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7,8-Dihydroxyflavone leads to survival of cultured embryonic motoneurons by activating intracellular signaling pathways

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ABSTRACT

Brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family and a ligand for the tropomyosin-receptor kinase B (TrkB), mediates neuronal survival, differentiation, and synaptic plasticity. However, BDNF is not used to treat neurodegenerative diseases because of its poor pharmacokinetic profile, side effects, and absence of survival properties in clinical trials. Consequently, alternative approaches such as TrkB receptor agonist application are gaining importance. 7,8-Dihydroxyflavone (7,8-DHF), a member of the flavonoid family, has been described as a robust TrkB receptor agonist in hippocampal neurons. Nevertheless, the influence of 7,8-DHF on motoneurons, one of the main targets of BDNF *in vivo*, is so far unknown. Therefore, we investigated the impact of 7,8-DHF treatment on primary cultured mouse motoneurons. Indeed, we found an activation of the TrkB receptor. Moreover, 7,8-DHF application promotes survival and neurite growth of cultured motoneurons and these effects appear dose-dependent. To investigate the PI3K/AKT and MAPK pathway activation in 7,8-DHF treated motoneurons, we developed a high-density culture system of primary mouse motoneurons. Analysis of both pathways demonstrated a PI3K/AKT but not MAPK pathway activation in cultured motoneurons. This is in contrast to previously published reports about BDNF-mediated activation of TrkB. The lack of MAPK pathway activation is also in contrast to what has been found for hippocampal neurons that indeed show MAPK activation after 7,8-DHF treatment. The ability of 7,8-DHF to imitate BDNF function in motoneurons by using Trk receptor signaling would provide a new approach for the treatment of motoneuron diseases, but needs a more detailed analysis of the activation profile of 7,8-DHF.

Introduction

The mammalian family of neurotrophins consists of four members (NGF, BDNF, NT-3, NT-4/5) and all of them are essential regulators of neuronal development and mediate neurogenesis, survival, growth, and plasticity in the vertebrate nervous system. BDNF is a non-glycosylated polypeptide of 119 amino acids that has been identified as the second member of the neurotrophin family (Barde et al., 1982). The actions of BDNF are mediated by two classes of cell surface receptors: the p75 neurotrophin receptor ($p75^{NTR}$) and the TrkB receptor. TrkB-dependent signaling has been shown to be important during both development and adulthood. Binding of BDNF to TrkB receptor elicits its dimerization and autophoshorylation and results in the activation of three major signaling pathways: PI3K/AKT, MAPK, and phospholipase C-γ1 pathway (Chao, 2003; Hempstead, 2002; Huang and Reichardt, 2001; Kaplan and Miller, 2000; Patapoutian and Reichardt, 2001). As a consequence of activating these pathways, BDNF affects synaptic transmission in hippocampal neurons (Binder and Scharfman, 2004), supports motoneuron survival (Sendtner et al., 1992), and reduces possible effects of ischemic injury (Schabitz et al., 2000). Because of its neurotrophic support of diverse neuronal cell populations that are involved in neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease (Zuccato and Cattaneo, 2009), or amyotrophic lateral sclerosis (Askanas, 1995), BDNF appears to be a powerful therapeutic tool. However, clinical trials using recombinant BDNF failed because of enzymatic degradation, poor delivery, and other limitations (Ochs et al., 2000; Thoenen and Sendtner, 2002). Many efforts have been directed at circumventing these problems and recently, an exogenous agent, so-called 7,8-DHF, was identified as a potent and selective TrkB receptor agonist in hippocampal neurons (Jang et al., 2010). Therefore, it could possibly serve as an alternative to BDNF.

7,8-DHF is a member of the flavonoid family, which is a diverse class of secondary plant metabolites, present in fruits and vegetables

Abbreviations: BDNF, brain derived neurotrophic factor; TrkB, tropomyosin-related kinase receptor tyrosine kinase B; 7,8-DHF, 7,8-dihydroxyflavone; $p75^{NTR}$, p75 Neurotrophin receptor; ALS, amyotrophic lateral sclerosis; SMA, spinal muscular atrophy; E, embryonic day; PFA, paraformaldehyde; PBS, phosphate-buffered saline; KRH, Krebs-Ringer solution; TBS, Tris-buffered saline; MN, motoneurons.

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(Spencer, 2008). Flavonoids exhibit several biological functions. They can act as cancer prevention agents, protect vulnerable neurons, and stimulate neuronal regeneration (Harborne and Williams, 2000; Spencer, 2008). It has been shown so far, that 7,8-DHF activates the TrkB receptor, protects against apoptosis, and activates the PI3K/AKT and MAPK pathway in hippocampal neurons (Jang et al., 2010). Therefore, 7,8-DHF appears to imitate BDNF, acts as a robust TrkB agonist, and seems to be a potential, therapeutic tool (Jang et al., 2010). Nevertheless, the influence of 7,8-DHF on motoneurons, which are major targets of BDNF *in vitro* as well as *in vivo* and which are affected in several motoneuron diseases such as amyotrophic lateral sclerosis (ALS) or spinal muscular atrophy (SMA), is still unexplained. Accordingly, motoneuron disorders such as ALS could be treated with 7,8-DHF.

In the present study, we investigated for the first time the influence of 7,8-DHF on primary cultured embryonic mouse motoneurons. Therefore, we elucidated whether 7,8-DHF treatment also influences the TrkB receptor in cultured embryonic motoneurons and found an activation of TrkB. Moreover, the effect of 7,8-DHF treatment on motoneuron survival and neurite outgrowth was analyzed and found similar to the supportive effect of BDNF. To elucidate underlying downstream pathways of TrkB after 7,8-DHF stimulation, we performed experiments with the MEK inhibitor PD98059 and the PI3K inhibitor LY294002 as well as we designed a high-density motoneuron culture to determine signaling effects on cultured motoneurons also by Western Blot. Based on these experiments motoneuron survival and outgrowth promoting effects of 7,8-DHF seem to be caused by PI3K/AKT pathway activation.

Results

TrkB receptor of motoneurons is activated by 7,8-DHF treatment

Treatment of hippocampal neurons with 7,8-DHF resulted in the activation of TrkB receptor in the absence of BDNF (Jang et al., 2010). To determine whether this response also occurs in cultured embryonic mouse motoneurons, we cultured isolated embryonic mouse motoneurons (Conrad et al., 2011; Wiese et al., 2010) in the presence of 7,8-DHF (40 pM), BDNF (40 pM, positive control), and in the absence of stimulation factor (control).

Immunohistochemical analyses of motoneurons, which were cultured without stimulation, displayed no phosphorylated TrkB receptor (pTrkB) (Fig. 1A). After a treatment with 40 pM BDNF for 3 min a dotted pTrkB staining pattern was visible over the whole surface of the motoneurons (Fig. 1B). Also a treatment with 7,8-DHF (40 pM) for 3 min revealed an activation of the TrkB receptor of motoneurons, which was observable as a dotted staining pattern of phosphorylated TrkB receptor at the cell surface (Fig. 1C).

7,8-DHF acts as a survival enhancer of embryonic motoneurons in vitro in a dose-dependent manner

Cultured motoneurons, grown in the presence of BDNF, showed reduced cell death after 5 days in culture (Wiese et al., 1999) and this effect is concentration dependent. Treatment of hippocampal neurons with 7,8-DHF resulted in an increased cell survival similar to that of BDNF treated cells (Jang et al., 2010). To investigate the

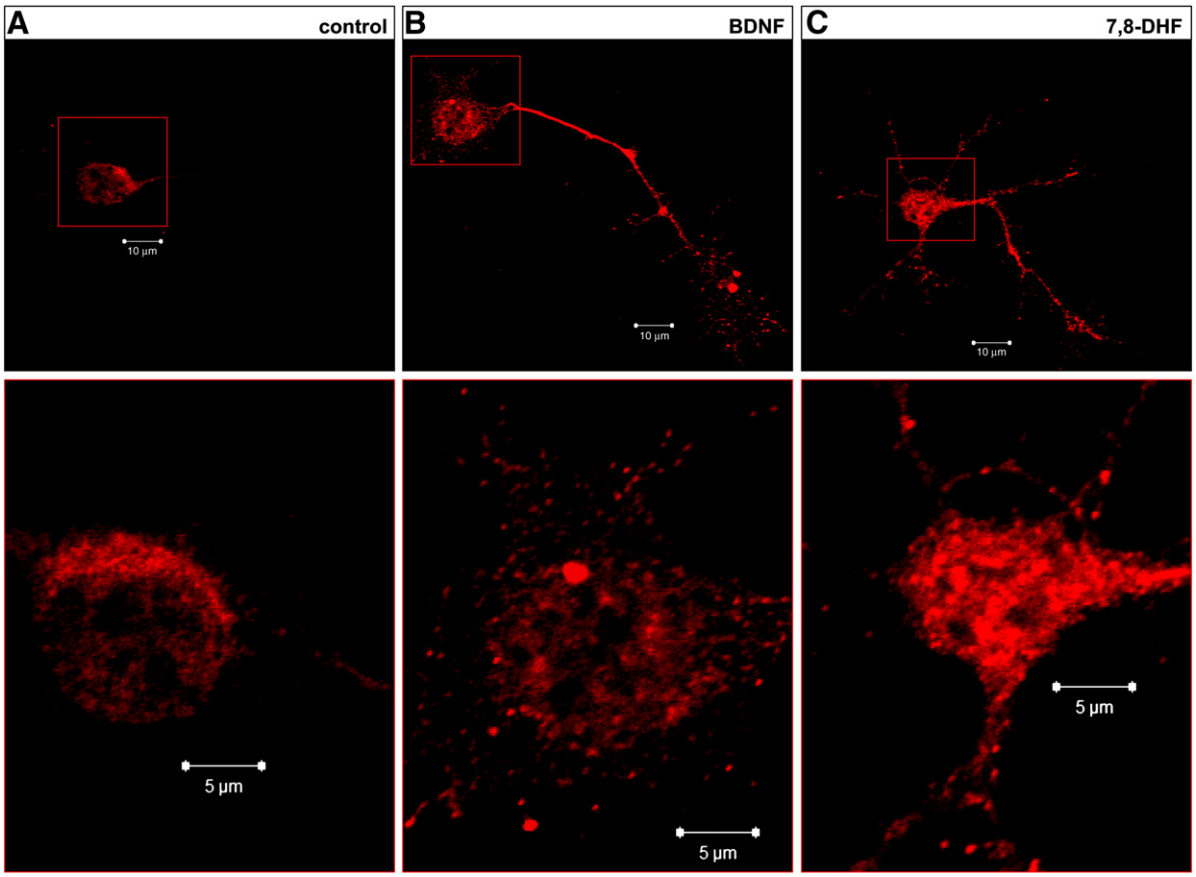

Fig. 1. 7,8-DHF elicits TrkB receptor phosphorylation in embryonic spinal cord motoneurons. Enriched isolated motoneurons from lumbar spinal cord were cultured for 2 days *in vitro*. Untreated motoneurons represented the control group (A). Motoneurons were treated for 3 min with 40 pM BDNF (B) or 7,8-DHF (C). Motoneurons stimulated with BDNF or 7,8-DHF elicited TrkB receptor phosphorylation at their cell surface.