mice (Fig. 5*D-F*). DA cell number in the SNpc was comparable between the genotypes (Fig. 5*G*) suggesting that the rescue in *Gdnf* 3'UTR-crR +/- mice occurs at the level of neuronal function.

Although ectopic GDNF application into the nigrostriatal DA system has beneficial dopaminotrophic effects in PD models and clinical trials, it also induces undesired effects such as hyperactivity (9, 10, 12, 13, 16), down-regulation of TH levels(13, 14), and reduction

in food-intake and bodyweight (9, 39). In Gdnf 3'UTR-crR +/- mice these features were not observed (Fig. 5*H-K*, Fig. S5*B-E*).

DISCUSSION

During the recent decade, 3'UTR-s have emerged as important sites of gene levels regulation by binding of micro-RNA-s, RNA binding proteins, etc but genetic studies have remained scarce (2). We found that *Gdnf* levels are negatively regulated via its 3'UTR. Our mutational analysis strongly suggests that miR-96 and miR-146a directly interact with *Gdnf* 3'UTR via the predicted binding sites. Online datasets from two genome wide screens for identifying miR target mRNA-s: i) Argonaute cross-linked immunopercipitation followed by RNA sequencing analysis i.e. miR CLIP-seq dataset; and ii) microarray analyses following miR transfection dataset, both suggested that miR-9 and miR-96 may directly interact with *Gdnf* 3'UTR in mouse brain and regulate *Gdnf* expression in cell-lines (40), http://servers.binf.ku.dk/antar/. Our data validates data from genome-wide screens suggesting functional interaction between miR-9 and miR-96 with *Gdnf*. In addition to miR-9, miR-96 and miR-146a, our data propose miR-133a, miR-133b, miR-125a-5p, miR-125b-5p, miR-30a and miR-30b as potential regulators of GDNF levels. Our results with miR-s also suggest that GDNF levels may be fine-tuned by a single miR, or more likely, by concerted action of several miR-s. Unlike in plants and insects, mammalian 3'UTR-s most often contain multiple conserved miR sites suggesting that the effect of miR-s on gene expression in mammals is additive and/or coordinated by several miR-s (26, 41). Which miR or combination of miR-s regulates *Gdnf* levels in PV+ striatal neurons remains to be identified. We also observed that *Gdnf* mRNA in *Gdnf* 3'UTR-crR +/- mice is significantly derepressed

only in the brain areas of higher *Gdnf* mRNA expression. The reason for this is unknown but may relate to relative concentration ratio of mRNA and inhibiting miR's and/or to the fact that miRs have different effects on their target mRNA-s depending on the tissue and developmental stage (42). A common notion that cellular environment is important in 3'UTR regulation is also illustrated by our finding that in human cells and mouse tissues, GDNF derepression is more pronounced in kidney than in brain cells. Future research will reveal the molecular mechanisms involved.

Classical GDNF KO mice die at birth due to the lack of kidneys with intact midbrain dopamine system which largely matures postnatally. Thus there is very little *in vivo* data on the postnatal function of endogenous GDNF in midbrain neurons. Despite clinical relevance, to date only a single study with conditional GDNF deletion in adult mice has been published (43). Because *Gdnf* 3'UTR-crR+/- mice are viable, we were able to analyze the role of GDNF in the function of postnatal DA system. We found that GDNF regulates DA cell number during development and adult SN, striatal tissue DA levels, DAT activity in a DA concentration-dependent manner, and prevents the decline in striatal DA levels in supra-nigrally injected lactacystin model of PD. Data on uncovered GDNF functions, and comparison to results from ectopic GDNF is summarized in Table 1.

Adverse effects associated with ectopic GDNF application such as downregulation of TH levels, loss of appetite and bodyweight, and hyperactivity were not observed in *Gdnf* 3'UTR-crR mice (Table 1). Interestingly, while application of low doses of ectopic GDNF into the DA system has no effect on DA system function, ectopic GDNF amounts exceeding hundreds of times the endogenous GDNF levels transiently increase DA turnover without affecting total striatal DA levels (9, 13, 16). We find that elevation of endogenous GDNF does the opposite -

augments tissue DA levels, not the turn-over (Table 1), suggesting a qualitative difference between the two sources of GDNF. Future studies using mice with conditional GDNF 3'UTR replacement (see below) will reveal the effects of GDNF derepression in adult animals.

We also compared *Gdnf* 3'UTR-crR+/- mice with constitutively active GDNF receptor Ret (Met919Thr, MEN2B) animals (44). While striatal DA levels, TH+ cell number and striatal DAT activity are increased in both mutants, elevation in DAT and TH protein levels, increased DA turn-over and reduced spontaneous locomotion was specific to MEN2B mice(45-47). Thus, derepression of endogenous GDNF is not equal to constitutively active Ret, a finding which may have broader implications for drug design and studies on receptor/ligand biology. Finally, in line with findings in MPTP model of PD in Ret conditional KO mice (48), we find that GDNF signalling is important in preserving striatal DA levels and function rather than TH+ cell bodies in proteasome inhibitor lactacystin induced PD model.

Sequence comparison of arbitrarily selected genes with known regulatory functions in brain development and plasticity revealed long 3'UTRs with the majority of miR sites conserved between mouse and man for many genes (Fig. 5*L*). Our results suggest that 3'UTR replacement can provide information inaccessible with, and complementary to the previously available tools. We propose a "Flex-cassette"(21) based approach enabling conditional 3'UTR replacement (Fig. 5*M*). Such animals would constitute an "opposite" system to classical conditional gene knock-out, which may enable to expand knowledge on gene function and regulation *in vivo*. Our results also indicate that the 3'UTR of GDNF and its regulators may be interesting drug targets for the development of treatment for PD.

Materials and Methods

Experiments were performed using routine methods applied in such studies. Details with appropriate references are provided in Online Materials and Methods.

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Author contributions

JOA conceived the GDNF miR regulation and GDNF 3' UTR replacement idea *in vitro* and *in vivo*, planned and executed experiments, provided funding and wrote the paper. AK and KV performed experiments with miR-s *in vitro*, JK and JM measured DA levels and counted DA neurons, MJ, RO, EP, KL and TT performed various *in situ* analysis of *Gdnf*, JK performed behavioural studies with amphetamine and corridor test, CA, NK and VV all other behavioural assays, LP measured DAT and TH levels with WB, KV and MAH *Gdnf* mRNA levels in various brain regions with qPCR, PM and NN performed amperometry, IS, ML and

PP planned experiments, MS initiated *Gdnf in vivo* studies, provided funding, planned experiments, and wrote the paper.

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FIGURE LEGENDS

Figure 1. Characterization of inhibitory property of *Gdnf* 3'UTR and design of the conditionally reversible Replacement (*Gdnf* 3'UTR-crR) allele.

(*A*) Mouse *Gdnf* 3'UTR was cloned to proceed Renilla (Ren) and Firefly (FF) luciferase coding sequence in the Dual-Luciferase Reporter Assay System (Fig. S1*A*). Comparison with SV40 late pA revealed a strong inhibitory property for *Gdnf* 3'UTR proceeding both reporter genes in human embryonic kidney HEK-298 cells and about twofold weaker inhibitory property in brain derived U87 glioblastoma cell line. **(*B*)** Schematic representation of a principle of the proposed conditionally reversible Replacement of a 3'UTR. **(*C*)** Expression from equimolar amounts of FF-*Gdnf* 3'UTR, FF-*Gdnf* 3'UTR-crR and FF-SV40 late pA constructs reveals eight fold derepression of FF-*Gdnf* 3'UTR-crR in human embryonic kidney cells (HEK293) and two fold derepression in brain derived glioblastoma U87 cells. N≥3 experiments with 3-4 repeats per construct/cell line per experiment; pooled data from all experiments is shown, Error bars indicate SEM; ** and *** P< 0.01 and 0.001 respectively, data was analyzed with Student's t-test. **(*D*)** Schematic representation of conditionally reversible Replacement allele for *Gdnf* 3'UTR (*Gdnf* 3'UTR-crR), *A* and *B* designate primers used in QPCR analysis. bGHpA-bovine Growth Hormone polyadenylation signal, PR/TK- bifunctional marker gene(20), black triangle-loxP site, F-FRT site.

Figure 2. Site and levels of GDNF expression in *Gdnf* 3'UTR-crR mice.

(*A*) QPCR analysis of *Gdnf* mRNA levels at post-natal day 7.5 (P7.5) in rostral brain; heterozygous *Gdnf* KO mice are known to harbor ~2-fold less *Gdnf* mRNA, serves as a control for QPCR sensitivity. **(*B*)** ELISA analysis of GDNF protein levels at post-natal day 7.5 (P7.5) in rostral brain. **(*C*)** QPCR analysis of *Gdnf* mRNA levels in adult brain, removal of the 3'UTR- crR cassette by crosses to Deleter-FLP line (Fig. 1*D*) resulted in restoration of wt *Gdnf* mRNA levels, *Gdnf* 3'UTR-rest, 4-10 animals per genotype always including controls from the same litter were analyzed in 2-8 experiments each containing 3-4 replicates. **(*D*)** ELISA analysis of GDNF protein levels in dorsal striatum of adult mice, N=5 per genotype. **(*E*)** *In situ* hybridization analysis of *Gdnf* mRNA expression in the striatum at P12.5; white triangles point cells with clearly detectable *Gdnf* expression in both genotypes, black triangles denote cells with "borderline" detection in the wt but clear signal in +/- animals; scale bar upper, 500µm and lower 50µm. **(*F*)** the number of parvalbumin (PV), marker of *Gdnf* expressing cells in the striatum is comparable between genotypes; N=4 +/+ and N=7 *Gdnf* 3'UTR+/- mice; two tissue slices and 45-55 microscopic fields per animal were analyzed. dSTR-dorsal striatum, OB-olfactory bulb, Hyp-hypothalamus, PFC-prefrontal cortex, NAc-Nucleus Accumbens, Hip-hippocampus, SN-substantia nigra, VTA-ventral tegmental area, RN- dorsal Raphe nucleus, CB-cerebellum. Error bars indicate SEM, data was analyzed with Student's t-test; +/+ indicates wt animals, *, ** and *** are $P<0.05$; 0.01 and 0.001 respectively.

Figure 3. Evolutionary conservation and regulation of *Gdnf* 3'UTR by miR-s.

(*A*) The 3'UTR of *Gdnf* mRNA is evolutionarily conserved. Exons and 3'UTR are drawn in scale. Percent of identities between human (Hs) and mouse (Mm) sequences is indicated, the conserved miR binding sites cluster within the areas conserved between Mm and Hs.

Shown are conserved sites for miRs broadly conserved among vertebrates; miRs underlined with a red bar were identified as "hits" in two existing genome wide screens i) Argonaute cross-linked immunopercipitation followed by RNA sequencing analysis (miR CLIP-seq dataset), and ii) microarray analyses following miR transfection dataset(40). Source: Blast, TragetScan. **(*B*)** The *Gdnf* 3'UTR analyzed using Dual-Luciferase Reporter Assay System in HEK-293 cells either with negative control pre-miRs (N1, N2) or putative *Gdnf* 3'UTR-regulating pre-miRs. **(*C*)** Two nucleotide mutation within miR-96 and miR-146a seed sequences in the *Gdnf* 3'UTR specifically abolishes their inhibitory properties. **(*D-E*)** Endogenous GDNF protein and mRNA in human U87 glioblastoma cells is suppressed by miRs in an additive manner. **(*F*)** Lack of miR mediated suppression from FF-*Gdnf*-3'UTR-crR construct (Fig. *S*1A) in HEK-293 cells. Experiments were repeated at least 3 times with 3-5 replicates within the experiment, error bars indicate SEM, data was analyzed with Student's t-test; *, ** and *** are P<0.05; 0.01 and 0.001.

Figure 4. Characterization of the nigrostriatal dopamine system at P7.5 and in adult mice

(*A*) Levels of phosphorylated ERK2 at P7.5 in the striatum of *Gdnf* 3'UTR-crR-/- mice is increased, N=4 animals per genotype, data averaged from two western blotting experiments, GAPDH and ERK were used for normalization, N=5 animals per genotype. **(*B*)** HPLC analysis of rostral brain DA levels at P7.5. Left: N=8 wt , N=5 *Gdnf* 3'UTR-crR +/- and N=8 *Gdnf* 3'UTR-crR -/-, and right: removal of the *Gdnf* 3'UTR replacement cassette from *Gdnf* 3'UTR-crR animals by crosses to Deleter-FLP generate *Gdnf* 3'UTR-restored mice (Fig. 1*D*) with wt DA levels at P7.5; N=5 wt and N= 5 Gdnf 3'UTR-rest -/- (F=7.44, P=0.016). **(*C*)** Unbiased stereological cell counts of TH immunoreactive neurons (F= 7.44, P=0.0048) and

(***D***) VMAT2+ neurons in substantia nigra pars compacta (SNpc) at P7.5 reveal increase in *Gdnf* 3'UTR-crR +/- and *Gdnf* 3'UTR-crR -/- animals, N=7 animals per genotype, except N=6 wt animals in TH staining and N=6 *Gdnf* 3'UTR-crR -/- animals in VMAT2 staining; $p<0.01$ only for TH+ cells. **(*E*)** DA levels in the dSTR of adult 3'UTR-crR +/- mice are elevated; DA levels are not elevated in the dSTR of *Gdnf* KO +/- mice; N=5-8 animals analyzed per group. **(*F*)** DA levels are not elevated in the dSTR of adult Gdnf 3'UTR-rest -/- mice, compared to wt littermate controls, N=6 animals per genotype. **(*G-H*)** Number of DA cells in the SNpc of adult *Gdnf* 3'UTR-crR +/- mice is increased as revealed by stereological countings of TH+ and VMAT2+ cells, N=8 animals per genotype, * $p<0.02$ only for VMAT2 positive neurons.

Figure 5. Effects of elevated endogenous GDNF on adult dopamine system function

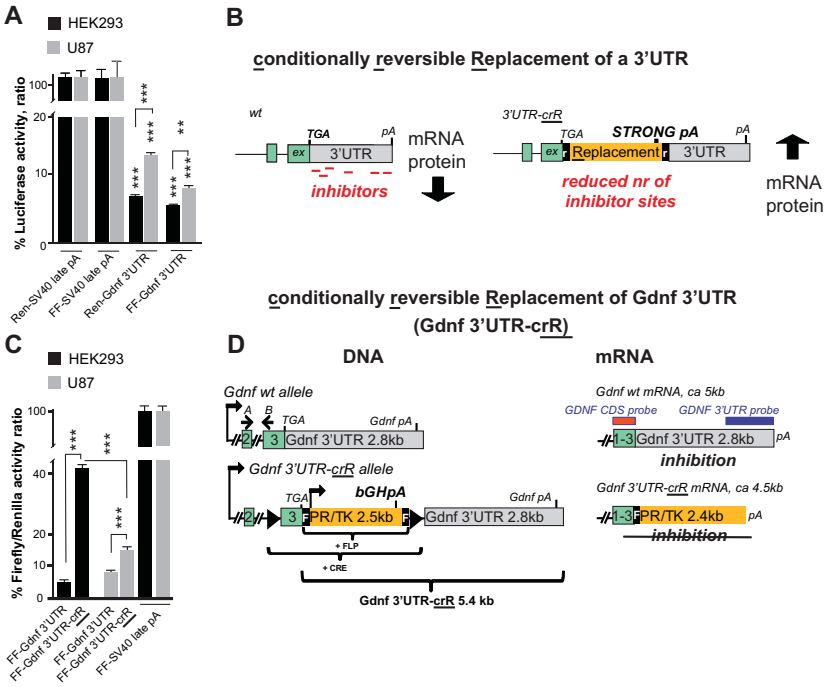
(*A*) *In vivo* amperometry; dopamine concentration-dependent increase in dopamine transporter (DAT) activity in *Gdnf* 3'UTR-crR +/- mice is revealed by intrastriatal DA injection. Dopamine peaks (µM) are separated into amplitude bins and plotted against uptake rate (µM/s; calculated using Michaelis-Menten first-order rate constant, k1; N=4 animals per genotype ($F=47,931$; * $P<0.05$; ** $P<0.01$; *** $P<0.001$ between wt and *Gdnf* 3'UTR-crR +/- mice). **(*B*)** Compared to wt gender matched littermate controls (N=10), amphetamine (1mg/kg, i.p.) increases locomotor activity in *Gdnf* 3'UTR-crR +/- mice (N=9); $F= 4.386$, $P= 0.040$). Experiment was repeated 3 times with similar results, representative experiment is shown. **(*C*)** Corridor test in a proteasome inhibitor lactacystin (LC) based Parkinson's disease (PD) model in 3 month old mice (N=7 *Gdnf* 3'UTR-crR +/- mice; N=5 wt mice) indicated a lesion in the wt but not in *Gdnf* 3'UTR-crR +/- mice right hemisphere at 5 weeks post LC injection ($F=6.087$, $P=0.033$). **(*D-F*)** At the endpoint at 5 weeks post LC injection, dorsal striatum (dSTR) DA and its metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) and

homovanillic acid (HVA) levels are better preserved in *Gdnf* 3'UTR-crR +/- mice, as revealed by HPLC analysis, expressed as a percent of the levels in the unlesioned side. **(*G*)** 5 weeks after LC injection, mean number of TH+ neurons in SNpc does not differ between the genotypes. **(*H-K*)** Evaluation of side effects associated with intracranial ectopic GDNF expression; **(*H*)** Spontaneous motor activity of *Gdnf* 3'UTR-crR +/- mice is not increased, N=31 +/- and N=34 wt mice. **(*I*)** Optical density of striatal TH immuno-staining is not changed *Gdnf* 3'UTR-crR +/- mice, N=10 wt, N=11 *Gdnf* 3'UTR-crR +/- animals. **(*J*)** Food intake in *Gdnf* 3'UTR-crR +/- mice is not changed N=10 wt, N=10 *Gdnf* 3'UTR-crR +/- animals **(*K*)** Bodyweight of *Gdnf* 3'UTR-crR +/- mice is not changed N=31 +/- and N=34 wt male mice; N=9 +/- and N=11 wt female mice. **(*L*)** Representative genes with known regulatory function in brain development and plasticity harboring long 3'UTRs with miR binding sites conserved between mouse (Mm) and man (Hs), bp-base pair, con-conserved. **(*M*)** a "Flex-cassette" (black and white triangles)(21) enables conditional 3'UTR replacement at the time and place of interest.

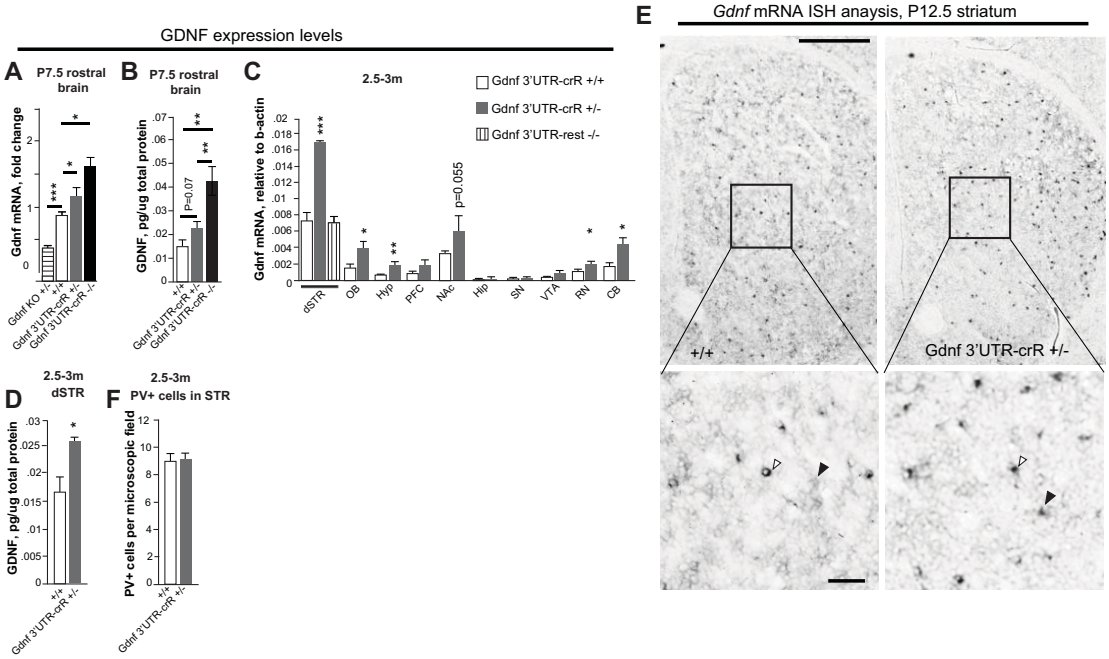
Table 1. GDNF function revealed by 3'UTR replacement method

Elevation of endogenous GDNF (eGDNF)	Ectopic GDNF expression	Method	Reference
elevated eGDNF increases DA neuron numbers in adult SNpc	GDNF does not control the number of dopamine neurons in the SNpc after two weeks of age	striatal transgenic overexpression of GDNF	(49)
elevated eGDNF augments DA concentration dependent increase in DAT activity	na	na	na
no effect on food intake and bodyweight at least until 3-4 months of age	reduction in food intake and weight loss within 1-2 weeks	nigrostriatal recombinant GDNF injection and viral gene delivery	(9, 39)
no effect on striatal TH levels at least until 3 months of age	downregulation of striatal tyrosine hydroxylase levels after 6 weeks	nigrostriatal viral gene delivery	(13, 14)
no effect on spontaneous motor activity	spontaneous motor hyperactivity	nigrostriatal recombinant GDNF injection	(9, 10, 12, 16)
no effect on DA turnover, elevation in total striatal DA levels	elevation in DA turnover, no effect on total striatal DA levels	nigrostriatal recombinant GDNF injection and viral gene delivery	(9, 13, 16)
Overlapping with ectopic GDNF expression			

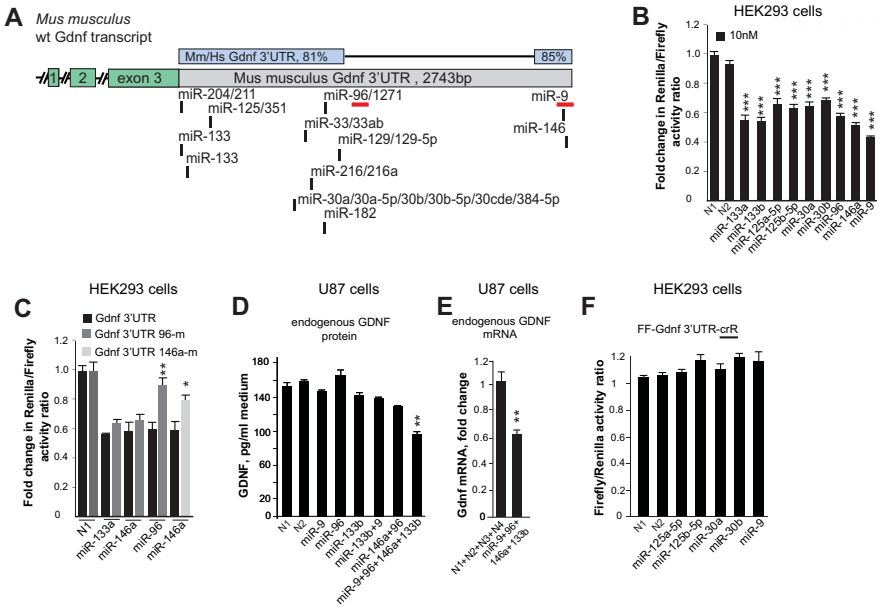
increased motor activity upon amphetamine injection	yes	nigrostriatal recombinant GDNF injection and viral gene delivery	(9, 13)
GDNF controls DA cell number at P7.5	yes	striatal transgenic overexpression of GDNF	(49)
maintains striatal DA levels upon supra-nigral LC injection	yes	striatal viral gene delivery	(50)



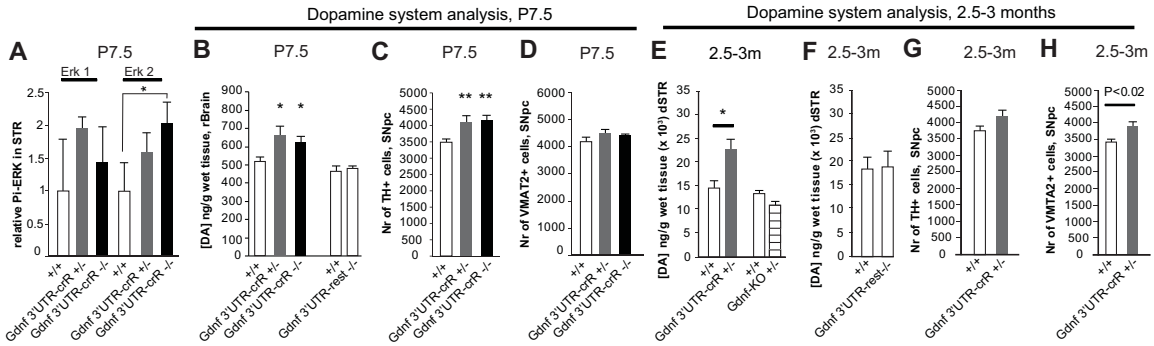
Andressoo, FIGURE 1



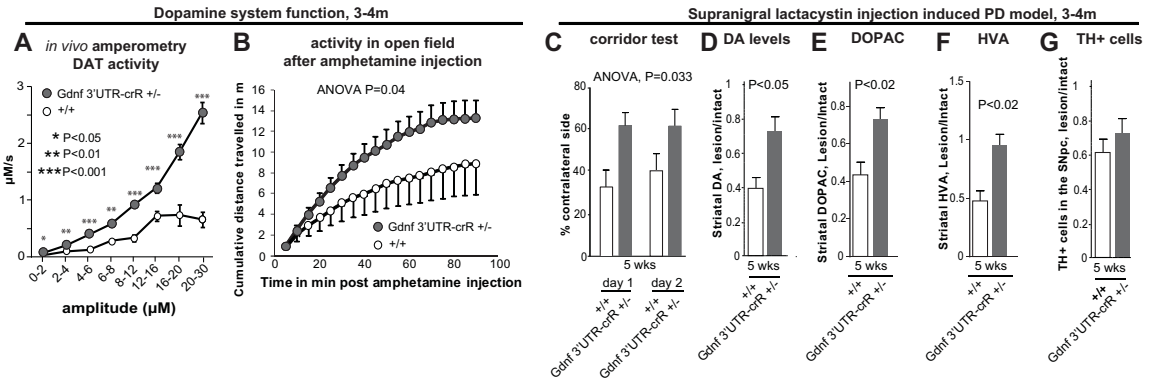
Andressoo, FIGURE 2

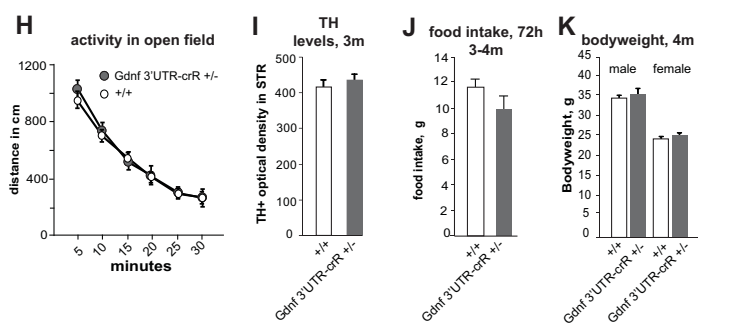


Andressoo, FIGURE 3



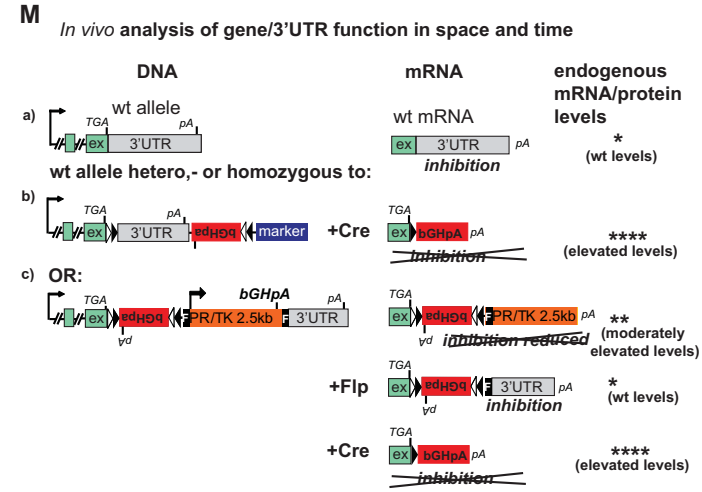
Andressoo, FIGURE 4

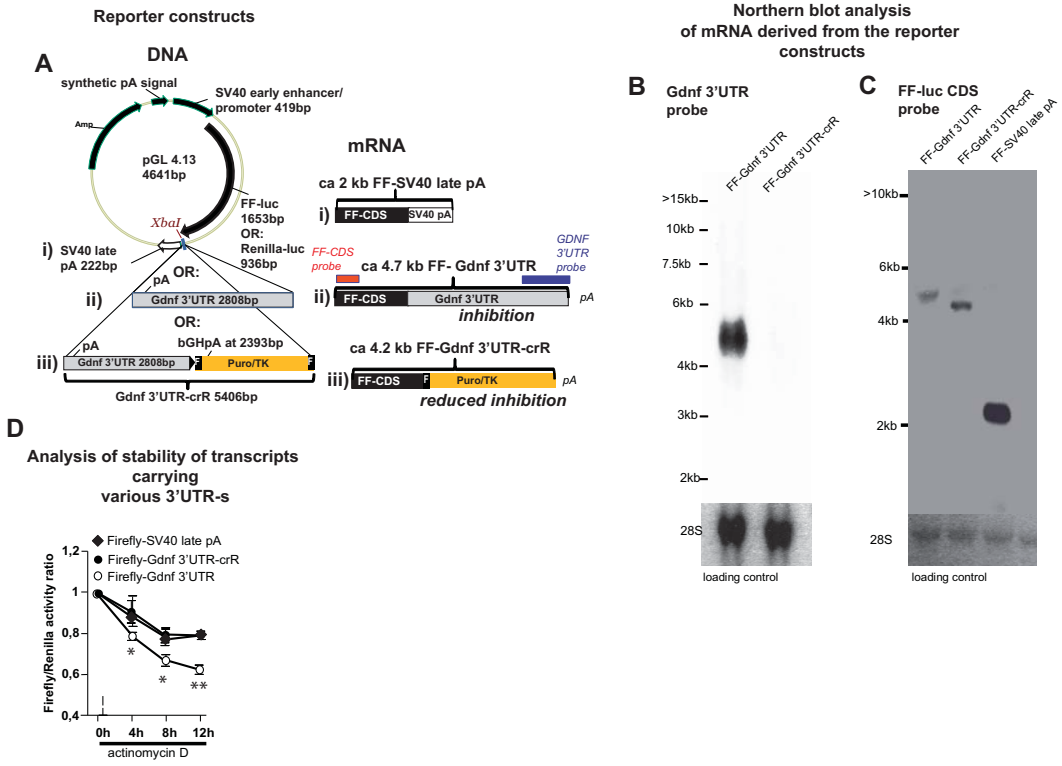




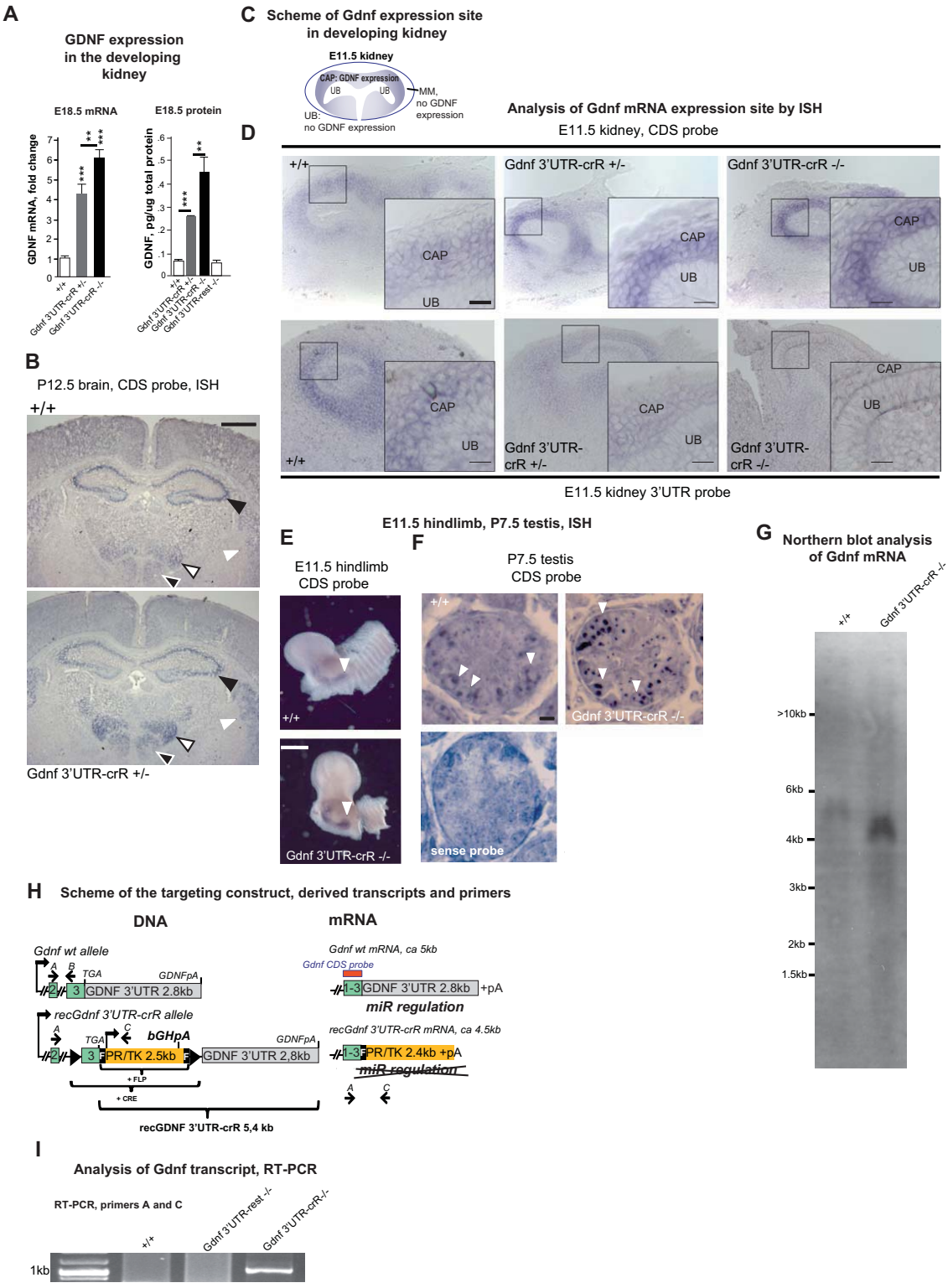
L

Gene	Gdnf	Bdnf	EfnA3	EfnB2	Ntn1	En2	Robo1	Sema3A	Ncam1
3'UTR bp	2743	2891	954	3141	3632	1764	1730	2954	3275
nr of con. miR sites	12	13	6	15	5	15	6	11	7
identical sites Mm/Hs	10	11	6	14	5	13	6	11	7



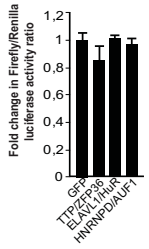


Andressoo, Supplementary FIGURE 1



Andressoo, SUPPLEMENTARY FIGURE 2

A



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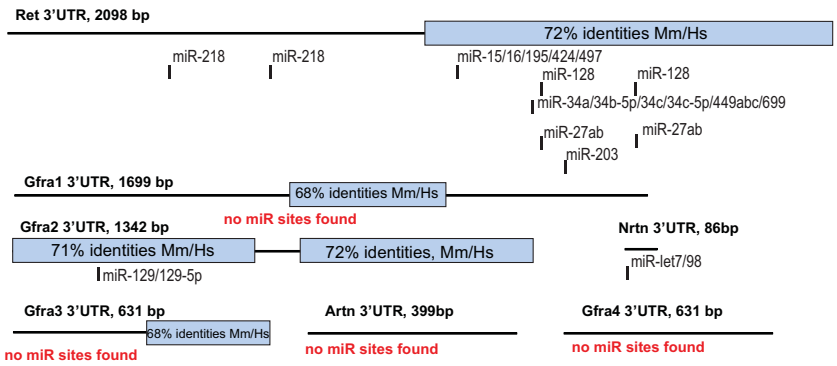


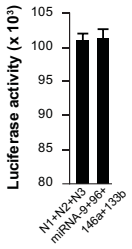
C

Dorsal striatal expression levels of mature miRs with conserved bindings sites in Gdnf 3'UTR

tissue	sno202	miR-9	miR-30a	miR-30b	miR-33a	miR-96	miR-125a-5p	miR-125b-5p	miR-129	miR-133a	miR-133b	miR-146a	miR-182	miR-204
Mm, dSTR 10w	1	0.97 ± 0.14	0.014 ± 0.006	0.16± 0.08	ne	ne	1.46 ± 0.33	0.088 ± 0.060	ne	0.067 ± 0.020	ne	0.067 ± 0.006	ne	0.35 ± 0.23

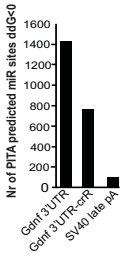
Survival of U87 cells

D



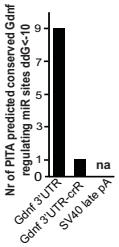
nr of total predicted miR sites

E

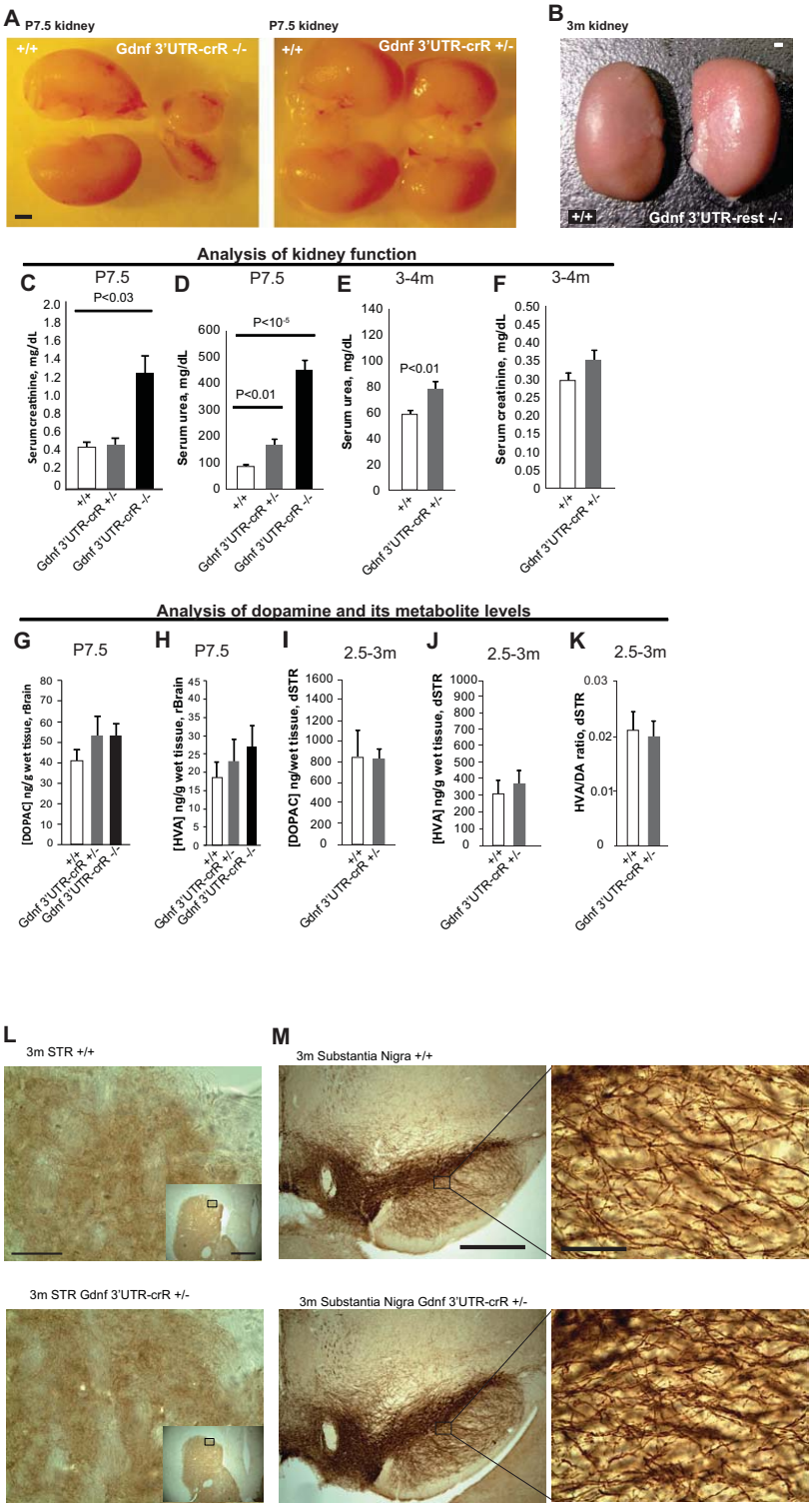


nr of Gdnf 3'UTR specific miR sites

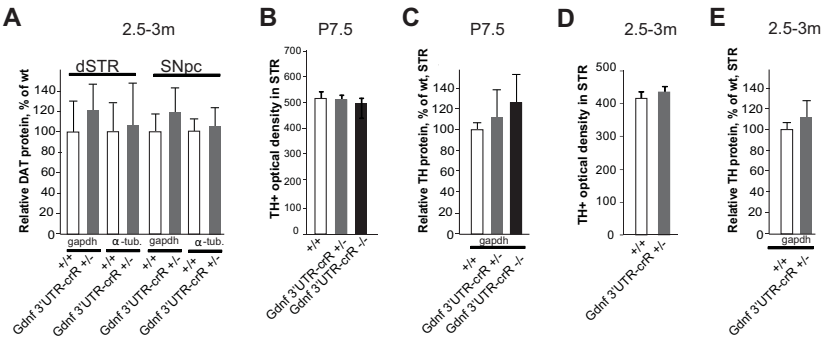
F



Andressoo, Supplementary FIGURE 3



Andressoo, SUPPLEMENTARY FIGURE 4



Andressoo, SUPPLEMENTARY FIGURE 5

Andressoo et al, Supplementary Information

Supplementary Figure 1, related to Figure 1

***In vitro* analysis of *Gdnf* 3'UTR.**

(*A*) Schematic representation of the reporter constructs and the derived mRNA used in this study. Bovine growth hormone polyadenylation signal (bGHpA) at the 3' end of the Puro-TK cassette is well established to efficiently induce termination of transcription in genome-wide gene-trap experiments in mice (Consortium; Schnutgen et al., 2005). **(*B*)** Northern analysis of expression from construct harbouring Firefly (FF) coding sequence upstream of the conditionally reversible Replacement cassette for *Gdnf* 3'UTR (Fig. 1*D* and Fig. S1*A*) using a probe complementary to *Gdnf* 3'UTR revealing a lack of transcription to *Gdnf* 3'UTR while the derived FF luciferase mRNA (Fig. S1*C*) and protein (Fig. 1*C*) are clearly detectable. 28S ribosomal RNA serves as loading control, experiment was repeated twice. **(*C*)** Northern analysis of total RNA from HEK293 cells transfected with constructs depicted on (*A*) using a probe complementary to Firefly luciferase coding sequence, experiment was repeated 2 times. **(*D*)** Compared to FF-*Gdnf* 3'UTR, a product derived from FF-*Gdnf* 3'UTR-crR or FF-SV40 late pA is more resistant to transcriptional inhibition (10 μm of actinomycin D), indicating negative regulation of *Gdnf*-3'UTR bearing transcripts at the post-transcriptional level. HEK-293 cells were transfected with Renilla-SV40 late pA together with either FF-*Gdnf* 3'UTR, FF-*Gdnf* 3'UTR-cr or FF-Sv40 late pA, data is derived from 9 biological replicates assayed in three experiments. * $p<0.05$; ** $p<0.01$, error bars represent SEM, FF-firefly luciferase, Renilla- Renilla luciferase.

Supplementary Fig. 2, related to Figure 2

Analysis of *Gdnf* mRNA expression in *Gdnf* 3'UTR-crR mice.

(*A*) QPCR and ELISA analysis of *Gdnf* mRNA and protein levels at embryonic day 18.5 (E18.5) in kidney. **(*B*)** *In situ* hybridization analysis of *Gdnf* mRNA expression in coronal cryo-sections from brain at P12.5 using a probe recognizing GDNF exons revealed no differences in the site of expression between genotypes, white triangle points striatum, triangle with black edge - ventromedial thalamus; triangle with white edge - thalamic nuclei; black triangle-hippocampus, scale bar 1 mm. **(*C*)** Scheme represents known location of *Gdnf* mRNA expression (blue) in developing kidney at E11.5. *Gdnf* mRNA is expressed at high levels in a structure called the cap (CAP) condensate of the metanephric mesenchyme (MM) sharply bordered by ureteric bud (UB), where *Gdnf* is not expressed. **(*D*)** *In situ* analysis of *Gdnf* mRNA expression (blue) on vibratome sections from the urogenital tract at E11.5. Site of *Gdnf* mRNA expression was indistinguishable between genotypes. Consistent with elevated GDNF levels in embryonic kidney (Fig. S2A), signal with a probe complementary to *Gdnf* exons as indicated with a red line in Fig. 1*D* is stronger in hetero- and homozygous *Gdnf* 3'UTR-crR mice. In accordance with *in vitro* results (Fig. S1*B-C*), and the established property of bGHpA to initiate termination of transcription *in vivo* (Consortium; Schnutgen et al., 2005) a probe recognizing the *Gdnf* 3'UTR gave a weaker signal in heterozygous and no signal in homozygous mice in the CAP (lower panel). The experiment was repeated 4 times, at least 4 animals were analyzed per genotype. Representative images are shown. Scale bar 10µm. **(*E*)** *In situ* hybridization analysis of *Gdnf* mRNA in *Gdnf* 3'UTR-crR -/- mice at the anatomical level in whole-mount preparations of E11.5 hind limb. Establishment of limb innervation is known to depend upon controlled temporospatial expression of GDNF(Kramer et al., 2006). *Gdnf* expression island (stained blue, indicated with white arrow heads) is comparable between genotypes, experiment was repeated 4 times with N=animals per genotype, scale bar 1.5 mm. **(*F*)** *In situ* hybridization analysis of *Gdnf* mRNA in *Gdnf* 3'UTR-crR -/- mice in a 5 µm paraffin slice

from P7.5 seminiferous tubule of the testis. *Gdnf* mRNA is mostly expressed by large cells believed to be Sertoli cells(Meng et al., 2000; Sariola and Saarma, 2003) aligned mostly in the periphery of the tubule (white arrow heads), *Gdnf* mRNA signal appears stronger in *Gdnf* 3'UTR-crR mice, scale bar 5µm, *Gdnf* CDS probe, experiment was repeated 2 times. **(*G*)** Northern blot analysis of *Gdnf* mRNA in E18.5 testis, a timepoint when *Gdnf* mRNA is known to be particularly abundant in this organ facilitating the analysis. As expected (Fig. S1A-C) *Gdnf* CDS probe hybridizes to about ~500 bp shorter transcript in *Gdnf* 3'UTR-crR -/- mice, experiment was repeated 2 times with RNA derived from different animals. **(*H*)** Schematic representation of conditionally reversible Replacment allele for *Gdnf*. bGHpA-bovine Growth Hormone polyadenylation signal, PR/TK- bifunctional fusion protein between puromycin N-acetyltransferase and a truncated version of herpes simplex virus type 1 thymidine kinase (TK)(Chen and Bradley, 2000), black triangle-loxP site, F-FRT site. *A* and *B* designate primers used in QPCR analysis of *Gdnf* mRNA levels in *Gdnf* 3'UTR-crR mice, primers *A* and *C* depict primers used in (*I*)**,** for *Gdnf* transcript sequence analysis. **(*I*)** cDNA from E18.5 testis was used as a template for PCR reaction with primers *A* and *C*. The observed PCR product in *Gdnf 3'UTR-crR -/-* mice using primers *A* and *C* was of the expected length (977bp) and was sequenced for validation, experiment was repeated three times. Cp values indicating cDNA quality from testis for *beta-actin* were 18.04 ±0.2, 18.5 ±0.08 and 18.8 ±0.08 for cDNA derived from wt, Gdnf 3'UTR-rest-/- and Gdnf 3'UTR crR-/- mice respectively. * $p<0.05$, ** $p<0.01$, *** $p<0.001$; error bars indicate SEM at all graphs.

Supplementary Fig. 3, related to Figure 3.

Analysis of 3'UTR-s of GDNF family members, RNA binding proteins, miR effect and expression.

(*A*) Analyses of the *Gdnf* 3'UTR revealed three conserved AU-rich elements (black triangles on Fig. 3*A*), the putative binding sites for RNA binding proteins (RBPs), which may regulate mRNA levels. We tested overexpression of the most common RBPs, tristetraprolin (TTP/ZFP36), embryonic lethal abnormal vision like protein 1 (ELAVL1/HuR) and

heterogeneous nuclear ribonucleoprotein D0 (HNRNPD/AUF1) in our reporter assays. Consistent with an independent, parallel study(Oh-Hashi et al., 2012), RBPs were found to have no or little effect. HEK293 cells were co-transfected with either negative control, green fluorescent protein (GFP), or putative *Gdnf* 3'UTR-regulating RBPs. TTP/ZFP36 reduced reporter construct levels by about 10% ($P<0.05$), data is derived from 9 biological replicates assayed in three experiments. **(*B*)** Depicted are the 3'UTR-s of neurturin (*Nrtn*), artemin (*Artn*), primary binding receptors *Gfrα1-4* and the common signaling receptor tyrosine kinase *Ret*. The most stringent default search modes in TargetScan for miR seed sequence identification, and Blast for homology search were used. Identical search criteria were used for the *Gdnf* 3'UTR (Fig. 3*A*). Note that besides *Gdnf* (Fig. 3*A*) only the 3'UTR of *Ret* contains multiple conserved miR target sites for miRs broadly conserved in vertebrates. *Nrtn* and its primary receptor *Gfrα2* harbor a single conserved miR site each and other family members harbor no conserved miR sites. Conserved sequence areas are depicted as colored bars, percentage indicates number of identities between *Mus musculus* and *Homo sapiens*. Relative lengths of 3'UTRs are drawn to scale. No data from the www was found for *Pspn* 3'UTR. **(*C*)** Levels of mature miRs expressed as fold difference relative to sno202 levels in adult (10weeks) mouse (Mm) dorsal striatum. Mature miR levels were assessed with TaqMan QPCR; 2 experiments were performed with 4 repeats per miR per QPCR run. **(*D*)** Co-transfection of a combination of pre-miRs is non-toxic to cells, as assayed with CellTiter-Glo Luminescent Cell Viability Assay (Promega), N-negative control, experiment was repeated 3 times. **(*E-F*)** Number of bioinformatics (PITA(Kertesz et al., 2007)) predicted miR sites in *Gdnf* 3'UTR, Puro/TK cassette (3'UTR-crR cassette) and bGHpA; and SV40 late pA, error bars represent SEM.

Supplementary Fig. 4, related to Figure 4

Analysis of kidneys and dopamine system in *Gdnf* 3'UTR-crR mice.

(*A*) Representative images of kidneys of *Gdnf* 3'UTR-crR -/- (N=10, left panel) and +/- (N=20, right panel) animals at P7.5. In about half of the *Gdnf* 3'UTR-crR +/- animals the size of the kidneys was reduced up to ~25%, whereas kidneys in all -/- animals were miniscule. **(*B*)** Kidneys in the *Gdnf* 3'UTR-rest -/- animals are normal, N=20 animals analyzed per genotype. Scale bars on *A* and *B* are 1 mm. (***C-D***) Kidneys of *Gdnf* 3'UTR-crR -/- function poorly, as revealed by measurements of serum urea (N= 7 *Gdnf* 3'UTR-crR -/-, N= 26 *Gdnf* 3'UTR-crR +/-, N= 16 wt) and creatinine (N= 3 *Gdnf* 3'UTR-crR -/-, N= 12 *Gdnf* 3'UTR-crR +/-, N= 7 wt) levels. **(*E*)** Serum urea levels in adult 4 month old animals were mildly increased in *Gdnf* 3'UTR-crR +/- animals. However, at least in humans, up to two-fold variation in serum urea levels between individuals is considered normal suggesting that kidney function in +/- animals is in a normal range (N= 16 wt, N= 19 *Gdnf* 3'UTR-crR +/-). **(*F*)** Consistent with (*E*), measurement of serum creatinine levels revealed no difference between genotypes at 4 months.

(***G-K***) Analysis of the dopamine system. We found that dopamine (DA) levels and dopaminergic neuron numbers were increased to an equal extent in the brain of both -/- and +/- *Gdnf* 3'UTR-crR mice (Fig. 4*B-C*) suggesting a lack of correlation between kidney and brain DA system development. To further address this, we performed correlation analyses of brain DA and serum urea levels in individual +/- animals at P7.5 and found that those parameters reflecting DA system and kidney function were indeed non-correlated (Supplementary Table 3).

(*G-H*) High performance liquid chromatography (HPLC) analysis of P7.5 rostral brain dopamine metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), N = 2 experiments, in the depicted experiment N=8 wt, N=5 *Gdnf* 3'UTR-crR +/-, N=8 *Gdnf* 3'UTR-crR -/-, no statistically significant difference was found **(*I-J*)** HPLC analysis of dopamine metabolites DOPAC and HVA in dSTR at 2.5-3 months of age reveals no difference between the genotypes. 5-8 animals were analysed per genotype. **(*K*)** HVA/DA ratio at 2.5-3 months of age reflecting DA turnover did not differ between the genotypes, 5-8 animals were analysed per genotype.

(*L-M*) TH+ immunostaining revealed no gross morphological changes in the STR (*L*) and midbrain of *Gdnf* 3'UTR-crR +/- mice, scale bar(*L)* 75µm, in inset 1mm, scale bar (*M*) 0.5 mm, in inset 30µm. Note that the arbitrary difference between genotypes on (*L-M*) reflects normal variation, N=8 animals per genotype.

Supplementary Fig. 5, related to Figure 5

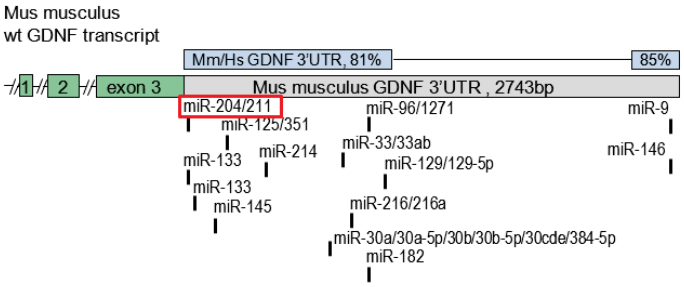
Analysis of DAT and TH protein levels in *Gdnf* 3'UTR-crR mice.

(*A*) DAT protein levels in dSTR and SNpc as measured by western blotting revealed no difference at 10 weeks of age, N= 5 animals per genotype, data averaged from two western blots, alpha-tubulin and gapdh were used to normalize loading. **(*B*)** Optical density (OD) measurements of striatal TH immunolabelling reflecting striatal TH levels and density of striatal dopaminergic innervation at the macroscopic level revealed no difference between genotypes at P7.5 (N=7 animals per genotype), OD of TH was analysed blind to genotype. **(*C*)** TH protein levels in dSTR as measured by western blotting revealed no difference at P7.5, N= 4 animals per genotype, data averaged from two western blots, gapdh was used to normalize loading. **(*D*)** OD measurements (as in *B*) of striatal TH immunolabelling in adult mice at 2.5-3 months of age did not differ between genotypes, N=10 wt, N=11 *Gdnf* 3'UTR-crR +/- animals. **(*E*)** TH protein levels in dSTR at 2.5-3months of age as measured by western blotting revealed no difference between wt and *Gdnf* 3'UTR-crR heterozygous mice, N= 5 animals per genotype, data averaged from three western blots, gapdh was used to normalize loading.

Supplementary Table 1, related to Figure 3
Alignment of conserved miR target sites within the 3'UTR of GDNF gene spanning 20bp 5' and 5bp 3' of the miR seed sequence.

Code: white-miR seed sequence, miR seed conservation, conservation outside miR seed

miR-204/204b/211



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M. mulatta (rhesus macaque)	AUUCCUGCUACAGUGCAAAGAAA----------GGGACCAAG
O. garnettii (bushbaby)	AUUCCUGCUACAGUGCAAAGAAA----------GGGACCAAG
T. belangeri (treeshrew)	AUUCCUGCUACAGUGCAAAGAAA----------GGGACCAAG
R. norvegicus (rat)	AUUCCUGCUACAGUGCGAAGAAA----------GGGACCAAG
C. porcellus (guinea pig)	AUUCCUGCUACAGUGCAAAGAAA----------GGGACCAAG
O. cuniculus (rabbit)	AUUCCUGCUACAGUGCAAAGAAA----------GGGACCAAG
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E. europaeus (hedgehog)	AUUCCUGCCACGGGACAGGGACG----------GGGACCGAG
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F. catus (cat)	AUUCCUGCUACAGUGCAAAGAAA----------GGGACCAAG
E. f. caballus (horse)	AUUCCUGCUACAGUGCAAAGAAA----------GGGACCAAG
B. taurus (cow)	AUUCCUGCUGCAGUGCAAAGAAA----------GGGACCAAG
D. rerio (zebrafish)	----------
L. africana (elephant)	AUUCCUGCUACAGUACAAAGUAA----------GGGACCAAG
E. telfairi (tenrec)	AUUCCGGCUACAGUACAAAGUCA----------GGGACCAAG
M. domestica (opossum)	AUUCCUGCUACAGUGCAAAGAAA----------GGGACCAAG
O. anatinus (platypus)	AUUCCUACGACAGCGCAGAGGAA----------GGGACCACG
A. carolinensis (lizard)	AUUCCUGCUAAAAUGGCAAAGAA----------GGGACCAAG
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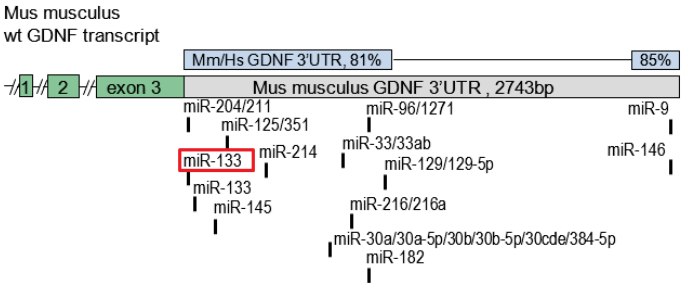
miR-133abc

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M. mulatta (rhesus macaque)        CUGCUACAGUGCAAAGAAA-----------------GGGACCAAGGUUC
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T. belangeri (treeshrew)           CUGCUACAGUGCAAAGAAA-----------------GGGACCAAGGUUC
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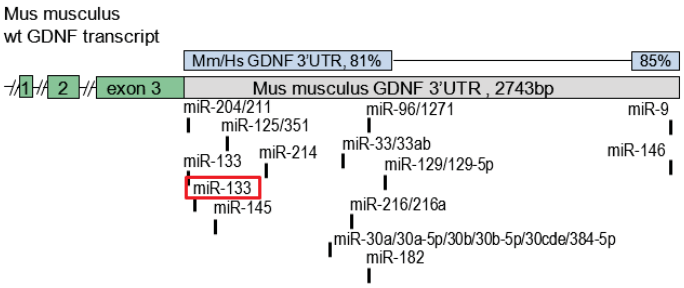


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T. belangeri (treeshrew)           UUGCUCAGAAUGGAAG-------AUGA--GGACCAAGGA---GG
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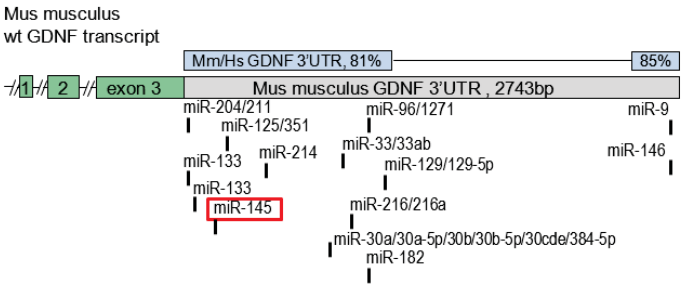


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E. europaeus (hedgehog)            AGG------GG----UC-------CGG-------------CCACAGACACCCGGACAGGA
C. l. familiaris (dog)             AGG------GA--GAUC-------CAG-------------CUACGGAGAAGCGGACGGGA
F. catus (cat)                     ------------------------------------------------------------
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D. rerio (zebrafish)               ------------------------------------------AACUGCACAG--
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O. anatinus (platypus)             AGA------GGAUGCUC-------CGG-------------CCACCGGGAAUGGGAUGGGG
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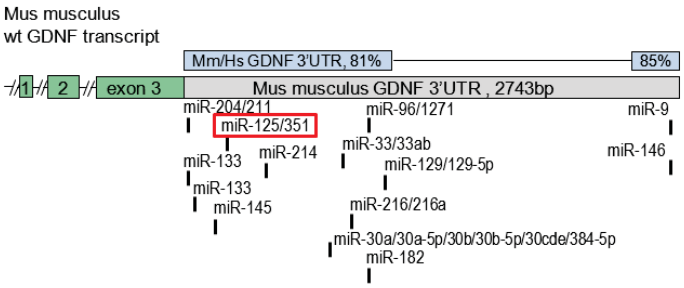


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F. catus (cat)                     ---------------------------------------------
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E. telfairi (tenrec)               GCAGC-CAA--CGGCAU------CCGGA---GCCCAGG-GCUGGU
M. domestica (opossum)             GCAACUUAA--AGGCAU------CACAA---GCACAGG-GCUGGU
O. anatinus (platypus)             GCAACUCGC--AGACAG------CCAAA---GUUCAGG-GCCGGG
A. carolinensis (lizard)           --------A--CGCAAACGGACAUCAAU---GCUCAGG-GCAGAC
G. gallus (chicken)                --------A--AGUAAA------UAAAA---GCUUAGGUGAGGAU
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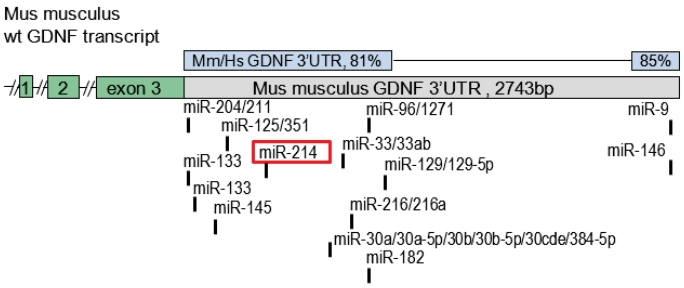


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M. mulatta (rhesus macaque)         UUCC--UUGUCAUUAUGUUUU-ACUGCUGAAAGUA
O. garnettii (bushbaby)             CUCC--UUGUUAUUACAUUUU-ACUGCUGAAAGGA
T. belangeri (treeshrew)            CUCC--UUGUCAUUACGUUUU-ACUGCUGAAAGUA
R. norvegicus (rat)                 CCUC--UUGUCACUACAUUUU-ACUGCUGAAAAUA
C. porcellus (guinea pig)           CUCC--UUGUGAUUACAUUCU-ACUGCUGAAAGUC
O. cuniculus (rabbit)               CUCC--UUGUCAUUAUGUUUU-ACUGCUGGAAGUC
S. araneus (shrew)                  UUCC--UUGUCAUUAUGUUUUUACUGCUGAAAGCA
E. europaeus (hedgehog)             --CC--UGGUCACUGUGUCGC--CUGCUGACAGCC
C. l. familiaris (dog)              UUCC--UUGU---GACGUUUC-ACUGCUGAAAGCA
F. catus (cat)                      UUCC--UUGUCGUGACGUUUU-ACUGCUGAAAGCA
E. f. caballus (horse)              UUCC--UUGCUGUUACAUUUU-ACCGCUGAAAGCA
B. taurus (cow)                     UUCC--UUGUCAUU--GUUUU-ACUGCUGAAAGCA
D. rerio (zebrafish)                GCCCACUUGUCCUUACGUUCU-ACUGCUGAAAGCA
L. africana (elephant)              GCCCACUUGUCAUGAUGUGUU-ACUGCUGAAAGCC
E. telfairi (tenrec)                GCCCACU-GCUGUGCCGCGUU-ACUGCGGCAAGUC
M. domestica (opossum)              ACUCGCGUGGCAUUAAGAGUC-AUUACUGAAAGUC
O. anatinus (platypus)              GCCCCUUUGUCAAGAAGCUUG-AUUAC-GGAAGUA
A. carolinensis (lizard)            ------------------------------------
G. gallus (chicken)                 GCCU--GUGCCCUCA-GCUCU-ACC----------
X. tropicalis (frog)                ------------------------------------
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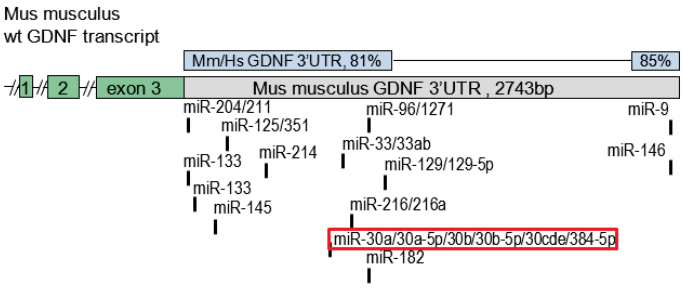


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H. sapiens (human)                 G-GAGAGC-CC-----------CUCAG-GCCU----GUUGGUUUACAGAGAGAC
P. troglodytes (chimpanzee)        G-GAGAGC-CC-----------CUCAG-GCCU----GUUGGUUUACAGAGAGAC
M. mulatta (rhesus macaque)        G-GAGAGC-AC-----------CUCGG-GCCU----GUUGGUUUACAGAGAGAC
O. garnettii (bushbaby)            G-GAGC-C-GC-----------CUCAG-GCCU----GUCAGUUUACAGAGAGAC
T. belangeri (treeshrew)           G-GAGACCUGC-----------CUCGG-GCCU----GUCGGUUUACAGAGAGAC
R. norvegicus (rat)                G-GAGACC--------------CCGGG-GCCU----GUUGGUUUACAGAGA--C
C. porcellus (guinea pig)          G-GAGACC-CAC----------CCCAGG-GCCU-----UCGGUUUACAGAGAGAC
O. cuniculus (rabbit)              G-GGGCAC-GGAGCCGGGC---CCUGG-GCCU----GUCGGUUUACAGAGAGAC
S. araneus (shrew)                 A-GACAGA-UG-------------------------GUC----------------
E. europaeus (hedgehog)            A-CACACA-UGACACACACCC-CAGUG-GGCU----GUCGGUC-ACAGAGAGAC
C. l. familiaris (dog)             G-GGGACC-CG-----------CCGG-GCCU----GUCGGUUUACAGAGAGAC
F. catus (cat)                     G-GACACC-CA-----------CUGG-GCCU----GUCGGUUUACAGAGAGAC
E. f. caballus (horse)             G-GCAACC-CG-----------CCGG-GCCU----GUCGGUUUACAGAGAGAC
B. taurus (cow)                    G-GAGGCC-G--------------CUG-GGCU----GUCGGUUUACAGAGAGAC
D. rerio (zebrafish)               G-GAGACU-UG-----------C-GGGUGCCA----GUCGGUUUACAGAGAGAC
L. africana (elephant)             G-GAGACC-CA-----------C-GUG-GCCU----GUCGGUUUACAGAAAGAC
E. telfairi (tenrec)               GAGAGACC-CG-----------G-GUG-GCCU----GUCGGUUUACAGAGAGAC
M. domestica (opossum)             A-GAGACG-GA-----------CAGGU-CUCU----UUCAGUU-------GGAC
O. anatinus (platypus)             A-GAGACG-AG-----------AUCCG-GUCUCUCUCGGUUUACAAAAGGAU
A. carolinensis (lizard)           ----------------------------CUU----UUUGGUUUACAAAAGGAC
G. gallus (chicken)                ----------------------------UCU----UUUGGUUUACAAA-GGAC
X. tropicalis (frog)               -----------------------------------UUGGUUUACAGAGGAAU
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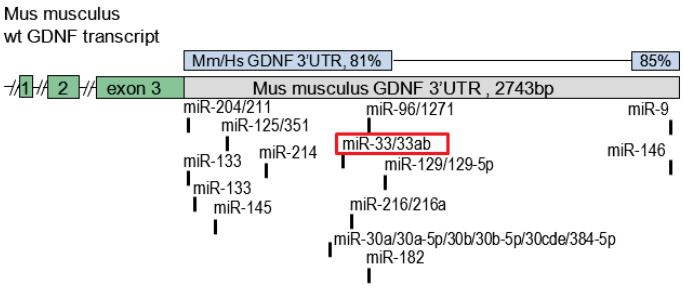


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H. sapiens (human)                 ACCAG-AGAAGCUACUCGAUGCAAUGCAUCUGU
P. troglodytes (chimpanzee)        ACCAG-AGAAGCUACUCGAUGCAAUGCAUCUGU
M. mulatta (rhesus macaque)        ACUAG-AGAAGCUACUCGAUGCAAUGCAUCUGU
O. garnettii (bushbaby)            ACCAG-AGAAGCUACUCGAUGCAAUGCAUCUGU
T. belangeri (treeshrew)           ACCAG-AGAAGCUACUCGAUGCAAUGCAUCUGU
R. norvegicus (rat)                ACCAG-AGAAGCUACUCGAUGCAAUGCAUCUGU
C. porcellus (guinea pig)          ACCAG-AGAAGCUACUCGAUGCAAUGCAUCUAU
O. cuniculus (rabbit)              ACCAG-AGAAGCUAAUCGAUGCAAUGCAUCUGU
S. araneus (shrew)                 ACCAG-AGAAGCUACUCGAUGCAAUGCAUCUGU
E. europaeus (hedgehog)            ACCAG-GAAGGAUACUCGAUGCAGUGCAUCUGU
C. l. familiaris (dog)             ACCAG-AGAAGCUACUCGAUGCAAUGCAUCUGU
F. catus (cat)                     ACCAG-AGAAGCUACUCGAUGCAAUGCAUCUGU
E. f. caballus (horse)             ACCAG-AGAAGCUACUCGAUGCAAUGCAUCUGU
B. taurus (cow)                    ACCAG-AGAAGCUACUCGAUGCAAUGCAUCUGU
D. rerio (zebrafish)               ACCAG-AGAAGCUACUCGAUGCAAUGCAUCUGU
L. africana (elephant)             ACCAG-AGAAGCUACUCGAUGCAAUGCAUCUGU
E. telfairi (tenrec)               ACCAG-AGAAGCUACUCGAUGCAAUGCAUCUGU
M. domestica (opossum)             GCCAG-AUAGACUACUUGAUGCAAUGCAUCCAU
O. anatinus (platypus)             GCCCA-ACAGACUACUUGAUGCGACGCAUCC-C
A. carolinensis (lizard)           GUGAGGAGAGACUACUUGAUGCAAUGCAUCC-A
G. gallus (chicken)                GUGAGCAGAGACUACUUGAUGCAAUGCAUCC-G
X. tropicalis (frog)               ACCAA-GCAGACUACUCGAUGCCAUGCAUCC-A
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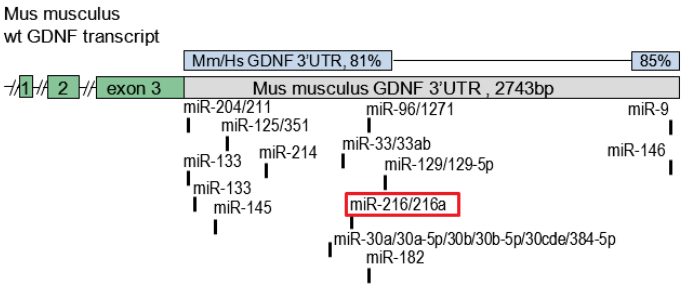
miR-216a

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H. sapiens (human)                 AAUAUAGAGAAGA-UAUUUAUUGAAAUUU-AAG
P. troglodytes (chimpanzee)        AAUAUAGAGAAGA-UAUUUAUUGAAAUUU-AAG
M. mulatta (rhesus macaque)        AAUAUAGAGAAGA-UAUUUAUUGAGAUUU-AAG
O. garnettii (bushbaby)            AAUAUAGAGAAGAAUAUUUAUUGAGAUUU-AAG
T. belangeri (treeshrew)           AAUAUAGAGAAGA-UAUUUAUUGAGAUUU-AAG
R. norvegicus (rat)                GAUAUAGAGAAGA-UAUUUAUUGAGAUUU-AAG
C. porcellus (guinea pig)          AAUAUAGAGAAGA-UAUUUAUUGAGAUUU-AAG
O. cuniculus (rabbit)              AAUAUAGAGAAGA-UAUUUAUUGAGAUUU-AAG
S. araneus (shrew)                 AUUAUAGAGAAGA-UAUUUAUUGAGAUUU-AAG
E. europaeus (hedgehog)            AAUAUAGAGAAGA-UAUUUAAUGAGAUCU-AAG
C. l. familiaris (dog)             AAUAUAGAGAAGA-UAUUUAUUGAGAUUU-AAG
F. catus (cat)                     AAUAUAGAGAAGA-UAUUUAUUGAGAUUU-AAG
E. f. caballus (horse)             AAUAUAGAGAAGC-UAUUUAUUGAGAUUU-AAG
B. taurus (cow)                    AAUAUAGAGAAGA-UAUUUAUUGAGAUUU-AAG
D. rerio (zebrafish)               AAUAUAGCGAAGA-UAUUUAUUGAGAUUU-AAG
L. africana (elephant)             AAUCUAGAGAAGC-UAUUUAUUGAGAUUU-AAG
E. telfairi (tenrec)               AAUAUAGAGAAGC-UAUUUAUUGAAAUUU-AAG
M. domestica (opossum)             AAUAUAUAGAAGA-UAUUUAUUGAGAUUU-AAG
O. anatinus (platypus)             AAUCUAUAGAAGA-UAUUUAUUGAGAUUUUAAG
A. carolinensis (lizard)           AAUCUAGAGAAAA-UAUUUAUUGAGAUUU-AAG
G. gallus (chicken)                AAUAUACAGAAGA-UAUUUAUUAAGAUU--AAG
X. tropicalis (frog)               AUUAUAUAAAGGA-UAUUUAUUGAGAAUU-AAG
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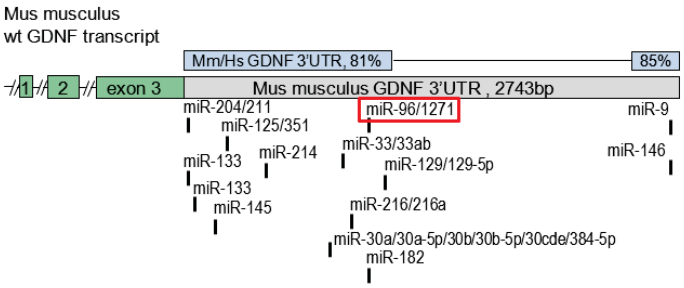
miR-96/507/1271

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H. sapiens (human)                 AUU-AAA-GUUUCUUUUC-----AAAGGUGCCAAAGUAUA
P. troglodytes (chimpanzee)        AUU-AAA-GUUUCUUUUC-----AAAGGUGCCAAAGUAUA
M. mulatta (rhesus macaque)        AUU-AAA-GUUUCUUUUC-----AAAGGUGCCAAAGUAUA
O. garnettii (bushbaby)            AUU-AAA-GUU-CUUUUC-----AAAGGUGCCAAAGUACA
T. belangeri (treeshrew)           AUU-AAA-GUUUCUUUAC-----AAAGGUGCCAAAGUAUA
R. norvegicus (rat)                AUU-AAA-GUCUCUUUUC-----AAAGGUGCCAAAGUAUA
C. porcellus (guinea pig)          AUU-AAA-GUUUCUUUUC-----AAAGGUGCCAAAGUAUA
O. cuniculus (rabbit)              AUU-AAA-GUUUCUUUUC-----AAAGGUGCCAAAGUAUA
S. araneus (shrew)                 AUU-GAA-GUUUCUUUUC-----AAAGGUGCCAAAGUAUA
E. europaeus (hedgehog)            AUA-AAA-GUUUCUUCUC-----CAAGGUGCCAAAGUAUA
C. l. familiaris (dog)             AUU-AAA-GUUUCUUUUC-----AAAGGUGCCAAAGUACA
F. catus (cat)                     AUU-AAA-GUUUCUUUUC-----GAAGGUGCCAAAGUAUA
E. f. caballus (horse)             AUU-AAA-GUUUCUUUUC-----AAAGGUGCCAAAGUAUA
B. taurus (cow)                    AUU-AAA-GUUUCUUUUC-----AAAGGUGCCAAAGUAUA
D. rerio (zebrafish)               AUU-AAA-GUAUCUUUUC-----AAAGGUGCCAAA---CA
L. africana (elephant)             AUU-AAAAGUUUCUUUUC-----AAAGGUGCCAAAGUAUA
E. telfairi (tenrec)               AUU-AAA-GUUUCUUUUC-----AAAGGUGCCAAAGUAUA
M. domestica (opossum)             AUU-AAA-AUUUCUUUUC-----AAAGGUGCCAAAGUGUA
O. anatinus (platypus)             AUUUGAA-GUUUCUCUUC-----AAAGGUGCCAAAGUGGA
A. carolinensis (lizard)           AUU-AAA-GCUUCUUUCUUCAAAACAGGUGCCAAAGU---
G. gallus (chicken)                AUU-AAA-GUUUUUUUC------AAAGGUGCCAAAGUAGG
X. tropicalis (frog)               AUU-AAA-CUUUCUCUUC-----CAUGGUGCCAAAGCAGA
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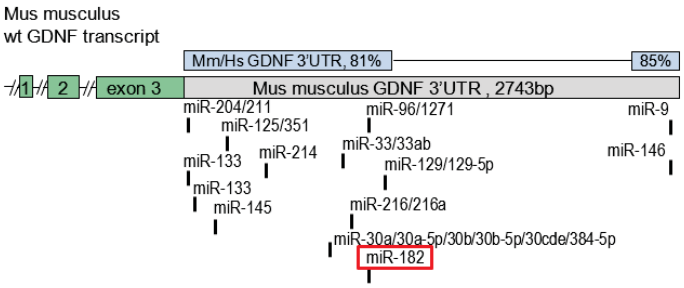
miR-182

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H. sapiens (human)                 UU-AAA-GUUUCUUUUC-----AAAGGUGCCAAAGUAUA
P. troglodytes (chimpanzee)        UU-AAA-GUUUCUUUUC-----AAAGGUGCCAAAGUAUA
M. mulatta (rhesus macaque)        UU-AAA-GUUUCUUUUC-----AAAGGUGCCAAAGUAUA
O. garnettii (bushbaby)            UU-AAA-GUU-CUUUUC-----AAAGGUGCCAAAGUACA
T. belangeri (treeshrew)           UU-AAA-GUUUCUUUAC-----AAAGGUGCCAAAGUAUA
R. norvegicus (rat)                UU-AAA-GUCUCUUUUC-----AAAGGUGCCAAAGUAUA
C. porcellus (guinea pig)          UU-AAA-GUUUCUUUUC-----AAAGGUGCCAAAGUAUA
O. cuniculus (rabbit)              UU-AAA-GUUUCUUUUC-----AAAGGUGCCAAAGUAUA
S. araneus (shrew)                 UU-GAA-GUUUCUUUUC-----AAAGGUGCCAAAGUAUA
E. europaeus (hedgehog)            UA-AAA-GUUUCUUCUC-----CAAGGUGCCAAAGUAUA
C. l. familiaris (dog)             UU-AAA-GUUUCUUUUC-----AAAGGUGCCAAAGUACA
F. catus (cat)                     UU-AAA-GUUUCUUUUC-----GAAGGUGCCAAAGUAUA
E. f. caballus (horse)             UU-AAA-GUUUCUUUUC-----AAAGGUGCCAAAGUAUA
B. taurus (cow)                    UU-AAA-GUUUCUUUUC-----AAAGGUGCCAAAGUAUA
D. rerio (zebrafish)               UU-AAA-GUAUCUUUUC-----AAAGGUGCCAAA---CA
L. africana (elephant)             UU-AAAAGUUUCUUUUC-----AAAGGUGCCAAAGUAUA
E. telfairi (tenrec)               UU-AAA-GUUUCUUUUC-----AAAGGUGCCAAAGUAUA
M. domestica (opossum)             UU-AAA-AUUUCUUUUC-----AAAGGUGCCAAAGUGUA
O. anatinus (platypus)             UUUGAA-GUUUCUCUUC-----AAAGGUGCCAAAGUGGA
A. carolinensis (lizard)           UU-AAA-GCUUCUUUCUUCAAAACAGGUGCCAAAGU---
G. gallus (chicken)                UU-AAA-GUUUUUUUC------AAAGGUGCCAAAGUAGG
X. tropicalis (frog)               UU-AAA-CUUUCUCUUC-----CAUGGUGCCAAAGCAGA
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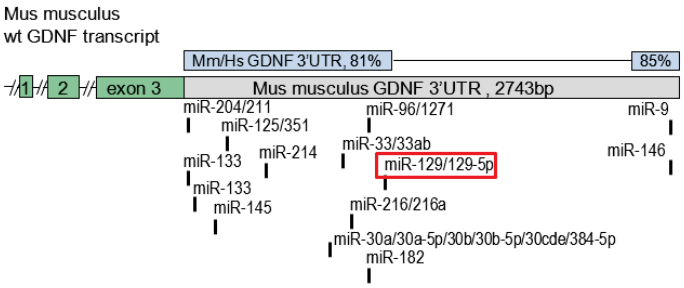
miR-129-5p/129ab-5p

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H. sapiens (human)                 AA---------AAAGUUUUUUGCCAGGC------AAAA-----------------A------UCACU
P. troglodytes (chimpanzee)        AA---------AAAGUUUUUUGCUAGGC------AAAA-----------------A------UCACU
M. mulatta (rhesus macaque)        AA---------AAAGUUUUUUGCUAGGC------AAAA-----------------A------UCACU
O. garnettii (bushbaby)            AA---------AAAGUUUUUUGCUAGGC------AAAA-----------------A------UCACU
T. belangeri (treeshrew)           AA---------AAAGUUUUUCACUAGGC------AAAA-----------------A------UCACU
R. norvegicus (rat)                AA---------GAGGGUUUUUGCUAGGC------AAAA-----------------A------UCACU
C. porcellus (guinea pig)          AA---------AAAGGUUUUCUCUAGGC------AAAA-----------------A------UCACU
O. cuniculus (rabbit)              AA---------CAAGUUUCUCGCUAGGC------AAAA-----------------A------UCACU
S. araneus (shrew)                 AA---------AAAGUUUUUCAUGAGGU------AAAA-----------------A------UCACU
E. europaeus (hedgehog)            ----------------------------------------------------------------------
C. l. familiaris (dog)             AA---------AAAGUUUCUCGCUAGGC------AAAA-----------------A------UCACU
F. catus (cat)                     AA---------AAAGUUUUUCGCUAGGC------AAAA-----------------A------UCACU
E. f. caballus (horse)             AA---------AAAGUUUUUCACCAGGC------AAAA-----------------A------UCACU
B. taurus (cow)                    AA---------AAAGACUUUCGCUAGGC------AAAA-----------------A------UCACU
D. rerio (zebrafish)               AAGUUGUGUUUCAUUUUGUUUGCUAGCC------AAAA-----------------A------UCACU
L. africana (elephant)             AAA--------AGGUCUUUUUGCUAGCC------AAAA-----------------A------UCACU
E. telfairi (tenrec)               AA----------GUCUUUUUACUAGCC------AAAA-----------------A------UCACU
M. domestica (opossum)             AA---------CA-GUUUUUGGGUAGCC------AAAA-----------------AAAAAAAUCACU
O. anatinus (platypus)             GA---------CA-GUGAUCUGCUAGCC------AAAA-----------------A------UCACU
A. carolinensis (lizard)           AA---------CAAGAAUGCA-------------AACACUUUUUUUAAAAAAAAAUA------UCACU
G. gallus (chicken)                AA---------CUUGAAUGCA-------------AAAA-----------------A------UCACU
X. tropicalis (frog)               AA---------AUAGAUGCUGG--AGGCCAACUGAAAA-----------------A------UCACA
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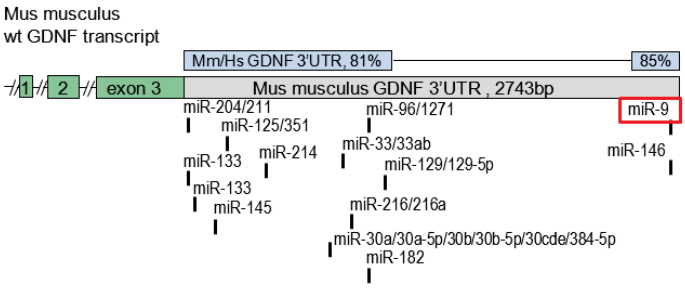
miR-9/9ab

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M. musculus (mouse)                UC---GGUCGUUUGUGUUAUACAACCAAAGUUCUC
H. sapiens (human)                 UU---GGUCGUUUGUGUUAUAAAACCAAAGUUCUC
P. troglodytes (chimpanzee)        UU---GGUCGUUUGUGUUAUACAACCAAAGUUCUC
M. mulatta (rhesus macaque)        UC---GGUCGUUUGUGUUAUACAACCAAAGUUCUC
O. garnettii (bushbaby)            UU---GGUCGUUUGUGUUAUACAACCAAAGUUCUC
T. belangeri (treeshrew)           UC---GGUCGUUUGUGUUAUACAACCAAAGUUCUC
R. norvegicus (rat)                UC---GGUCGUUUGUGUUAUACAACCAAAGUUCUC
C. porcellus (guinea pig)          UC---GGUCAUUUGUGUUAUACAACCAAAGUUCUC
O. cuniculus (rabbit)              UU---GGUCGUUUGUGUUAUACAACCAAAGUUCUC
S. araneus (shrew)                 UU---GGUCGUUUGUGUUAUACAACCAAAGUUCUC
E. europaeus (hedgehog)            ----------------------------------
C. l. familiaris (dog)             UC---AGUCGUUUGUGUUAUACAACCAAAGUUCUC
F. catus (cat)                     ----------------------------------
E. f. caballus (horse)             UU---GGUCGUUUGUGUUAUACAACCAAAGUUCUC
B. taurus (cow)                    UC---AGUCGUUUGUGUUAUACAACCAAAGUUCUC
D. rerio (zebrafish)               UC---GGUCGUUUGUGUUAUGCAACCAAAGUUCUC
L. africana (elephant)             UU---GGUCGUUUGUGUUAUACAACCAAAGUUCUC
E. telfairi (tenrec)               UC---CGUCAUUUGUGUUAUACAACCAAAGUUCUC
M. domestica (opossum)             UC---AGUCAUUUGUGUUAUACAACCAAAGUUCUC
O. anatinus (platypus)             UC---AAUCAUUUGUGUUCUACAACCAAAGUUCUC
A. carolinensis (lizard)           UC---AGACAUUUGUGUUAACCAACCAAAGUUCUC
G. gallus (chicken)                UC---AGUCAUUUGUGUUAAUCAACCAAAGUUCUC
X. tropicalis (frog)               UCUCAAGUCAUUUGUGUUAUCAACCAAAGUUCCU
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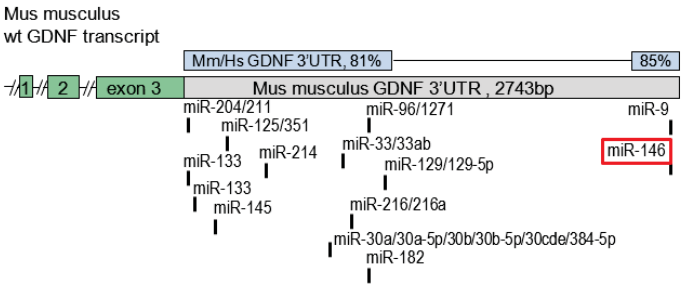
miR-146ac/146b-5p

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M. musculus (mouse)                CGUUUGUGUUAUACAACCAAAGUUCUCUACAA
H. sapiens (human)                 CGUUUGUGUUAUAAAACCAAAGUUCUCUACAG
P. troglodytes (chimpanzee)        CGUUUGUGUUAUACAACCAAAGUUCUCUACAG
M. mulatta (rhesus macaque)        CGUUUGUGUUAUACAACCAAAGUUCUCUACAG
O. garnettii (bushbaby)            CGUUUGUGUUAUACAACCAAAGUUCUCUACAG
T. belangeri (treeshrew)           CGUUUGUGUUAUACAACCAAAGUUCUCUACAG
R. norvegicus (rat)                CGUUUGUGUUAUACAACCAAAGUUCUCUACAG
C. porcellus (guinea pig)          CAUUUGUGUUAUACAACCAAAGUUCUCUACAG
O. cuniculus (rabbit)              CGUUUGUGUUAUACAACCAAAGUUCUCUACAG
S. araneus (shrew)                 CGUUUGUGUUAUACAACCAAAGUUCUCUACAG
E. europaeus (hedgehog)            --------------------------------
C. l. familiaris (dog)             CGUUUGUGUUAUACAACCAAAGUUCUCUACAG
F. catus (cat)                     --------------------------------
E. f. caballus (horse)             CGUUUGUGUUAUACAACCAAAGUUCUCUACAG
B. taurus (cow)                    CGUUUGUGUUAUACAACCAAAGUUCUCUACAG
D. rerio (zebrafish)               CGUUUGUGUUAUGCAACCAAAGUUCUCUACAG
L. africana (elephant)             CGUUUGUGUUAUACAACCAAAGUUCUCUACAG
E. telfairi (tenrec)               CAUUUGUGUUAUACAACCAAAGUUCUCUACAG
M. domestica (opossum)             CAUUUGUGUUAUACAACCAAAGUUCUCUACAA
O. anatinus (platypus)             CAUUUGUGUUCUACAACCAAAGUUCUCUACAG
A. carolinensis (lizard)           CAUUUGUGUUAACCAACCAAAGUUCUCUACAG
G. gallus (chicken)                CAUUUGUGUUAAUCAACCAAAGUUCUCUACAG
X. tropicalis (frog)               CAUUUGUGUUAUUCAACCAAAGUUCCUUACAA
```

Supplementary Table 2A, related to Figure 4. GDNF levels regulate kidney size and number.

genotype, P7.5	nr of animals analysed	two kidneys	one kidney	no kidneys	kidney size
+/+	141	141	-	-	normal
***Gdnf* 3'UTR-crR +/-**	60	60	-	-	normal or mildly reduced
***Gdnf* 3'UTR-crR -/-**	14	14	-	-	severely reduced
***Gdnf* KO +/-**	34	26	8	-	normal
***Gdnf* KO -/- (E18.5)**	7	-	-	7	na

Gdnf KO animals were obtained by crossing *Gdnf* 3'UTR-crR animals to Deleter-Cre mice, which results in deletion of GDNF protein-coding exon 3 (Fig. 1*D*).

Supplementary Table 2B, related to Figure 4. *Gdnf* 3'UTR-crR -/-mice die before weaning, *Gdnf* 3'UTR-crR +/- mice are produced in Mendelian ratios.

developmental stage	nr of animals analysed	*Gdnf* 3'UTR-crR +/- expected	*Gdnf* 3'UTR-crR +/-found	*Gdnf* 3'UTR-crR -/- expected	*Gdnf* 3'UTR-crR -/- found
E10-E18	116	58	62	29	26
P7.5	105	53	60	26	14
P18	22	11	14	6	0
2-4 months	101	50	53	25	0

Supplementary Table 3, related to Figure 4. Lack of correlation between serum urea and rostral brain dopamine levels in individual *Gdnf*3'UTR-crR +/- mice.

dopamine levels in rostral brain, ng/g wet tissue	genotype	serum urea, mg/dL
273.2	+/+	78.3
265.9	+/+	68.8
291.4	+/+	92.4
275.3	+/+	72.9
329.6	+/+	69
254.5	+/+	103.4
203.3	+/+	107.6
239.5	+/+	107.6
284.9	+/+	73.9
correlation function=-0.73		
282.1	*Gdnf* 3'UTR-crR +/-	197.7
167.8	*Gdnf*3'UTR-crR +/-	88.4
291.4	*Gdnf*3'UTR-crR +/-	90.2
318.9	*Gdnf*3'UTR-crR +/-	96.8
269.1	*Gdnf*3'UTR-crR +/-	104.2
299.7	*Gdnf*3'UTR-crR +/-	234.2
194.5	*Gdnf*3'UTR-crR +/-	266.9
244.4	*Gdnf*3'UTR-crR +/-	259.5
297.8	*Gdnf*3'UTR-crR +/-	208.9
278.1	*Gdnf*3'UTR-crR +/-	114.2
395.8	*Gdnf*3'UTR-crR +/-	110.4
420.9	*Gdnf*3'UTR-crR +/-	224.6
correlation function=-0.037		

Correlational analyses of rostral brain dopamine and serum urea levels in individual animals in 10 WT and 12 *Gdnf* 3'UTR-crR +/- animals at P7.5 using the Correlation function in Microsoft Excel was -0.037 for *Gdnf* 3'UTR-crR +/- animals for those parameters and -0.73 in wt mice.

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Supplementary Materials and Methods

Cell culture Human Embryonic Kidney 293 (HEK293) cells and human glioblastoma-astrocytoma epithelial-like cell line U87 MG were from ATCC and were cultured at 5% CO_2 , 37^0 C in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen/Gibco) supplemented with 10% Fetal Bovine Serum (FBS, HyClone) and 100µg/ml Normocin^{TM} (InvivoGen), if not indicated otherwise. Cells were not allowed to reach confluency beyond 70% at any point during culturing and were split one day before plating for an experiment.

Luciferase reporter assay

PAC RP21-583-K20 containing mouse *Gdnf* gene was identified from RPCI 129S6/SvEvTac mouse genomic library using routine radioactive DNA hybridization screening with a probe complementary to *Gdnf* exon 3 and obtained from CHORI. 2.8 kb mouse *Gdnf* 3'UTR and *Gdnf* 3'UTR-crR were cloned into *XbaI* site as indicated on Fig. S1*A* in Dual-Luciferase® Reporter Assay System vectors (Promega). Constructs were verified by sequencing. Actinomycin D (Sigma) was used at 2µg/ml. cDNAs encoding for indicated human RBPs were obtained from ORFeome Collaboration in pDEST 40 vector (Invitrogen) generated by Genome Biology Unit cloning service (Biocenter Finland, University of Helsinki) and sequenced. To reduce putative nonspecific effects stemming from DNA isolation related impurities, DNA constructs compared in this study were isolated twice from parallel maxi-preparations. Mutations in *Gdnf* 3'UTR in miR-146 and miR-96 seed sequences were introduced with PCR (see Primers) and verified by sequencing. HEK-293 cells (15.000 cell per well) were seeded per well to 96- well plate, pre-coated with 0.1% gelatin one day before transfection. Reporter plasmids were transfected along with pre-miRs (Ambion) as indicated according to standard protocol recommended for lipofectamine 2000 (Invitrogen), Cy3-

or FAM-labeled negative controls alongside with non-labeled negative controls were used to verify transfection. Transfection efficiency of pre-miRs and anti-miRs was estimated to be about 95%. The medium was replaced 3-4 hours after transfection, cells were lysed 24 hours later using passive lysis buffer as recommended by the manufactuer (E1960, Promega). Luciferase activity was measured with Dual-Luciferase® Reporter Assay System (E1960, Promega) with GlomaX 20/20 luminometer (Promega).

GDNF mRNA measurements in U87 MG cells

Cells were plated on 12-well plates (Cellstar) at 10^5 cells/well, indicated pre-miRs (Ambion) were transfected 24h later with lipofectamine 2000 (Invitogen) as recommended by the manufacturer. The medium was replaced with fresh cell culture medium after 3-4 hours. Cells were washed with PBS and harvested in TRI Reagent® (Molecular Research Center) after 48 hours for total RNA isolation. See QPCR section for gene/miR levels measurements.

GDNF protein measurements in U87 MG cells

24h before transfection 10^5 cells were plated on 12 well plates (Cellstar). Pre-miRs (Ambion) were transfected as indicated, medium was replaced after 3-4 hours after the transfection, after 3 hours medium was replaced with 600µl of serum free OptiMEM (Invitrogen/Gibco) supplemented with 0.5 % BSA (Sigma, A-9576). The plate was incubated at 8% CO_2 and 37^0 for 48h as described in[4], the medium was collected and centrifuged at $+4^0$C at 2000rpm for 3 min. GDNF levels were measured using GDNF E_{max}® ImmunoAssay System (Promega,USA) according to the manufacturer's protocol.

Northern blotting

Total RNA from cells or mouse tissues was isolated using TRI Reagent® (Molecular Research Center), Northern blotting was performed using standard procedures. Briefly, RNA was electrophoresed in 1% denaturing agarose gel, transfered to the nylon membrane, specific RNAs were detected using indicated probes with DIG-based nucleotide detection system *(*Roche).

Cell survival assay

Survival of U87 cells was assayed using CellTiter-Glo® Luminescent Cell Viability Assay (Promega) according to manufacturer's protocol.

Public databases

Evolutionary conservation of miR seed sequences in *Gdnf* 3'UTR was evaluated using the most stringent conditions of Targetscan (http://www.targetscan.org/). Blast was used to assess the overall sequence conservation of *Gdnf* 3'UTR. miR-96 and miR-9 were identified to interact with *Gdnf* 3'UTR in two genome wide screens, i) miR CLIP-seq dataset using Argonate cross-linked immunopercipitation followed by RNA sequencing analysis indicating direct interaction, and ii) microarray analyses following miR transfection indicating functional interaction[5] http://servers.binf.ku.dk/antar/. Prediction of AU-rich elements as potential RNA binding protein binding sites in *Gdnf* 3'UTR were performed using ARESite (http://rna.tbi.univie.ac.at/cgi-bin/ARESite.cgi). Number of potential miR binding sites in *Gdnf* 3'UTR, Puro/TK cassette (GenBank: CR847878.1.), bovine growth hormone polyadenylation signal (bGHpA) and SV40 late pA was performed using PITA[6].

Animals

Gdnf 3'UTR-cR mice were generated using routine methods. Briefly, 5668bp 5' and 6055bp 3' homologous arms and *Gdnf* exon 3 until, and including the stop codon were amplified with PCR from *Gdnf* containing RP21-583-K20 (CHORI, see above) and cloned into *PmeI*, *NotI* and *HindIII* sites in pFlexible respectively (GenBank: CR847878.1.) to generate *Gdnf* allele as depicted on Fig. 1D. ES (IB10) clones that had undergone homologous recombination (17%) were identified using standard Southern blotting with 5' and 3' probes located outside the homologous arms. Standard karyotyping was performed prior morula aggregations. Mice were maintained in 129Ola/ICR/C57bl6 mixed genetic background, housed in 12/12 light/dark cycle with "lights-on" at 06.00h, at ambient temperature 20-22°C, 2-5 animals per cage with *ad libitum* access to standard chow and water. All animal experiments were authorized by the national Animal Experiment Board of Finland.

Tissue dissection

Mice were killed by decapitation, brains were removed in about 60 seconds, cooled in ice cold saline and placed on an ice-cold brain matrix (Stoelting, Wood Dale, IL). Indicated brain areas were isolated on ice. The brains of the P7.5 animals were cut in half with a razor blade at -0.2 mm from bregma and the dorsal part of the brain was collected. Samples were stored at -80°C until assayed. Tissues were either snap frozen on dry ice immediately after separation for qPCR or Western blotting analysis or fixed with 4 % paraformaldehyde for GDNF *in situ* hybridization or immunohistochemistry (see below).

Estimation of monoamines and their metabolites

Dopamine and its metabolites were analyzed as previously described in[7] using HPLC with electrochemical detection.

Immunohistochemistry (IHC) for light microscopy

The mice were anesthetized with sodium pentobarbital (100 mg/kg, i.p.) and intracardially perfused with PBS followed by 4 % paraformaldehyde (PFA) in 0.1 M phosphate buffer, pH 7.4. The brains were postfixed in PFA for 4 h, and stored in phosphate buffer containing 20 % sucrose at 4°C. In an alternative "light" perfusion method brains were cooled after PBS perfusion and dSTR was dissected from the rostral part, while the posterior part containing the midbrain was fixed overnight in 4% PFA for IHC. The latter method was used to reduce the number of experimental animals in experiments with PD model. Coronal striatal and nigral sections were cut and saved in serial order at -20°C until immunostained.

TH and VMAT2 immunohistochemistry. The striatal (30 µm) and nigral (40 µm) freefloating sections were stained using standard immunohistochemical procedures. Briefly, after quenching with 3 % hydrogen peroxide (H_2O_2) and 10 % methanol for 5 min sections were preincubated in 2 % normal goat serum (NGS; Vector Laboratories, Burlingame, CA) and 0.3 % Triton X-100 for 60 min followed by incubation with rabbit anti-TH polyclonal antibody (AB 152, 1:2000, Millipore, Bedford, MA) overnight, followed by incubation for 2h with the biotinylated goat anti-rabbit antibody (BA1000, 1:200, Vector Laboratories) the next day. Vectastain Elite ABC peroxidase kit (Vector Laboratories) was used for visualization using 0.06 % diaminobenzidine (or 0.025 % for P7.5 sections) and H_2O_2. The sections were mounted on gelatin/chrome alume-coated slides, air-dried, dehydrated, cleared and mounted using Pertex mounting medium (Cellpath, Hemel

Hempstead, UK). VMAT2 staining was done similarly except for the following differences: quenching period was 15 min, normal horse serum was used for pre-incubation, primary antibody was goat anti-VMAT2 polyclonal antibody (ab87594, 1:4000, Abcam) and secondary antibody was biotinylated horse anti-goat antibody (PI-9500, 1:200, Vector Laboratories). See [8] for further details.

Stereological analysis of TH- and VMAT2-positive cells

The number of TH- and VMAT2-positive neurons in the SNpc was assessed by a person blinded to the identity of the samples. Briefly, TH- and VMAT2-positive cell counts were assessed at medial levels of the SNpc, around the medial terminal nucleus (MTN). From each adult animal, every third section between levels −3.08 and −3.28 mm from the bregma was selected (3 sections per animal). From each P7.5 animal, every second section between levels −2.92 and −3.16 mm from the bregma was selected (3 sections per animal). StereoInvestigator (MBF Bioscience, Williston, VT) was used to outline the SNpc, and positively stained cells were counted within the defined outlines according to optical dissector rules[9]. Cells were counted at regular predetermined intervals (x = 100 µm; y = 80 µm) within the counting frame (60 µm × 60 µm) superimposed on the image using a 60× oil objective [Olympus BX51 (Olympus Optical, Tokyo, Japan) equipped with an Optronics camera]. The counting frame positions within the SNpc were randomized by the software, which created a systematic random sample of the area. The coefficient of error was calculated as an estimate of precision and values <0.1 were accepted. Failure in staining or perfusion resulting in spoiled sections was an exclusion criterion. Please see [8] for further details.

Striatal densitometry measurements

The OD analysis was performed under blinded condition on coded slides. Striatal TH-positive fiber immunostaining optical density (OD) measurements were performed using an Optronics (Goleta, CA) digital camera and Image-Pro Plus software (Version 3.0.1; Media Cybernetics, Silver Spring, MD) from three striatal sections from each animal and the final reading was calculated as an average. The nonspecific background correction in each section was done by subtracting the OD value of the corpus callosum from the striatal OD value of the same section. Failure in staining or perfusion resulting in spoiled sections was an exclusion criterion. Please see [8] for further details.

Amphetamine induced locomotor activity

Mice were individually placed in an open-field activity monitor (MED Associates, St. Albans, GA), and habituated for about 15 min before injection of D-amphetamine-sulphate (1 mg/kg, i.p; University Pharmacy, Helsinki, Finland). Locomotor activity was monitored for 60 min. Animals that gave no locomotor response (<2000 cm in 60 min) to amphetamine were excluded from the experiment.

Lactacystin model of PD

Lactacystin (A.G. Scientific/Nordic Biosite) (4µg in 4 µl of PBS in 5 minutes) was injected just above substantia nigra (AP -3.3; ML -1.2 and DV -4.6) of 3 months old male mice. The animals were subjected to corridor[11] test prior sacrificing at 5 weeks post injection. Analyses were performed by experimenters blinded to the genotype. Exclusion criteria were: histological analysis revealing a needle puncture or other physical damage in the SNpc; or the lesion in terms of reduction in TH positive cell number in the SN was completely absent indicating failure in LC injection.

Whole-mount* in situ *hybridization was performed with InSituPro Automate (Intavis, Cologne, Germany) as described previously[12]. The *in situ* hybridization protocol for the P12.5 brain tissue was modified from the routine as follows. The brains were fixed in 4% PFA/PBS at 4°C for 3-7 days and cryoprotected overnight with 20% sucrose in 4% PFA/PBS (4°C), frozen at -80°C. Freely floating cryosections (40μm) were washed for 20 min in ice cold PBS/0.25% TritonX-100, followed by 5 min incubation with 5x SSC (pH5; RT). The prehybridization was carried out for 2 h and hybridization overnight (with 1 μg/ml Dig-labelled RNA probe) at 65°C in 50% formamide, 5x SSC (pH5), 2% blocking reagent (BR), (Roche). Posthybridization washes were as follows: i) 50% formamide, 5x SSC (pH5), 1% SDS for 30 min at 65°C; ii) 50% formamide, 2x SSC (pH5) for 30 min at 60°C; followed by triple washes with TBST. Immunohistochemistry was carried out according to Wilkinson, 1993[13]; BM Purple AP Substrate (Roche) was used as substrate. All the steps were performed under shaking. The stained sections were transferred onto slides in 0.5% gelatine, air dried and mounted with Pertex (Histolab, Malmö, Sweden).

In vivo chronoamperometry

In vivo chronoamperometry with second-by-second quantitative detection of dopamine levels was performed with the Fast Analytical Sensing Technology (FAST-16) system (Quanteon, Nicholasville, KY, USA)12 using single carbon fiber electrodes (Quanteon, Nicholasville, KY, USA) coated with Nafion (Sigma, Stockholm, Sweden). Electrodes were calibrated in phosphate buffered saline (0.05 M PBS, pH=7.4), 20 mM ascorbic acid and dopamine (2 mM) were added during the calibration procedure. Only electrodes with selectivity of more than 200:1 for DA over ascorbic acid, a limit of detection below 0.05 μM, and linear response to DA (R2 >0.995) were used. Following calibration, the electrode was mounted parallel with a micropipette used for application of dopamine with a

distance of 130-160µm between the tips. Mice were anesthetized with an intraperitonal injection of urethane (1.7-1.9 g/kg body weight, Sigma) and fixed in a stereotaxic frame on a heating pad. An incision was made in the scalp and the bone overlying the striatum was removed, and an additional single hole was made caudally for implantation of an Ag/AgCl reference electrode. The electrode/micropipette-assembly was lowered into the striatum, using a microdrive, at stereotaxic coordinates anterior-posterior +0.3 and +1 mm and medio-lateral ±1.8 mm, calculated from bregma level. Recordings were performed at two distinct rostrocaudal striatal tracks in each hemisphere. At each recording site, data was collected from three depths below the dura: at -2.0, -2.5, and -3.0, mm. The ejected volume (25-75nl) was monitored using a scale fitted in the ocular of an operation microscope. Dopamine (200 µM, in saline containing 20 µM ascorbic acid) was locally applied to evaluate dopamine clearance.

During recordings, a square wave potential of 0.55 and 0 V (against an Ag/AgCl reference electrode) was applied over the electrode at a frequency of 5 Hz. Current produced from the oxidation and reduction reactions were integrated, giving an average signal per second for each reaction. Intrastriatally injected DA (200µM) produced a range of amplitudes at each stereotaxic co-ordinate (AP +0,3mm; +1,0mm; ML ±1,8mm, DV-2,0; -2,5; -3,0; -3,5); data points were pooled for analysis and DA peaks (µM) were separated into amplitude bins and plotted against uptake rate, µM/s; calculated using Michaelis-Menten first-order rate constant, k1.

GDNF protein levels measurements in tissues

GDNF protein levels in E18.5 kidney lysate were analyzed using GDNF E_{max}® ImmunoAssay System (Promega, USA) according to the manufacturer's protocol. GDNF protein levels in brain lysate was assessed as follows. Animals were decapitated; brain was isolated and cooled for 3 minutes in ice-

cold PBS. Striata were isolated on ice, snap frozen on dry ice and stored at -70^{o}C. GDNF protein level was analyzed using GDNF ELISA from (R&D Systems, USA) with acidification step included as suggested by the manufacturer. After preparation of the striatal lysate as suggested by the manufacturer the brain lysate was aliquoted and stored at -70^{o}C. Aliquots were thawed only once. Total protein concentration was established using Lowry method (Bio-Rad) prior loading on ELISA. 100µg of total protein was loaded per well. Brain lysate from *Gdnf* full knockout mice was used for defining the background signal. Tissue lysates for each mouse were analyzed with at least three total protein concentrations in duplicates.

Serum urea and creatinine were measured with standard kits (BioAssay Sytems).

Quantitative PCR (qPCR)

DNAse (Promega) treated RNA was used for reverse transcription (RT) with RevertAid reverse transcriptase (Fermentas) as recommended by the manufacturer. The LightCycler® 480 Real-Time PCR System (Roche) was used for routine quantitative PCR, beta-actin served as a reference gene. Each cDNA was analyzed in at least three qPCR runs with 3-4 technical repeats per run using the LightCycler® 480 Real-Time PCR System (Roche). For statistical analysis, average *beta-actin* Cp value (obtained from the Absolute Derivative Max function with the Lightcycler® 480 Software Release 1.5.0 SP1 software) was subtracted from *Gdnf* Cp value and the resulting dCt value was used to calculate fold difference relative to the reference gene (2^{-dCt}). Primer pairs used for qPCR are indicated below. For miR expression analysis, cDNA from 0.35-1 µg RNA was synthesized with TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems) using Megaplex™ RT Primers,

Rodent Pool A or B (Applied Biosystems) without preamplification in a final volume of 7.5 µl. The cDNA product was diluted 1:30 and 2.5 µl of the diluted cDNA was used for each real-time PCR reaction in a final volume of 10 µl in 384-well plates. Each sample was run in duplicate. microRNA expression from mouse-derived cells was normalized to sno202.

Western blotting

Dorsal striatum and substantia nigra were isolated at indicated ages as described above, homogenized on ice and total protein levels were measured using Lowry method (Bio-Rad, USA). Following blotting and incubation with antibodies, nylon membranes were washed in TBS-T (TBS+0.1% Tween20) for 3x 15 minutes. Blocking was performed in 5 % non-fat milk in TBS-T 1 h RT. 1:3000 mouse anti-TH (Chemicon MAB318) for o/n at +4 °C in blocking solution followed by anti-mouse-HRP (DAKO P0449) for 2h at RT was used to detect TH. Rat anti-DAT (Chemicon MAB369) 1:2500 for o/n at +4°C in blocking solution followed by 1:1000 biotinylated anti-rat for 2h at RT followed by 2h RT incubation with 1:2500 streptavidin-HRP (Molecular Probes S-911) (Vector BA-4000), were used to detect DAT. 1:1000 mouse anti p-ERK (sc 7383 Santa Cruz) for o/n at +4°C followed by 2h at RT incubation with donkey anti-mouse-HRP 1:3000 (Dako, P0449) was used to detect phospho-Erk (Pi-Erk), rabbit anti-Erk 1:1000 for o/n at +4°C (sc94 Santa Cruz) followed by 2h at RT incubation with HRP conjugated anti-rabbit 1:3000 (NA9340 GE Healthcare) was used to detect Erk.

Blots were stripped 15 min at +70 °C (50 mM DTT, 50 mM Tris-HCl, 2 % SDS) followed by washing and blocking as described above. Gapdh 1:10 000 mouse anti-GAPDH (Millipore MAB374) o/n +4 °C followed by 2 h RT 1:3000 anti-mouse-HRP (DAKO P0449); alpha-tubulin 1:30 000 mouse anti-alpha-tubulin (Sigma T9026) o/n +4 °C, followed by 2 h RT 1:3000 anti-mouse-HRP (DAKO P0449). Signal on WB-s was visualized with Pierce ECL western blotting kit #32106 followed by film

exposure, gapdh or alpha-tubulin and gene of interest and Pi-Erk/Erk signal ratio was calculated using ImageJ software.

Behavioral and CLAMS analysis

For open field test, non-lesioned mice were tested in three independent cohorts of comparable size (N=10-12 male animals per genotype in each experiment). Male wt animals from the same litter served as controls. Experiment was performed by three different experimenters blind to the genotypes. Randomized animals were tested during the light period of the day between 9.00am and 4pm. Metabolic monitoring (food intake), was perfromed as described in detail in [16-18] using Comprehensive Lab Animal Monitoring System (CLAMS).

Primers

Mutation primers:

miR-146mut agttctc -> agtctct

F: agtctcttacaaactttatttttgtacaatatc

R: ttggttgtataacacaaacgac

miR-96mut gtgccaaa -> cgaaagtc

F: cgaaagtcgtatatgtgctcacaaaatacaaag

R: ctttgaaaagagactttaataaataag

qPCR primers:

Gdnf

F: cgctgaccagtgactccaatatgc

R: tgccgcttgtttatctggtgacc

beta-actin

F: ccagttcgccatggatgac

R: gagccgttgtcgacgacc

Statistical analysis

Data from biological repeats, i.e. cDNA or tissue/cell lysates derived from different animals or cell culture dishes was subjected for statistical analysis. In case of technical repeats (qPCR, ELISA, luciferase assays) mean values were used in calculations. Statistical analysis for pairwise comparisons was performed using Student's t-test with two tailed distribution using the unequal variance option. When not noted otherwise, the indicated p value reflects the above Student's t-test.

Data from amperometry was analysed by one way ANOVA followed by Bonferroni test.

The behavioural data were analysed using a factorial ANOVA design with genotype and cohort as between-subject factors where appropriate. Post-hoc analysis after significant ANOVA was carried out by means of Student-Newman-Keuls test. All numerical results are reported as mean $\pm$ standard error of mean. SPSS for Windows 20 software (IBM Corp., Armonk NY, USA) or STATISTICA 11 software (StatSoft Inc., Tulsa) were used for analysis.

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