Neurobiology of Disease

Decreased BDNF Levels Are a Major Contributor to the Embryonic Phenotype of Huntingtin Knockdown Zebrafish

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Huntington's disease (HD) is an autosomal dominant, neurodegenerative condition caused by a CAG trinucleotide repeat expansion that is translated into an abnormally long polyglutamine tract in the protein huntingtin. Genetic and transgenic studies suggest that the mutation causes disease predominantly via gain-of-function mechanisms. However, loss of normal huntingtin function resulting from the polyglutamine expansion might also contribute to the pathogenesis of HD. Here, we have studied the effects of huntingtin knockdown in zebrafish using morpholino antisense oligonucleotides, as its huntingtin orthologue has 70% amino acid identity with the human protein. Reduced huntingtin levels did not impact on gastrulation and early development, but caused massive apoptosis of neuronal cells by 24 hpf. This was accompanied by impaired neuronal development, resulting in small eyes and heads and enlargement of brain ventricles. Older huntingtin knockdown fish developed lower jaw abnormalities with most branchial arches missing. Molecular analysis revealed that BDNF expression was reduced by ~50%. Reduction of BDNF levels by injection of a BDNF morpholino resulted in phenotypes very similar to those seen in huntingtin knockdown zebrafish. The phenotypes of both huntingtin- and BDNF-knockdown zebrafish showed significant rescue when treated with exogenous BDNF protein. This underscores the physiological importance of huntingtin as a regulator of BDNF production and suggests that loss of BDNF is a major cause of the developmental abnormalities seen with huntingtin knockdown in zebrafish. Increasing BDNF expression may represent a useful strategy for Huntington's disease treatment.

Key words: **huntingtin; BDNF; zebrafish; knockdown; animal model; neurotrophic factor**

Introduction

Huntington's disease (HD) is a devastating, autosomal dominant, neurodegenerative condition caused by a CAG trinucleotide repeat expansion that is translated into an abnormally long polyglutamine tract in the protein huntingtin (Htt). Genetic and transgenic studies suggest that the mutation causes disease predominantly via gain-of-function mechanisms. However, loss-of-function effects resulting from the polyglutamine expansion or the reduced wild-type alleles may also contribute to HD pathogenesis and disease progression (for review, see Zhang et al., 2003; Cattaneo et al., 2005; Imarisio et al., 2008).

Htt is a large protein (>3100 aa residues) which is ubiquitously expressed with highest expression in the brain and testes. In addition to the polyglutamine and neighboring polyproline stretches, its most noticeable structural feature is the presence of multiple HEAT repeats, which are typically involved in mediating protein-protein interactions (Li et al., 2006). This feature in conserved across vertebrates, including zebrafish Htt (Takano and Gusella, 2002).

Htt interacts with dozens of different proteins, and has possible roles in a range of cellular processes, including vesicle trafficking, protection against apoptosis, and regulation of transcription (Imarisio et al., 2008). One process that is dependent on multiple roles of Htt is brain-derived neurotrophic factor (BDNF) function. Wild-type, but not mutant Htt increases the production of BDNF at the transcriptional level (Zuccato et al., 2003; Cattaneo et al., 2005), and BDNF transcript and protein levels are reduced in HD models (Zuccato et al., 2001, 2005). Interestingly, mutant Htt appears to impair BDNF transcription (Zuccato et al., 2001). In addition, wild-type Htt also enhances the transport of vesicles containing BDNF along microtubules, which eventually impacts on the secretion of this neurotrophic factor (Gauthier et al., 2004). However, it is not known to what extent (if at all) isolated downregulation of wild-type Htt would impact on processes dependent on BDNF *in vivo*.

At the organismal level, loss of Htt causes embryonic lethality in mice (Duyao et al., 1995; Nasir et al., 1995; Zeitlin et al., 1995; Dragatsis et al., 2000) and has dramatic effects on embryogenesis in zebrafish (Lumsden et al., 2007). The zebrafish Htt orthologue

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is of similar size to human Htt and shares 70% amino acid identity (Karlovich et al., 1998). It is not clear which physiological processes are major contributors to the developmental effects of Htt loss. However, recent data suggest that Htt-deficient zebrafish have decreased hemoglobin production, which may lead to defects in iron utilization and oxidative energy production (Lumsden et al., 2007).

Here, we have used zebrafish to study wild-type Htt function. Our data suggest that loss of BDNF function is a major contributor to many of the developmental defects seen when Htt levels are knocked down. BDNF levels were reduced in the Htt-knockdown zebrafish and these fish had phenotypes that were very similar to those we observed in BDNF-knockdown fish. Finally, we showed a significant rescue of the phenotypes of both Htt- and BDNF-knockdown zebrafish, when exogenous BDNF was administered. This underscores the physiological importance of Htt as a regulator of BDNF production.

Materials and Methods

Zebrafish strain husbandry and in situ *hybridization.* Wild type zebrafish of the AB strain were maintained at 28.5°C under standard conditions (Westerfield, 2000) in compliance with UK Home Office regulations. Embryos were collected after natural spawning, staged as previously described (Kimmel et al., 1995) and raised in embryo medium (Westerfield, 2000). Whole-mount *in situ* hybridization was performed as previously described (Jowett and Lettice, 1994). Antisense DIG-labeled RNA probes were generated using a cDNA of zebrafish *huntingtin* (zfHtt; Karlovich et al., 1998).

Morpholino injections and BDNF treatment. Two non-overlapping MOs (Htt_ATG-MO and Htt_UTR-MO) were designed to specifically target the zebrafish *huntingtin* translation initiation codon and the 5′UTR, respectively (ATG-targeting: 5′-GCC ATT TTA ACA GAA GCT GTG ATG A- 3′ (+5 to −20); 5′-UTR targeting: 5′-AGA TAT AAT CTG ATC GGA GAT AGG G - 3′ (-23 to - 47; obtained from Gene Tools) and dissolved in 1× Danieau medium (58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$ 0.6 mM Ca(NO3), 2, 5 mM HEPES, pH 7.6). Morpholinos with the same sequence have already been used and proved to be specific for the downregulation of Huntington expression in zebrafish (Lumsden et al., 2007). As a negative control, a mispair control morpholino with 5 base modifications out of 25 (Htt-5mis-MO: 5′-GCg Ata TTA ACA cAA cCT GTc ATG A- 3′) was used and did not produce any phenotype upon injection. One-cell stage embryos were injected either with Htt_ATG-MO, Htt-5mis-MO or Htt_UTR-MO at 3–5 ng. Since Htt_ATG-MO and Htt_UTR-MO gave the same phenotype (data not shown), only results from injection of Htt_ATG-MO are presented (Htt-MO). A morpholino targeting zebrafish BDNF translation (BDNF-MO; PZF1245–8858255) was bought from Open Biosystems, dissolved in 1× Danieau medium and 11 ng was injected into one-cell stage embryos.

For BDNF rescue experiments, human recombinant BDNF (100 ng/ml; Millipore) was dissolved in water and added to the embryonic media 10 h after morpholino injection. Fish were analyzed 1, 2, or 5 d after injection.

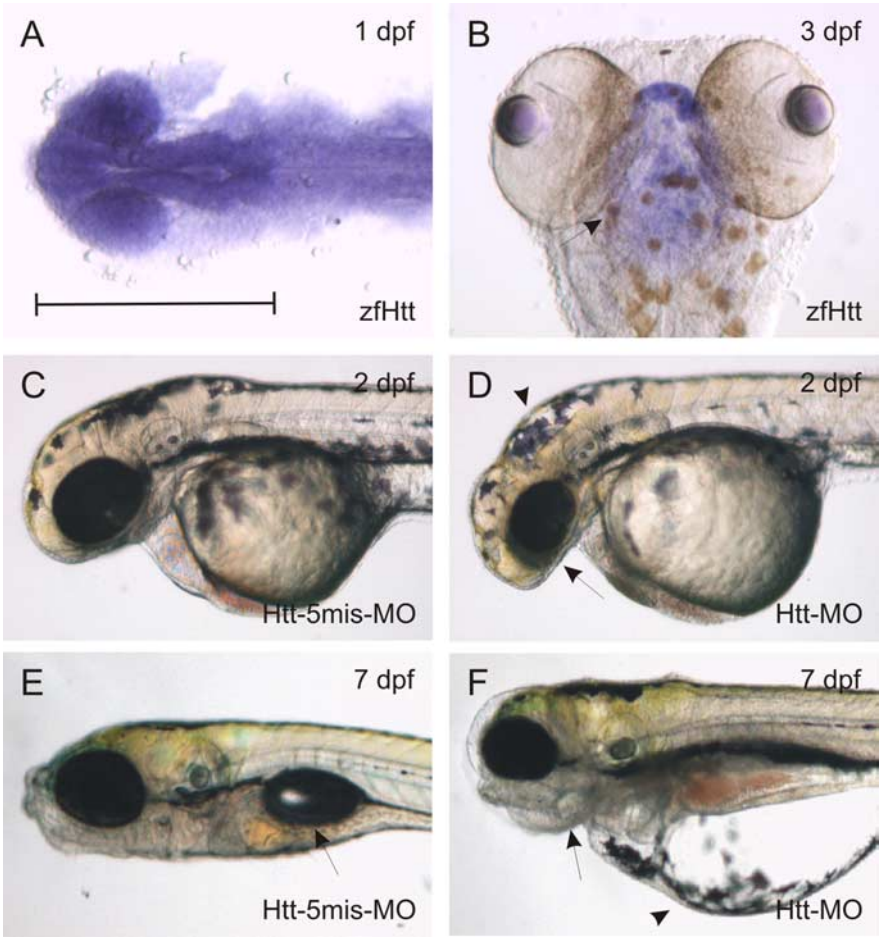

Figure 1. zfHtt expression and Htt morpholino phenotype. ***A***, *In situ* hybridization detects zfHtt mRNA expression predominantly in the brain and eyes (bracket) at 1 dpf. ***B***, zfHtt mRNA is expressed in the pharyngeal cartilage (arrow) at 3 dpf. Control hybridizations with Htt sense probe yield no signal at all embryonic stages analyzed (data not shown). ***C***, Control zebrafish injected with 3 ng of Htt-5mis (control morpholino which has 5 bp mismatch compared with Htt-MO morpholino) show the same morphology as non-injected wild-type zebrafish at 2 dpf. ***D***, Embryos injected with 3 ng of Htt-MO develop slightly smaller eyes (arrow) and small heads with enlarged brain ventricles (arrowhead) at 2 dpf. ***E***, Control zebrafish injected with 3 ng of Htt-5mis-MO look normal and have developed a swim bladder (arrow) by 7 dpf. ***F***, At 7 dpf, 3 ng of Htt-MO injected larvae have small eyes, a prominent lower jaw defect (arrow), no swim bladder and fail to resorb the yolk sac membrane (arrowhead). Orientation in ***A*** is anterior left, dorsal up; ***B*** is a dorsal view with anterior to the top and ***C–F*** are lateral views with anterior to the left.

Immunohistochemistry and Western blots. Immunohistochemistry for acetylated tubulin (Sigma-Aldrich), was performed as described earlier (Kaslin and Panula, 2001). Images were taken using an Olympus-SZX12 or BX51 fluorescent microscope equipped with digital camera, and processed using AnalySIS ^{D} 5.0 software (Soft Imaging System).

For Western blotting, dechorionated and deyolked (Link et al., 2006) zebrafish embryos were homogenized in RIPA buffer with protease inhibitors. Ten microliters of homogenate were separated per lane on 12% SDS-polyacrylamide gels and transferred to Hybond C Super nitrocellulose membranes (GE Healthcare). After blocking with 3% milk powder/0.05% Tween20/350 mM NaCl/PBS, anti-BDNF mAB248 (R&D Systems) and anti-actin mAb (loading control) were added. The mAB248 was made against recombinant human BDNF aa129–247 and recognizes both mature and pro-BDNF. As described before (Lum et al., 2001), detection of mature BDNF was much weaker than for pro-BDNF which is why this isoform was used to quantify BDNF levels. Immunosignals were visualized with HRP-coupled anti-mouse antibody and ECL substrate (GE Healthcare). Films were scanned using a desk-top scanner HP ScanJet 3800, and densitometric analysis of blots was performed using NIH ImageJ software. The background intensity of the film was subtracted from the band intensity. At least three separate experiments were

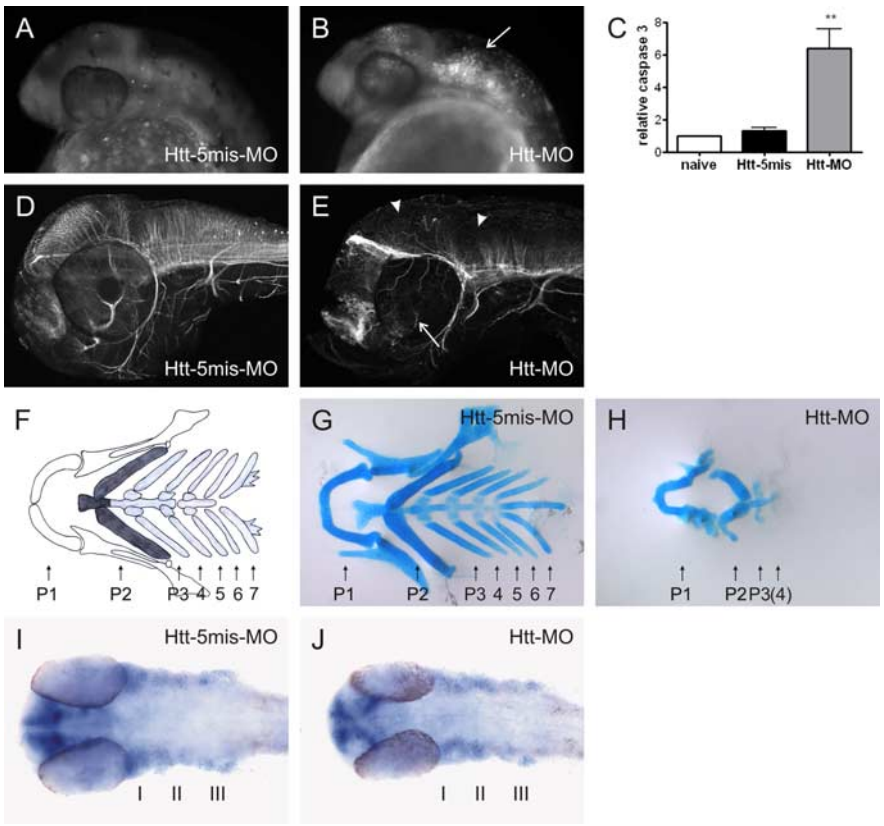

Figure 2. Analysis of Htt-MO phenotype. ***A***, Control embryo injected with 3 ng of Htt-5mis-MO show only sparse and sporadic staining with Acridine Orange at 1 dpf. ***B***, Acridine Orange staining indicative of dead cells is dramatically increased in brains of 1dpf zebrafish injected with 3 ng of Htt-MO (arrow). ***C***, Caspase 3 activity is increased approximately five-fold in Htt-MO injected zebrafish at 1 dpf compared with uninjected and Htt-5mis-MO injected control embryos. Values are two independent experiments, each done with three samples of 15 zebrafish. $^{**}p < 0.01$ by one way ANOVA. ***D***, Acetylated tubulin immunostaining detects normal axonal patterning in the head of a control embryo (3 ng of Htt-5mis-MO injected) at 2 dpf. ***E***, Axonal projections into the tectum and hindbrain (arrowheads) and the optic nerves (arrow) are missing in zebrafish injected with 3 ng of Htt-MO at 2 dpf. ***F***, Diagram of the pharyngeal skeleton in ventral view which consists of the first or mandibular arch (P1, white), the second or hyoid arch (P2, dark gray) and five brachial arches (P3–7, light gray). ***G***, Htt-5mis-MO control injected zebrafish have normal pharyngeal cartilage with the prominant first/mandibular arch (P1), the hyoid arch (P2) and 5 consecutive branchial arches (P3–7). ***H***, Larvae injected with 3 ng of Htt-MO have massively reduced pharyngeal cartilage. The mandibular and hyoid arches (P1, P2) are reduced in size and ventrally orientated (instead of anterior). Most or sometimes all of the branchial arches are missing. ***I***, Three streams of undifferentiated cranial neural crest cells (I, II, III) are detected in Htt-5mis-MO injected control zebrafish at 24 hpf by dlx2 *in situ* hybridization. ***J***, The dlx3 mRNA expression pattern is unchanged in embryos injected with 3 ng of Htt-MO. ***A–E*** are lateral views with anterior to the left; ***F–J*** are dorsal views, anterior to the left; ***F–H*** are dissected lower jaws.

analyzed, and band intensities were normalized to the loading control band intensity.

Detection of cell death. Apoptotic/necrotic cells were detected by incubation of live 24 hpf old embryos in acridine orange solution (5 μg/ml) (Furutani-Seiki et al., 1996). Cells loaded with the dye were visualized using FITC-filter on an Olympus-SZX12 fluorescent microscope.

To detect the activity of caspase-3 in zebrafish embryos, fish were homogenated in ice-cold RIPA buffer and the homogenate was mixed with an equal volume of 0.39 mM of acetyl Asp-Glu-Val-Asp 7-amido-4-methylcoumarin (Sigma-Aldrich), a selective caspase-3 substrate, resulting in the release of the fluorescent 7-amino-4-methylcoumarin (AMC). The excitation and emission wavelengths of AMC (360 and 460 nm, respectively) were detected using Victor^3 plate reader (PerkinElmer Life And Analytical Sciences). Control wells containing fluorescent substrate without lysate, were used to assess spontaneous oxidation of the substrate, and the levels of fluorescence did not change over the incubation time. The reactions were normalized against total protein concentrations.

QPCR. Total RNA was prepared from control or Htt-MO injected zebrafish at 1 dpf and 3 dpf, using the RNeasy mini kit (Qiagen). Following DNase treatment (Invitrogen), 1 μg of RNA was reverse transcribed using the Superscript III cDNA synthesis kit (Invitrogen). Q-PCR was performed using 4 μl of cDNA, 375 nM final concentration of each primer and 2X SYBR green mastermix (ABI systems), in a total of 20 μl per well, on an Applied Biosystems 7900 HT fast real-time PCR system. RNA levels from control zebrafish were normalized to a cDNA standard curve and to the house-keeping gene actin. Oligonucleotides used were: BDNF 5′-ATAGTAACGAACAGGATGG-3′; 5′-GCTCAGTCATGGGAGTCC-3′ and TrkB1 5′-GGTTTATTAGACGAGCAACCC-3′; 5′-GGCAGCGATTCCCACGAC-3′.

Cartilage staining. After fixation in 4% PFA, 5 dpf zebrafish were stained in Alcian Green (0.1% w/v in 0.37% HCl/70% EtOH) at room temperature overnight. Zebrafish were washed in 0.37% HCl/70% EtOH, rehydrated and digested with trypsin for ~2 h at 37°C. Pigment was bleached in 3% H_2O_2 in 0.5% KOH for ~30 min and fish were stored in 100% glycerol at 4°C. Lower jaws were dissected using Tungsten needles and photographed with an Olympus SZX12 microscope at the same magnification.

Results

Htt morpholino phenotype

In this study, we aimed to analyze the effect of reduced Htt expression on the development of zebrafish embryos. To determine where in the zebrafish we might see a morphological change due to Htt knockdown, we first analyzed its mRNA distribution. During the preparation of this manuscript, another description of the zebrafish Htt expression pattern up to 48 hpf was published (Lumsden et al., 2007). Consistent with these results, we detected Htt mRNA predominantly in the head region of 1 dpf zebrafish (Fig. 1*A*). By 3 dpf, however, Htt mRNA expression was detected only in the pharyngeal arches of the lower jaw (Fig. 1*B*). Given the early ubiquitous expression of zebrafish Htt, its knockdown using morpholino oligonucleotides (MO) was expected to have a detrimental effect on zebrafish development. Surprisingly, Htt-MO injected embryos appeared similar to control injected zebrafish during early development (data not shown; Lumsden et al., 2007). For our experiments, we used Htt-MOs identical to those that were previously validated for specificity and reduction of Htt protein (Lumsden et al., 2007). Control fish were injected with MOs that had 5 bp mismatches compared with Htt-MO and did not show a phenotype in any of our assays compared with uninjected wild-type larvae. By 2 dpf, however, a striking phenotype was obvious. The heads and eyes of Htt-MO zebrafish were smaller, while the brain ventricles were enlarged compared with control embryos (Fig. 1*C*,*D*). This phenotype was very similar to that described previously with the same Htt-MO (Lumsden et al., 2007). During subsequent development, the morphological abnormalities became more pronounced. At 7 dpf, zebrafish larvae injected with Htt-MO showed an even more prominent small eye phenotype and failed to inflate their swim bladder. In addition, the lower jaw appeared grossly malformed (Fig. 1*E*,*F*).

More detailed analysis revealed further striking features of the

Htt-MO phenotype. In normally developing control zebrafish, only a few dead cells can be detected at 24 hpf (Fig. 2*A*). Htt-MO injected zebrafish, however, show massively increased cell death as detected by acridine orange incorporation, especially in the midbrain/hindbrain region of the developing zebrafish embryo (Fig. 2*B*). In addition, caspase 3 activity was upregulated approximately five-fold in Htt-MO injected 24hpf embryos compared with control injected zebrafish (Fig. 2*C*). This massive apoptosis was accompanied by severe under-development of the CNS. At 48 hpf, immunostaining with anti-acetylated tubulin antibody revealed a dense network of neurites and prominent optic nerves in control injected zebrafish (Fig. 2*D*). In Htt-MO injected embryos, however, the midbrain and hindbrain were hardly innervated and the optic nerves were missing (Fig. 2*E*), in accordance with death of the respective neurons.

At later stages of development, Htt-MO injected zebrafish seemed to have somewhat recovered from the early defects caused by reduced Htt expression (Fig. 1, compare *D*, *F*), but the lower jaw was still prominently malformed. Cartilage staining in these fish revealed severe deformation of the mandibular and hyoid arches (P1, P2) while most of the branchial arches (P3–7) were completely missing (Fig. 2*F–H*). Injection of the control MO (Htt-5mis) had no effect on the formation of the lower jaw (Fig. 2*G*). *In situ* hybridization using a *dlx2* probe to detect undifferentiated cranial neural crest cells that subsequently form the cartilage (Akimenko et al., 1994), indicated that the early developmental steps in cartilage formation were unaffected by reduced Htt levels. Neural crest cell formation and migration in three distinct streams occurred normally in both Htt-MO and control-injected embryos at 24 hpf (Fig. 2*I*, *J*). Therefore, we suspect that Htt knockdown may impair the differentiation of neural crest cells into cartilage-forming osteoblasts.

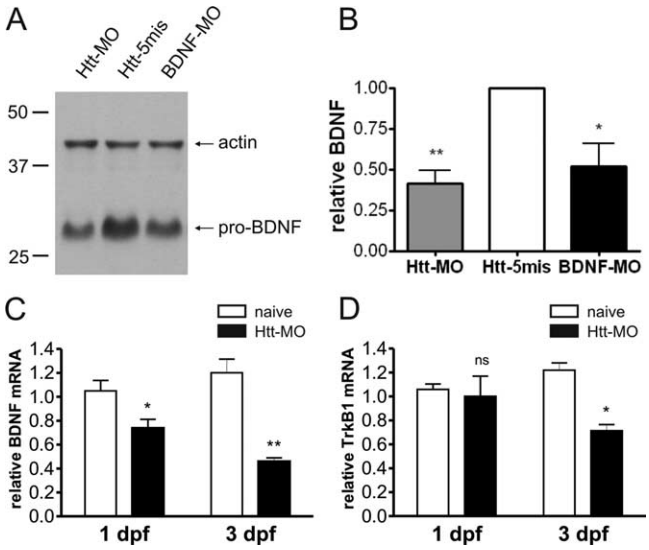

Figure 3. Reduced BDNF levels in Htt-MO injected zebrafish. ***A***, Western blot of zebrafish lysates show reduced pro-BDNF levels in Htt-MO (lane 1) and BDNF-MO (lane 3) injected embryos compared with Htt-5mis-MO injected controls (lane 2). The blot is a representative of three independent experiments. ***B***, Quantification of BDNF expression in Htt-MO and BDNF-MO injected embryos compared with Htt-5mis-MO controls at 1 dpf. Blots obtained from three independent experiments were used for quantification and the levels of pro-BDNF were normalized to the actin loading control. $^{**}p < 0.01$, $^{*}p < 0.05$ as analyzed by two-tailed *t* test. ***C***, Q-PCR assessment of BDNF mRNA expression in Htt-MO injected zebrafish,compared with uninjected controls, at 1 dpf and 3 dpf. cDNA levels from four independent experiments were obtained and normalized to actin levels. $^{*}p < 0.05$, $^{**}p < 0.01$, as analyzed by unpaired *t* test. ***D***, Q-PCR assessment of TrkB1 mRNA expression in Htt-MO injected zebrafish, compared with uninjected controls at 1 dpf and 3 dpf. cDNA levels from four independent experiments were obtained and normalized to actin levels. ns, non-significant; $^{*}p < 0.05$ as analyzed by unpaired *t* test.

Htt-MO phenotype is caused by reduced BDNF levels

Given the atrophic phenotype of Htt knockdown embryos, we speculated that growth factor expression might be reduced in these fish. Since wild-type Htt has previously been shown to induce BDNF (brain derived neurotrophic factor) expression (Zuccato et al., 2001), we next analyzed BDNF levels in zebrafish with reduced Htt expression. BDNF protein was reduced by ~60% in Htt-MO injected zebrafish compared with Htt-5mis control fish (Fig. 3*A*,*B*). Injection of a morpholino directly targeting BDNF translation (BDNF-MO) resulted in a similar decrease in BDNF protein levels (Fig. 3*A*,*B*). Although we quantified the levels of pro-BDNF protein, the mature form showed the same tendency with lower levels in Htt-MO and BDNF-MO injected fish compared with controls (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). However, as described previously by Lum et al. (2001), the detection of mature BDNF was much weaker, so it was not feasible to reliably quantify this band.

To show that loss of wild-type Htt reduces BDNF levels by acting on BDNF transcription, we also performed quantitative PCR (QPCR) experiments. Quantification of BDNF mRNAs revealed a reduction of 25% at 1 dpf and of 60% at 3 dpf in Htt-MO injected zebrafish compared with controls (Fig. 3*C*), consistent with our Western blot results (Fig. 3*A*,*B*). The mRNA levels of TrkB1, the high-affinity receptor through which BDNF exerts its effect on cells, were unchanged at 1 dpf, but were reduced by 40% at 3 dpf (Fig. 3*D*). Thus, it appears that after prolonged BDNF reduction, zebrafish downregulate the expression of the TrkB1 receptor.

Zebrafish injected with BDNF-MO at a dose causing ~50% BDNF protein reduction (11 ng) (Fig. 3*A*,*B*) looked remarkably similar to Htt knockdown fish. Higher BDNF-MO concentrations expected to abolish BDNF expression caused early death, precluding analyses of developmental phenotypes (data not shown). Fish injected with 11 ng of BDNF-MO developed small eyes and heads with enlarged brain ventricles at 2 dpf (Fig. 4*A*). At 5 dpf, BDNF-MO injected larvae fail to inflate their swim bladder and to resorb their yolk sac, as well as showing a prominent lower jaw defect (Fig. 4*B*). Similar to Htt-MO injection, BDNF reduction caused severe brain atrophy with the midbrain and hindbrain lacking most of their neurites at 48 hpf (Fig. 4*C*), together with a approximately six-fold increase in caspase 3 activity (Fig. 4*D*). In addition, the mandibular and hyoid arches were deformed and the number of branchial arches was variably reduced at 5 dpf (Fig. 4*E*). Supplementation of the growth medium with recombinant human BDNF protein partially rescued the pharyngeal arch phenotype of BDNF-MO injected zebrafish, increasing cartilage stain intensity (Fig. 4, compare *E*, *F*) and arch number (Fig. 4*G*).

BDNF rescues the Htt-MO phenotype

The phenotype observed by the downregulation of BDNF expression in zebrafish was remarkably similar to the effects seen with Htt-MO injection and could be attenuated by the supplementation of the fish growth medium with recombinant BDNF protein. If the defects seen in zebrafish with Htt knockdown were largely due to reduced BDNF levels (Fig, 3*A–C*), then application of BDNF protein should rescue these defects. After Htt-MO injection, embryos were allowed to develop in fish water until 10 hpf

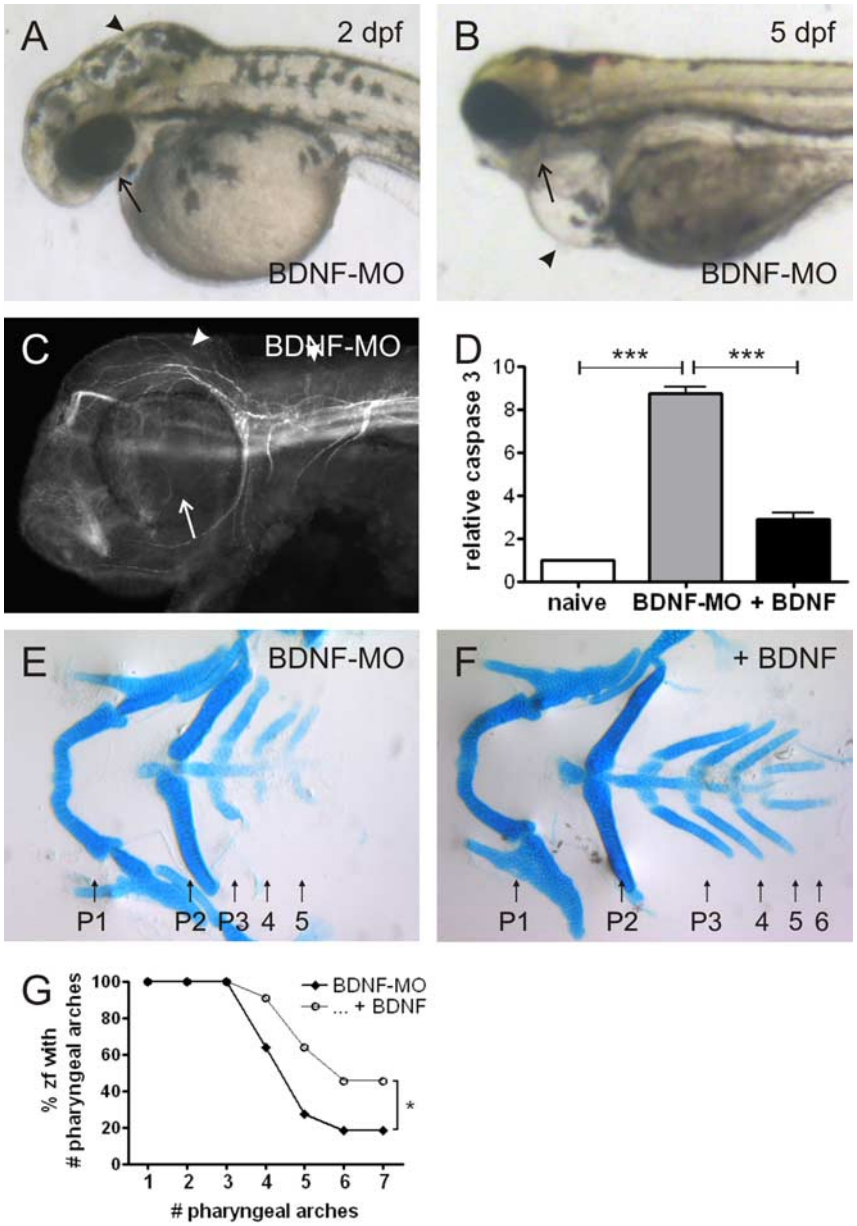

Figure 4. BDNF-MO zebrafish show comparable phenotype to Htt-MO. ***A***, Zebrafish injected with 11 ng of BDNF-MO develop small eyes (arrow) and small heads with enlarged brain ventricles (arrowhead) at 2 dpf, similar to Htt-MO injected embryos. ***B***, At 5 dpf, BDNF morpholino injected zebrafish have small eyes, a prominent lower jaw defect (arrow), no swim bladder (which should have developed by this time) and fail to resorb the yolk sac (arrowhead). ***C***, As in Htt-MO injected zebrafish, axonal projections into the tectum and hindbrain (arrowheads) and the optic nerves (arrow) are missing in BDNF-MO embryos at 2 dpf. ***D***, Caspase 3 activity is increase approximately six-fold in zebrafish embryos injected with 11 ng of BDNF-MO compared with uninjected controls at 24 hpf. Supplementation of the fish medium with recombinant BDNF protein rescues these fish from increased caspase 3 activity levels. Bars represent means of three data points from total lysates of 50 embryos at 100 min incubation time. $^{***}p < 0.001$ as analyzed by one way ANOVA test. ***E***, BDNF-MO injected zebrafish have similar pharyngeal cartilage reduction as seen in Htt-deficient larvae. The mandibular and hyoid arches (P1, P2) are reduced and misorientated and most if not all branchial arches (P3–7) are reduced in size or missing. ***F***, Application of 100 ng/ml human recombinant BDNF into the fish water partially rescues the pharyngeal arch phenotype of BDNF-MO injected zebrafish. Cartilage is stronger and the number of branchial arches is slightly increased. ***G***, Quantification of pharyngeal arches in BDNF-MO injected zebrafish with and without BDNF supplementation. The figure depicts the fraction of zebrafish with # or more pharyngeal arches. At 5dpf, all wild-type zebrafish have 7 pharyngeal arches. The data are a representative of three independent experiments, each with 10–15 fish. $^{*}p < 0.05$ as analyzed by Mann–Whitney test. ***A–C*** are lateral views with anterior to the left; ***E***, ***F***, are dorsal views of dissected lower jaws, anterior to the left.

when their chorions were ruptured to allow access of the added BDNF. These fish were then compared with identically manipulated Htt-MO injected fish without BDNF treatment. At 5 dpf, Htt-MO injected larvae treated with BDNF (Fig. 5*C*) were generally more normal than untreated Htt-knockdown fish (Fig. 5, compare *A*, *B*). BDNF treated Htt knockdown fish had straighter bodies, were more pigmented and had less severe cardiac edema than untreated siblings. The cartilage stained more intensely and the number of pharyngeal arches was slightly increased in Htt-MO injected fish supplemented with BDNF compared with untreated larvae (Fig. 5*D–F*). In addition, caspase 3 activity was attenuated by BDNF supplementation in Htt-MO injected zebrafish, in a manner similar to what we observed in BDNF-MO larvae (Figs. 4*D*, 5*G*). A stronger rescue effect due to BDNF protein supplementation might have been averted by attenuated TrkB receptor expression (Fig. 3*D*). Nevertheless, these results indicate that reduction in BDNF levels is a major factor in the phenotype caused by downregulation of Htt expression.

Discussion

In this study, we have analyzed the effects of Htt knockdown on zebrafish development. Consistent with data from Htt knock-out mice (Nasir et al., 1995), zebrafish injected with high concentrations of Htt-MO (aimed to eradicate Htt expression, Lumsden et al., 2007) did not develop beyond gastrulation, but died. Therefore, we analyzed the phenotypes of fish with reduced huntingtin protein levels (injected with moderate amounts of morpholino). At the time-point of neuron differentiation (1 dpf), we noted obvious external malformations and a failure of proper head development, which is in agreement with what was observed in Htt knock-out mice (Dragatsis et al., 2000; Woda et al., 2005)). Coinciding with this, we detected a dramatic increase in apoptosis in the Htt knockdown fish. The improvement of some morphological abnormalities at 2 dpf was not due to death of the most severely affected fish, but may have been due to dilution of the non-metabolized morpholino oligonucleotides (Summerton, 1999), with successive cell divisions and/or the regenerative capacity of the zebrafish embryos (Abdelilah et al., 1996; Ekker and Larson, 2001).

Htt is a large protein with a multitude of protein interactors and biological functions. One of the challenges with such proteins is to discern the biological consequences of these interactions and ultimately investigate their physiological relevance *in vivo*. Reduction of Htt in zebrafish resulted in approximately five-fold upregulation of caspase 3 activity at 1 dpf, together with massive cell death. This is in agreement with previous *in vitro* experiments showing a physical interaction of Htt and caspase 3 and the inhibition of caspase

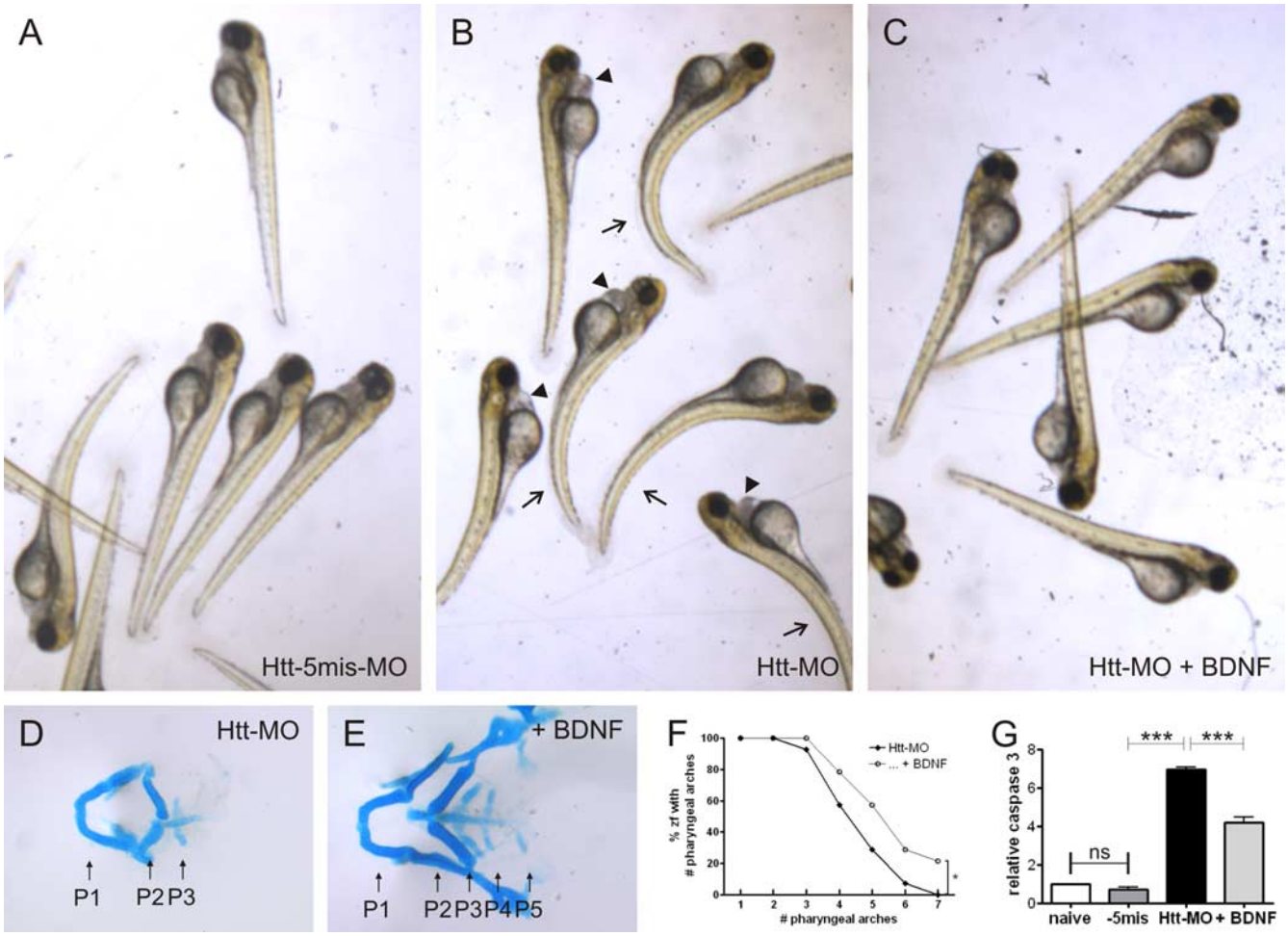

Figure 5. Htt-MO phenotype is partially rescued by BDNF application. ***A***, Htt-5mis-MO control injected zebrafish have normal morphology. ***B***, Zebrafish injected with 3 ng of Htt-MO show prominently bent tails (arrows), small eyes and heart edema (arrowheads). ***C***, Overall morphology of Htt-MO zebrafish is rescued by application of 100 ng/ml recombinant human BDNF at 5 dpf. ***D***, Htt-MO injected zebrafish (without added BDNF) develop deformed lower jaws with reduced branchial arches. ***E***, Application of 100 ng/ml human recombinant BDNF into the fish water partially rescues the pharyngeal arch phenotype of Htt-MO injected zebrafish. Overall, the cartilage looks stronger and the number of branchial arches is slightly increased. ***F***, Quantification of pharyngeal arches in Htt_ATG injected zebrafish with and without BDNF supplementation. The figure depicts the fraction of zebrafish with # or more pharyngeal arches. At 5dpf, all wild-type zebrafish have seven pharyngeal arches. The data are a representative of five independent experiments, each with 10–15 fish. $*p < 0.05$ as analyzed by Mann–Whitney test. ***G***, The increase in caspase 3 activity in Htt-MO injected zebrafish at 1 dpf is attenuated to ~60% by the supplementation of the fish medium with recombinant human BDNF protein. Control Htt-5mis-MO injected embryos show low caspase 3 activity identical to uninjected zebrafish which is not affected by BDNF application (data not shown). Bars represent means of three data points from total lysates of 50 embryos at 100 min incubation time. ns: non-significant, $***p < 0.001$, as analyzed by one way ANOVA test. ***D***, ***E*** are dorsal views of dissected lower jaws, anterior to the left.

3 activation by Htt (Zhang et al., 2006). Mutant huntingtin also interacts with caspase 3, albeit with lower affinity. This relative loss of protective function may contribute to the ultimate neuronal loss observed in HD.

A prominent feature of HD pathology is reduced levels of the pro-survival neurotrophin, BDNF. Previous studies have shown that Htt increases BDNF transcription by sequestering the inhibitory transcription factor REST/NRSF in the cytoplasm, preventing it from binding to the NRSF binding site (NRSE) on exon II of the BDNF promoter (Zuccato et al., 2001, 2003). The relevance of this neurotrophin to HD has been strengthened by recent striatal gene expression profiling studies that suggested that BDNF knock-out mice have mRNA expression patterns more similar to human HD than other HD mouse models (Strand et al., 2007). Our data corroborate previous findings that loss of wild-type Htt results in decreased BDNF mRNA and protein levels (Zuccato et al., 2005, 2008). We show that BDNF depletion is an important contributor to the developmental abnormalities seen in Htt-knockdown zebrafish, since these fish showed phenotypes that were very similar to those observed in BDNF-knockdown fish. In addition, BDNF administration resulted in significant rescue of the phenotypes of both Htt- and BDNF-knockdown zebrafish. The moderate rescue effect observed could be explained by the concomitant downregulation of TrkB, the high affinity receptor for BDNF.

BDNF, like nerve growth factor (NGF), are secondary factors involved in the differentiation of sensory and sympathetic neurons. In other words, they do not initiate the differentiation but are required to enhance a differentiation process that has already been initiated by other factor(s) (Hall and Ekanayake, 1991). Sensory and sympathetic neurons are derived from the neural crest. This ectodermal derivative also gives rise to a range of other cell types including the craniofacial skeletal and connective tissues. However, previous studies have not demonstrated the importance of BDNF in the development of the hyoid, mandibular and branchial arches. BDNF is expressed in mandibular chondrocytes and in bone in mammals (Yamashiro et al., 2001). In zebrafish embryos, BDNF is expressed in the brain and eye at

early stages and in cells lining the gill arches and the jaw cartilages at 4 dpf (Lum et al., 2001). Our data show that BDNF is critical for normal development of the cartilage in the mandibular, hyoid and branchial arches.

In conclusion, loss of BDNF function appears to be a major contributor to many of the developmental defects seen in zebrafish with reduced Htt expression. While these data do not exclude other distinct Htt interacting pathways from important roles in development, our data do show that the ability of Htt to regulate BDNF expression is a crucial component of its normal function and could be a crucial component of HD progression.

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