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RESEARCH ARTICLE

# Neuron-type-specific signaling by the p75$^{NTR}$ death receptor is regulated by differential proteolytic cleavage

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## ABSTRACT

Signaling by the p75 neurotrophin receptor (p75$^{NTR}$, also known as NGFR) is often referred to as cell-context dependent, but neuron-type-specific signaling by p75$^{NTR}$ has not been systematically investigated. Here, we report that p75$^{NTR}$ signals very differently in hippocampal neurons (HCNs) and cerebellar granule neurons (CGNs), and we present evidence indicating that this is partly controlled by differential proteolytic cleavage. Nerve growth factor (NGF) induced caspase-3 activity and cell death in HCNs but not in CGNs, whereas it stimulated NFκB activity in CGNs but not in HCNs. HCNs and CGNs displayed different patterns of p75$^{NTR}$ proteolytic cleavage. Whereas the p75$^{NTR}$ carboxy terminal fragment (CTF) was more abundant than the intracellular domain (ICD) in HCNs, CGNs exhibited fully processed ICD with very little CTF. Pharmacological or genetic blockade of p75$^{NTR}$ cleavage by γ-secretase abolished NGF-induced upregulation of NFκB activity and enabled induction of CGN death, phenocopying the functional profile of HCNs. Thus, the activities of multifunctional receptors, such as p75$^{NTR}$, can be tuned into narrower activity profiles by cell-type-specific differences in intracellular processes, such as proteolytic cleavage, leading to very different biological outcomes.

**KEY WORDS: Cell death, γ-secretase, Nerve growth factor, NF-κB, Presenilin-1**

## INTRODUCTION

The p75 neurotrophin receptor (p75$^{NTR}$, also known as NGFR), a member of the tumor necrosis factor (TNF) receptor superfamily, can regulate a number of different biological activities in response to ligand binding, including cell death and/or survival, axonal growth and synaptic plasticity (Dechant and Barde, 2002; Roux and Barker, 2002; Underwood and Coulson, 2008). The specific biological outcomes mediated by p75$^{NTR}$ in a given cell depend in part on the type of ligand engaging the receptor. Initially, p75$^{NTR}$ was characterized as a receptor for members of the neurotrophin family, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 and neurotrophin-4 (NT-3 and NT-4, respectively). These neurotrophins can also signal through TrkA, B and C (also known as NTRK1, 2 and 3, respectively), members of the receptor tyrosine kinase superfamily (Reichardt, 2006). The two receptor systems can function synergistically, antagonistically or independently of each other in different cell types (Lu et al., 2005). In addition to its function as a receptor for mature neurotrophins, p75$^{NTR}$ has been shown to bind and mediate biological effects when stimulated with unprocessed neurotrophins (pro-neurotrophins), an interaction that is thought to preferentially result in cell death (Lee et al., 2001). In addition to mature and pro-neurotrophins, a number of different ligands are known to engage or signal through p75$^{NTR}$, including myelin-derived polypeptides, such as myelin-associated glycoprotein (MAG) or Nogo (also known as reticulon-4; Wang et al., 2002; Wong et al., 2002), the rabies virus glycoprotein (RVG) (Tuffereau et al., 1998) and the β-amyloid peptide, a major component of the amyloid plaques in Alzheimer's disease (Knowles et al., 2009; Perini et al., 2002). Some of these ligands, such as mature neurotrophins, RVG and β-amyloid, can bind p75$^{NTR}$ directly, whereas others require co-receptor proteins for either binding or downstream signaling. Pro-neurotrophins engage p75$^{NTR}$ together with members of the Vps10 family, including sortilin, SorL1 and SorCS2 (Nykjaer and Willnow, 2012; Nykjaer et al., 2004). Myelin-derived ligands require expression of Nogo receptor (NgR, also known as RTN4R) and Lingo-1 for signaling through p75$^{NTR}$ (Mi et al., 2004; Wang et al., 2002; Wong et al., 2002).

p75$^{NTR}$ is known to engage different signaling pathways in the cell, of which the three most important ones are the JNK or caspase pathway, which mediates cell death (Friedman, 2000; Yoon et al., 1998), the RhoA pathway, which regulates axon growth, collapse and degeneration (Park et al., 2010; Yamashita and Tohyama, 2003; Yamashita et al., 1999) and the NF-κB pathway, which has been implicated in cell survival (Carter et al., 1996; Khursigara et al., 2001). Structural determinants involved in the regulation of each of these pathways have been mapped onto non-overlapping epitopes in the p75$^{NTR}$ intracellular death domain, demonstrating that different signaling outputs can be genetically separated in p75$^{NTR}$ (Charalampopoulos et al., 2012). Using this approach, p75$^{NTR}$ mutants that are selectively deficient in one pathway but not others have been generated. As the intracellular domain (ICD) of p75$^{NTR}$ lacks catalytic activity, the receptor engages with downstream signaling pathways by recruitment and release of intracellular components. Several proteins that interact with the p75$^{NTR}$ ICD have been shown to link the receptor to well-known signaling pathways, such as RIP2 (also known as RIPK2), for the NF-κB pathway (Khursigara et al., 2001), and RhoGDI, for the RhoA pathway (Yamashita and Tohyama, 2003). However, most of the intracellular proteins that are known to interact with p75$^{NTR}$, such as NRIF (Casademunt et al., 1999), NRAGE (also known as MAGED1; Salehi et al., 2000), SC-1 (Chittka and Chao, 1999), Bex1 (Vilar et al., 2006) and several others, are not members of previously established signaling pathways. Moreover, p75$^{NTR}$ undergoes proteolytic

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cleavage at the plasma membrane. Extracellular cleavage by membrane metalloproteases (often referred to as 'α-cleavage') is followed by intramembrane cleavage by γ-secretase (referred to as 'γ-cleavage'), leading to release of the $p75^{NTR}$ ICD in the cytoplasm (Jung et al., 2003; Kanning et al., 2003). Proteolytic processing of $p75^{NTR}$ occurs constitutively in cells, but some studies have indicated that it might also be regulated by ligand binding. Pharmacological experiments have indicated that proteolytic cleavage contributes to $p75^{NTR}$-mediated cell death in sympathetic and hippocampal neurons (Kenchappa et al., 2006; Volosin et al., 2008), but the broader significance of $p75^{NTR}$ cleavage for its activities in different neuronal subpopulations is not fully understood.

In summary, a variety of ligands and co-receptors, together with proteolytic cleavage, can contribute to the diversity of $p75^{NTR}$ signaling. In addition to these mechanisms, several studies have described the activities of $p75^{NTR}$ as being cell-context dependent, meaning that the receptor is able to elicit distinct responses in different types of cells. However, the extent to which distinct pathways can be regulated by $p75^{NTR}$ in different cell types has not been systematically investigated, and the molecular bases for cell-type-specific functions of $p75^{NTR}$ have not been defined. In this study, we investigated signaling and biological activities regulated by $p75^{NTR}$ in response to NGF in two populations of neurons in the developing mammalian brain, namely hippocampal neurons (HCNs) and cerebellar granule neurons (CGNs). Both of these neuronal types express $p75^{NTR}$ in the absence of TrkA receptors. The NGF responses that we have studied are $p75^{NTR}$-mediated, as they are abolished in $p75^{NTR}$-knockout neurons. We found striking differences in the ability of $p75^{NTR}$ to induce cell death and activation of the NF-κB pathway in these two neuronal types. We also found important differences in the proteolytic processing of $p75^{NTR}$ between the two classes of neurons, and we present genetic and pharmacological evidence indicating that differences in proteolytic processing machinery contribute to the different biological responses elicited by $p75^{NTR}$ in the two neuronal types.

## RESULTS

### $p75^{NTR}$ differentially activates cell death and NF-κB pathways in hippocampal and cerebellar granule neurons

We investigated the ability of $p75^{NTR}$ to induce cell death, assessed by TUNEL assay, in embryonic (E17.5) HCNs and early postnatal (P7) CGNs. Cultures were stimulated with NGF for 24 h, fixed and stained for TUNEL and the neuronal marker β-III tubulin (Fig. 1A). NGF was able to induce cell death in HCNs but not in CGNs (Fig. 1B). Next, we investigated the ability of $p75^{NTR}$ to engage the NF-κB and JNK signaling pathways in response to NGF in cultured HCNs and CGNs. For the NF-κB pathway, we assessed NGF-induced recruitment of RIP2 to $p75^{NTR}$, degradation of IκBα (also known as NFKBIA), a negative regulator of the pathway, and nuclear translocation of the p65 NF-κB subunit ($p65^{NFκB}$). RIP2–$p75^{NTR}$ interaction was assessed by proximity ligation assay (PLA) in HCNs and CGNs after 0, 15 or 30 min of NGF stimulation. RIP2 was robustly recruited to $p75^{NTR}$ in CGNs but not in HCNs (Fig. 2A). Moreover, NGF stimulated IκB degradation in CGNs but not in HCNs (Fig. 2B). In agreement with these results, NGF induced nuclear translocation of $p65^{NFκB}$ in CGNs but not in HCNs (Fig. 2C). However, interleukin-1β (IL-1b) readily induced $p65^{NFκB}$ nuclear translocation in HCNs (Fig. 2C), indicating that the pathway can be activated by means other than NGF–$p75^{NTR}$ signaling in these neurons. For the JNK pathway, we assessed the ability of NGF to induce phosphorylation of c-jun kinase (JNK) in cultured HCNs and CGNs. Although NGF was capable of inducing JNK phosphorylation in both neuronal types, this was most prominent on the p54 isoform in HCNs, but on the p46 isoform in CGNs (Fig. 2D). Taken together, these data demonstrate a differential ability of $p75^{NTR}$ to induce cell death, NF-κB activity and phosphorylation of JNK isoforms in HCNs and CGNs.

### $p75^{NTR}$-mediated NF-κB activity regulates cell survival in cerebellar granule neurons

Activation of the NF-κB pathway has previously been linked to cell survival in different cell types, including neurons (Beg and Baltimore, 1996; Mattson et al., 1997; Middleton et al., 2000). We speculated that the inability of NGF to induce cell death through $p75^{NTR}$ in CGNs might have been due to activation of the NF-κB pathway in these cells. Sustained NF-κB activity is important for CGN cell survival, as blockade of NF-κB signaling with JSH-23 [which inhibits nuclear translocation of $p65^{NFκB}$ and its transcriptional activity (Shin et al., 2004)] induced caspase-3 cleavage in cultured CGNs (Fig. 3A). Our laboratory has recently elucidated a structure-function map of the $p75^{NTR}$ death domain and identified residues that are important for the selective activation

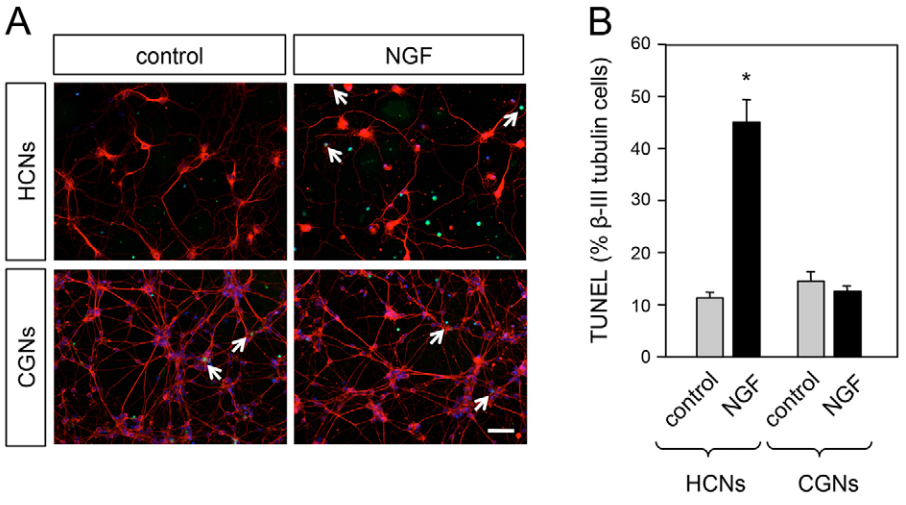


**Fig. 1. $p75^{NTR}$ can induce cell death in hippocampal neurons but not in cerebellar granule neurons.** (A) Cell death in response to NGF treatment was assessed in hippocampal neurons (HCNs) and cerebellar granule neurons (CGNs), identified by β-III tubulin immunostaining (red), using the TUNEL method (green). Nuclear staining by DAPI is shown in blue. Arrows indicate β-III tubulin and TUNEL double-positive cells. Scale bar: 50 µm. (B) Quantification of TUNEL-positive neurons in HCN and CGN cultures after 24 h treatment with NGF. Results are expressed as the mean±s.e.m. (relative to control) of three independent experiments each performed in triplicate. *$P<0.05$ versus control (Student's *t*-test).

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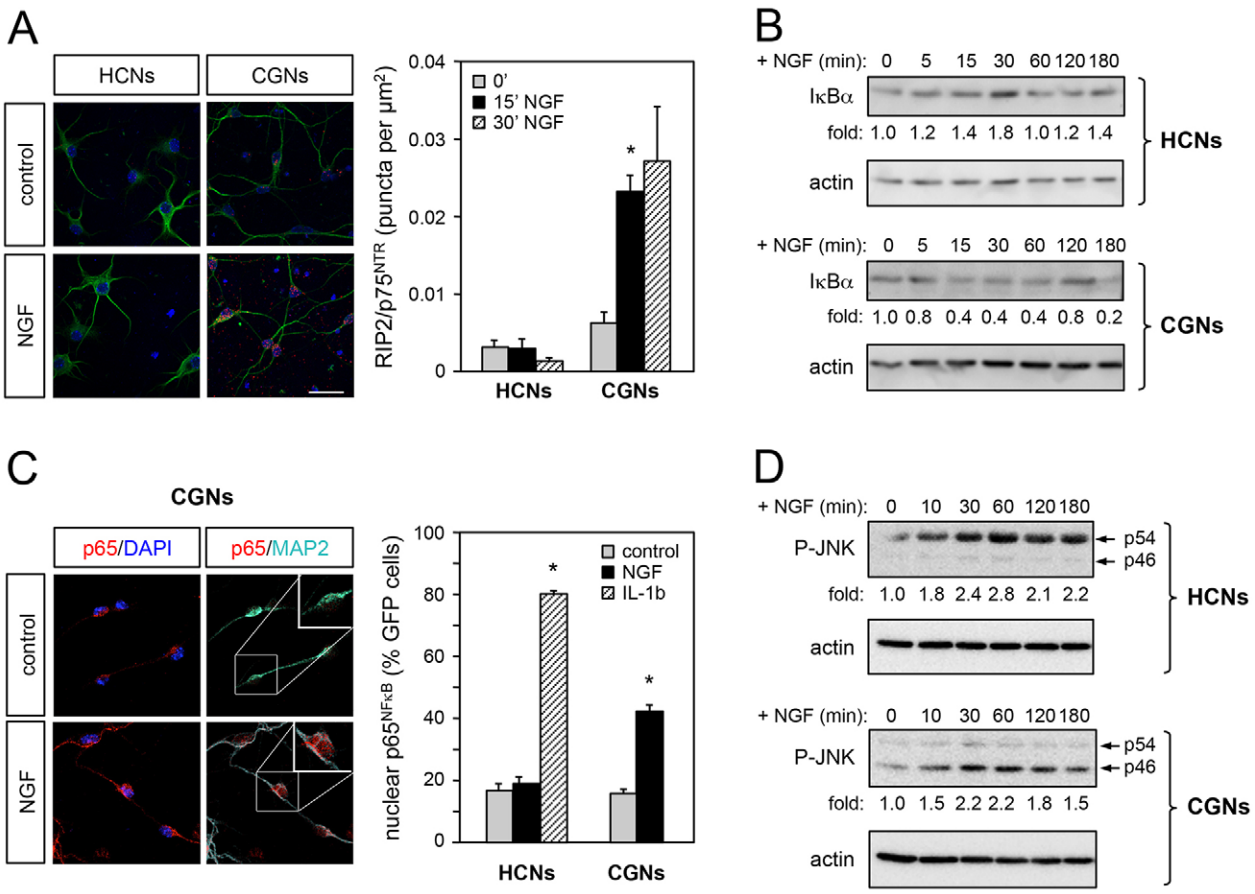


**Fig. 2. Differential p75$^{NTR}$-mediated activation of NF-κB and JNK signaling in hippocampal and cerebellar granule neurons.** (A) Recruitment of RIP2 to p75$^{NTR}$ in HCNs and CGNs following NGF treatment as assessed by proximity ligation assay (PLA). PLA signal appears as red puncta, neurons were identified by MAP2 staining (green) and nuclei were counterstained with DAPI (blue). Scale bar: 30 µm. The graph shows quantification of PLA puncta density under basal conditions (0') and after 15 and 30 min of NGF treatment. Results are expressed as the mean±s.e.m. of three independent experiments each performed in triplicate. *$P$<0.05 versus 0' (Student's $t$-test). (B) Levels of IκBα in HCNs and CGNs following NGF treatment for the indicated times. Fold change was calculated by densitometric scanning of IκBα signals normalized to actin levels. Results shown are representative of three independent experiments. (C) Nuclear translocation of p65$^{NFkB}$ (red) in CGNs identified by MAP2 staining (green) in response to NGF (representative micrographs shown in upper panel). The histogram shows quantification of the proportion of cells showing prominent nuclear p65$^{NFkB}$ signal in CGNs and HCNs following stimulation with NGF or interleukin-1β (IL-1b). Results are expressed as the mean±s.e.m. of three independent experiments each performed in triplicate. *$P$<0.05 versus control (Student's $t$-test). (D) Levels of phosphorylated c-jun kinase (P-JNK) in HCNs and CGNs following NGF treatment for the indicated times. p54 and p46 JNK isoforms are indicated. Fold change was calculated by densitometric scanning of IκBα signals normalized to actin levels. Results shown are representative of two independent experiments.

of JNK–caspase-3 and NF-κB pathways (Charalampopoulos et al., 2012). p75$^{NTR}$ molecules carrying mutations in these residues can be used to genetically dissect the contribution of different pathways to p75$^{NTR}$ physiology by reconstitution experiments in p75$^{NTR}$-deficient cells. As shown in Fig. 3B, a p75$^{NTR}$ mutant with a selective defect in NFκB signaling (p75$^{NFkB}$) was unable to induce p65$^{NFkB}$ nuclear translocation in CGNs derived from p75$^{NTR}$-knockout mice in response to NGF. In contrast, both wild-type p75$^{NTR}$ and a mutant deficient in JNK signaling (p75$^{JNK}$) readily induced nuclear translocation of p65$^{NFkB}$ after NGF treatment in these cells (Fig. 3B). We took advantage of the p75$^{NFkB}$ mutant to investigate the contribution of p75$^{NTR}$-mediated NF-κB activity to the regulation of cell death pathways by NGF in HCNs and CGNs. As expected, NGF induced caspase-3 activation in HCNs (Fig. 4A) but not in CGNs (Fig. 3C) isolated from wild-type mice, in agreement with the TUNEL assay. p75$^{NTR}$-knockout CGNs reconstituted with wild-type p75$^{NTR}$ behaved as CGNs isolated from wild-type mice and were refractory to activation of caspase-3 by NGF (Fig. 3C). In contrast, knockout CGNs reconstituted with a p75$^{NFkB}$ mutant deficient in NF-κB signaling displayed a significant increase in caspase-3 activation, which was further enhanced by stimulation with NGF (Fig. 3C). Thus, absence of receptor coupling to the NF-κB pathway unmasked the ability of p75$^{NTR}$ to induce cell death in CGNs. We speculated that activation of NF-κB signaling might limit the ability of p75$^{NTR}$ to induce cell death in CGNs, perhaps by inducing a parallel survival pathway in response to NGF. NF-κB has been shown to induce the expression of several genes encoding a variety of anti-apoptotic proteins, including many from the Bcl-2 and IAP families (Karin and Lin, 2002; Kucharczak et al., 2003; Perkins, 2007; Wang et al., 1998). We therefore investigated the induction of a number of anti-apoptotic NF-κB target genes in CGNs following stimulation with NGF. We found that NGF treatment for 24 h greatly induced the expression of NIAP (neuronal inhibitor of apoptosis protein, also known as NAIP) and Livin (also known as BIRC7), two anti-apoptotic genes regulated by the NF-κB pathway (Fig. 3D;



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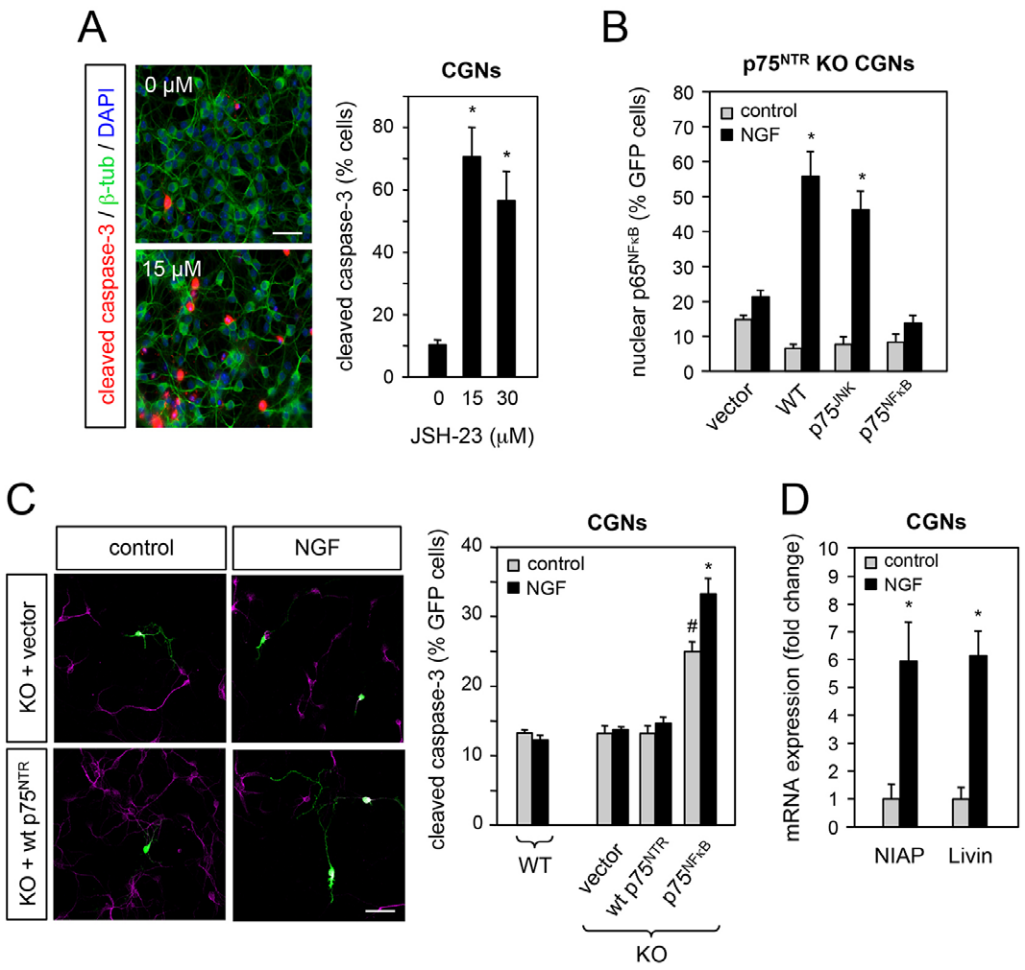


**Fig. 3. p75$^{NTR}$-mediated NF-κB activity regulates cell survival in cerebellar granule neurons.** (A) Activated caspase-3 in CGNs after overnight treatment with NF-kB inhibitor JSH-23. The micrographs show immunofluorescence images of cleaved caspase-3 (red) and β-III tubulin (green) with DAPI as nuclear counterstaining (blue). Scale bar: 30 μm. The graph depicts the percentage of cells showing activated caspase-3 under control conditions (0) and after treatment with two doses of JSH-23. Results are expressed as the mean±s.e.m. of four independent experiments each performed in triplicate. *$P$<0.05 versus 0 μM. (B) Nuclear translocation of p65$^{NFkB}$ in p75$^{NTR}$ knockout (KO) CGNs reconstituted with the indicated constructs in response to NGF treatment. p75$^{JNK}$ and p75$^{NFkB}$ denote mutant p75$^{NTR}$ constructs deficient in JNK and NF-κB signaling, respectively. EGFP plasmid was co-transfected to allow the recognition of transfected neurons. The graph shows the percentage of GFP-positive cells showing nuclear p65$^{NFkB}$ staining. Results are expressed as the mean±s.e.m. of three independent experiments each performed in triplicate. *$P$<0.05 versus control. (C) Activated caspase-3 in wild-type (WT) and p75$^{NTR}$ knockout CGNs in response to NGF. Knockout neurons were reconstituted with the indicated constructs. The micrographs show examples of the responses of knockout neurons. Scale bar: 30 μm. The graph shows the percentage of GFP-positive cells showing activated caspase-3. Results are expressed as the mean±s.e.m. of three independent experiments each performed in triplicate. #$P$<0.05 versus wild type; *$P$<0.05 versus control. (D) Induction of mRNAs encoding pro-survival NF-κB target genes NIAP and Livin in CGNs in response to NGF was analyzed by Q-PCR. Results are expressed as the mean±s.e.m. ($N$=5). *$P$<0.05

Busuttil et al., 2002; Zhang et al., 2014). NGF did not induce the expression of Bcl-2, Bcl-xL (encoded by *BCL2L1*), cIAP1 (also known as BIRC2), cIAP2 (also known as BIRC3), XIAP, Bruce (also known as BIRC6) or survivin (also known as BIRC5) in cultured CGNs (data not shown).

In HCNs derived from p75$^{NTR}$-knockout mice, reconstitution with wild-type p75$^{NTR}$ or the p75$^{NFkB}$ mutant resulted in comparable activation of caspase-3 in response to NGF (Fig. 4A), demonstrating that cell death of HCNs induced by NGF is indeed mediated by p75$^{NTR}$ and that it is unaffected by the mutation that impairs receptor coupling to NF-κB signaling. In view of the pro-survival effects of NF-κB in CGNs, we investigated whether activation of this pathway might have similar effects in HCNs; in particular, whether it could counteract the induction of caspase-3 activity by NGF in these neurons. To this end, we used IL-1b, a known stimulator of the NF-κB pathway in HCNs, in the presence or absence of the NF-κB inhibitor JSH-23. As expected, pharmacological blockade of NF-κB had no effect on the ability of NGF to induce caspase-3 cleavage in cultured HCNs (Fig. 4B). On its own, IL-1b had no major effect on caspase-3 activity, and blockade of NF-κB promoted a small increase, although still within the range of basal activity levels of naive cells (Fig. 4B). Interestingly, IL-1b attenuated the activation of caspase-3 in response to NGF, and this could be reverted by JSH-23, indicating that the anti-apoptotic effect of IL-1b in HCNs was mediated by the NF-κB pathway (Fig. 4B). These results indicate that, as in CGNs, activation of the NF-κB pathway is able to intercept the pro-apoptotic effects of NGF in HCNs. The inability of p75$^{NTR}$ to effectively engage this pathway thereby allows

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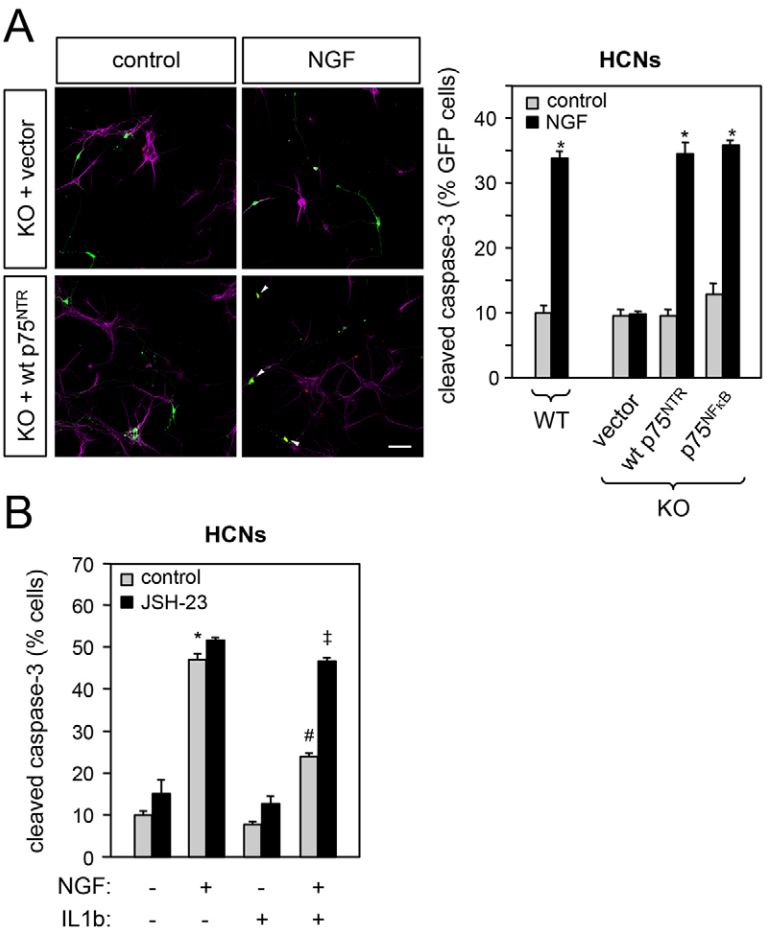


**Fig. 4. p75$^{NTR}$-mediated apoptosis in hippocampal neurons can be attenuated by concomitant activation of NF-κB.** (A) Activated caspase-3 (red) in wild-type (WT) and p75$^{NTR}$-knockout (KO) HCNs in response to NGF. Knockout neurons were reconstituted with the indicated constructs. The micrographs show examples of the responses of knockout neurons. Neurons were identified by MAP2 staining (purple). EGFP plasmid was co-transfected to allow recognition of transfected neurons (green). Scale bar: 30 μm. The graph shows the percentage of GFP-positive cells showing activated caspase-3. Results are expressed as the mean±s.e.m. of three independent experiments each performed in triplicate. *$P$<0.05 versus control (Student's $t$-test). (B) Activated caspase-3 in wild-type HCNs following NGF treatment in the presence or absence of IL-1b and the NF-κB inhibitor JSH-23 (10 μM). The graph shows the percentage of neurons showing activated caspase-3. Results are expressed as the mean±s.e.m. of three independent experiments each performed in triplicate. *$P$<0.01 versus no NGF; $^{\#}P$<0.01 versus no IL-1b; $^{‡}P$<0.01 versus control (Student's $t$-test).

apoptosis and cell death to proceed following NGF stimulation in these neurons.

### HCNs and CGNs display different patterns of p75$^{NTR}$ proteolytic cleavage

We compared the patterns of p75$^{NTR}$ cleavage in HCNs and CGNs to assess the possible contribution of this process to the differential activities of p75$^{NTR}$ in these cells. In tissue extracts, as well as in cell culture, the carboxy-terminal fragment (CTF) of p75$^{NTR}$ – i.e. the product of α-cleavage in the absence of γ-cleavage – was significantly more enriched in hippocampus (and cultured HCNs) than in cerebellum (and cultured CGNs) (Fig. 5A,B). Conversely, the intracellular domain (ICD) – i.e. the product of both α- and γ-cleavage − was more abundant in cerebellar extracts and cultured CGNs (Fig. 5A,B), suggesting higher γ-secretase activity in cerebellar cells compared with hippocampal cells. In agreement with this, cerebellar tissue and cultured cells displayed higher levels of full-length and proteolytically activated presenilin-1 (PS-1) – the catalytically active subunit of the γ-secretase complex – than their hippocampal counterparts (Fig. 5C,D). These data suggest that differential proteolytic processing of p75$^{NTR}$ in HCNs and CGNs might underlie the different activities of the receptor in these two neuronal cell types.

### Proteolytic processing by γ-secretase regulates p75$^{NTR}$ activities in cerebellar granule neurons

In order to assess the importance of proteolytic processing for the activities of p75$^{NTR}$ in HCNs and CGNs, we first utilized a pharmacological approach based on selective inhibition of α- or γ-cleavage with TAPI or DAPT, respectively. These compounds have previously been reported to effectively disrupt cleavage of p75$^{NTR}$ in sympathetic neurons (Kenchappa et al., 2006; Kraemer et al., 2014), CGNs (Ceni et al., 2010) and HCNs (Volosin et al., 2008). Neither TAPI or DAPT were able to abolish NGF-induced activation of caspase-3 in HCNs (Fig. 6A), although a limited attenuating effect of TAPI was observed, in agreement with previously reported results (Volosin et al., 2008). These data suggest that proteolytic cleavage of p75$^{NTR}$ is not obligatory for the induction of cell death by NGF in hippocampal neurons. In CGNs, NGF did not induce caspase-3 activity, as expected, and TAPI had no effect (Fig. 6B). Surprisingly, however, treatment with DAPT allowed NGF to induce activation of caspase-3 in CGNs (Fig. 6B). Interestingly, DAPT also prevented NGF-mediated activation of the NF-κB pathway in these cells, whereas TAPI had no effect (Fig. 6C), complementary to the observed effects on cell death. Moreover, the activation of caspase-3 by NGF in the presence of DAPT could be abolished by JNK-IN-8 (Fig. 6D), a specific JNK inhibitor (Zhang et al., 2012), indicating the involvement of the JNK pathway in the activation of caspase-3 in CGNs. Taken together, these results suggest that deficient γ-cleavage after α-cleavage, but not proteolytic processing per se, of p75$^{NTR}$ prevents activation of NF-κB by NGF and allows the induction of cell death in CGNs.

A limitation of pharmacological inhibition of α- or γ-secretases is its relative lack of specificity. To address this problem, we developed a genetic approach based on variants of p75$^{NTR}$



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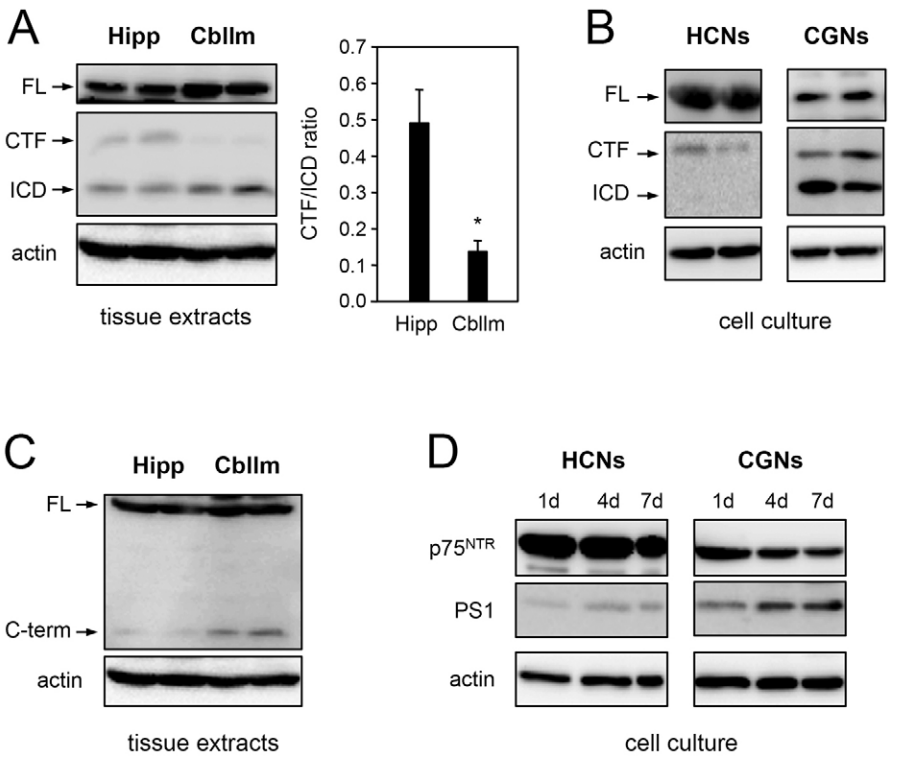


**Fig. 5. Different patterns of p75$^{NTR}$ proteolytic cleavage in hippocampal and cerebellar granule neurons.** (A) Expression of p75$^{NTR}$ and its proteolytic products in tissue extracts of P7 hippocampus (Hipp) and cerebellum (CbIlm). A representative example of a western blot is shown with duplicate samples from two different animals run in parallel for each brain structure. FL, full length; CTF, C-terminal fragment; ICD, intracellular domain. The blot was reprobed for actin as the loading control. The graph shows quantification of the CTF:ICD ratio. Results are expressed as the mean±s.d. of three independent experiments. *$P$<0.05 versus Hipp (Student's $t$-test). (B) Expression of p75$^{NTR}$ and its proteolytic products in cultured HCNs and CGNs after 7 days *in vitro* in the presence of epoxomicin. (C) Expression of presenilin-1 (PS-1) [full length and carboxy-terminal fragment (C-term)] in tissue extracts of P7 hippocampus and cerebellum. A representative example of a western blot is shown with duplicate samples from two different animals run in parallel for each brain structure. (D) Expression of p75$^{NTR}$ and PS-1 in cultured HCNs and CGNs after the indicated days (d) *in vitro*.

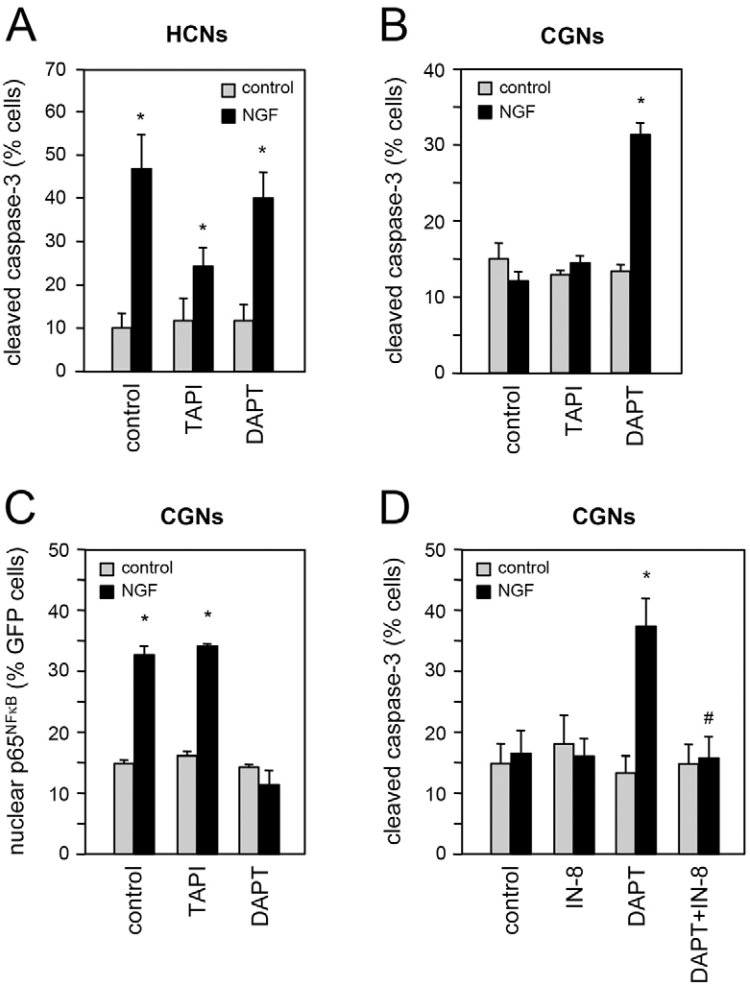


**Fig. 6. Pharmacological inhibition of p75$^{NTR}$ proteolytic cleavage affects receptor activities in cerebellar granule neurons but not in hippocampal neurons.** (A) Effects of TAPI and DAPT on NGF-mediated activation of caspase-3 in wild-type hippocampal neurons (HCNs). The graph shows the percentage of neurons showing activated caspase-3. (B) Effects of TAPI and DAPT on NGF-mediated activation of caspase-3 in wild-type cerebellar granule neurons (CGNs). The graph shows the percentage of neurons showing activated caspase-3. (C) Effects of TAPI and DAPT on NGF-mediated nuclear translocation of p65$^{NFkB}$ in wild-type CGNs. The graph shows the percentage of GFP-positive cells showing nuclear p65$^{NFkB}$ staining. (D) Effects of DAPT and JNK-IN-8 (IN-8, 3 μM) on NGF-mediated activation of caspase-3 in wild-type CGNs. The graph shows the percentage of neurons showing activated caspase-3. Results in A–D are expressed as the mean±s.e.m. of three independent experiments each performed in triplicate. *$P$<0.05 versus control; $^{\#}P$<0.05 versus no IN-8 (Student's $t$-test).



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carrying point mutations that interfere with α- and γ-cleavage of the receptor. In earlier work, Coulson and colleagues reported that introduction of an N-glycosylation site at position 243 of the stalk region of the p75$^{NTR}$ extracellular domain effectively blocks α-cleavage of the receptor (Coulson et al., 2008; Underwood et al., 2008), possibly through a steric hindrance mechanism. In transfected COS-7 cells, a p75$^{NTR}$ construct carrying this mutation (Val$^{243}$ to Asn, herein referred to as N-glyco) was completely resistant to proteolytic cleavage induced by phorbol 12-myristate 13-acetate (PMA), in agreement with previous studies (Fig. 7A). As γ-cleavage is dependent upon a prior α-cleavage, neither ICD nor CTF can be produced by the p75$^{NTR}$ N-glyco construct. We also generated a γ-cleavage-resistant variant by replacing intramembrane residue Val$^{264}$ with Ala (herein referred to as γsec$^{mut}$). This construct can generate the p75$^{NTR}$ CTF but not the ICD in transfected COS-7 cells (Fig. 7A). In subsequent experiments, constructs expressing wild-type p75$^{NTR}$, N-glyco, γsec$^{mut}$ or empty vector were transfected into neurons derived from p75$^{NTR}$-knockout mice. In agreement with the pharmacological experiments, reconstitution of p75$^{NTR}$-knockout HCNs with wild type, N-glyco mutant or γsec$^{mut}$ (but not empty vector) restored the ability of NGF to induce activation of caspase-3 in these cells (Fig. 7B). This result confirms that proteolytic cleavage of p75$^{NTR}$ is not necessary for hippocampal neuron death in response to NGF. As expected, wild-type p75$^{NTR}$ did not induce cell death in CGNs and the N-glyco mutant had no effect on cell death rate (Fig. 7C). In contrast, γsec$^{mut}$ induced cell death of knockout CGNs, both at basal levels and to an even greater extent after stimulation with NGF (Fig. 7C), a result that resembles the activity of the NF-κB-deficient p75$^{NTR}$ mutant (compare to Fig. 3C). Taken together, these results confirmed those obtained with the pharmacological approach, indicating that

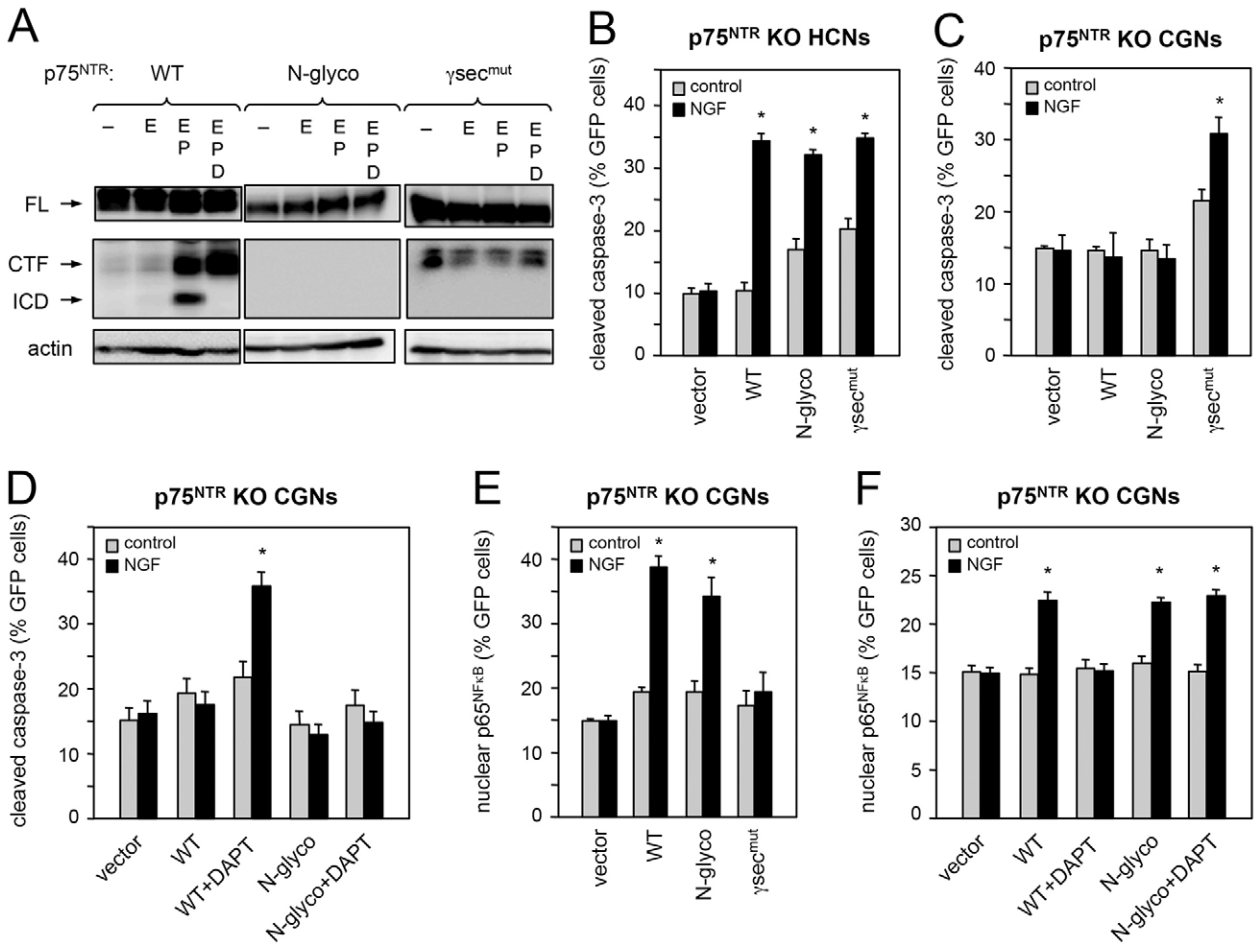


**Fig. 7. Proteolytic processing by γ-secretase regulates p75$^{NTR}$ activities in cerebellar granule neurons.** (A) Proteolytic cleavage of wild-type (WT) p75$^{NTR}$, N-glyco and γsec$^{mut}$ mutant variants in transfected COS cells. FL, full length; CTF, C-terminal fragment; ICD, intracellular domain. Epoxomicin ('E') is a proteasomal inhibitor that facilitates detection of the p75$^{NTR}$ ICD. PMA ('P') was used to induce p75$^{NTR}$ proteolytic cleavage. DAPT ('D') blocks γ-secretase and prevents formation of the ICD. The blot was reprobed for actin as loading control. (B) Activation of caspase-3 in p75$^{NTR}$-knockout HCNs reconstituted with the indicated constructs in response to NGF treatment. The graph shows the percentage of GFP-positive cells showing activated caspase-3. (C) Activation of caspase-3 in p75$^{NTR}$-knockout CGNs reconstituted with the indicated constructs in response to NGF treatment. The graph shows the percentage of GFP-positive cells showing activated caspase-3. (D) NGF-mediated activation of caspase-3 in p75$^{NTR}$-knockout CGNs reconstituted with the indicated constructs and treated with DAPT or TAPI as indicated. The graph shows the percentage of GFP-positive cells showing activated caspase-3. (E) Nuclear translocation of p65$^{NFkB}$ in p75$^{NTR}$-knockout CGNs reconstituted with the indicated constructs in response to NGF treatment. The graph shows the percentage of GFP-positive cells showing nuclear p65$^{NFkB}$ staining. (F) NGF-mediated nuclear translocation of p65$^{NFkB}$ in p75$^{NTR}$-knockout CGNs reconstituted with the indicated p75$^{NTR}$ constructs and treated with DAPT or TAPI as indicated. The graph shows the percentage of GFP-positive cells showing nuclear p65$^{NFkB}$ staining. Results in B–F are expressed as the mean±s.e.m. of three independent experiments each performed in triplicate. *$P$<0.05 versus control (Student's $t$-test).

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deficient γ-cleavage after α-cleavage, but not proteolytic processing per se, of $p75^{NTR}$ allows induction of cell death in CGNs by NGF. As γ-cleavage depends on prior α-cleavage, DAPT should not have any effect on knockout CGNs reconstituted with the N-glyco mutant. Indeed, although DAPT treatment of $p75^{NTR}$-knockout CGNs reconstituted with wild-type $p75^{NTR}$ allowed NGF-induced cell death, in agreement with our findings on wild-type neurons, this was not the case if the N-glyco mutant was introduced instead (Fig. 7D). Finally, we tested whether these results could be explained by the status of NF-κB activation in these cells. In fact, whereas both wild-type $p75^{NTR}$ and the N-glyco mutant were able to reconstitute NGF-mediated nuclear translocation of $p65^{NFkB}$ in knockout CGNs, $\gamma sec^{mut}$ was unable to do so (Fig. 7E), paralleling the ability of this construct to induce caspase-3 activity in these cells. Moreover, although DAPT inhibited NGF-mediated $p65^{NFkB}$ nuclear translocation in knockout neurons reconstituted with wild-type $p75^{NTR}$, the N-glyco mutant retained its ability to activate NF-κB in the presence of DAPT in (Fig. 7F). We conclude from these results that, although full proteolytic cleavage does not per se affect $p75^{NTR}$ activities in CGNs, incomplete cleavage, i.e. α- without γ-cleavage, inhibits the ability of the receptor to couple to the NF-κB pathway and allows the induction of cell death in response to NGF, presumably as a result of diminished pro-survival NF-κB activity.

## DISCUSSION

This study describes differential responses to $p75^{NTR}$ activation in two subpopulations of central nervous system neurons and provides mechanistic evidence for the contribution of proteolytic processing to the regulation of cell-type-specific activities in this receptor. In HCNs, $p75^{NTR}$ was able to induce cell death, but not activation of NF-κB, when stimulated with NGF, in agreement with previous observations (Volosin et al., 2008). RIP2 was previously shown to link $p75^{NTR}$ to the NF-κB pathway (Khursigara et al., 2001), and we found that NGF induced RIP2 recruitment to the receptor in CGNs but not in HCNs, providing a possible explanation for the inability of $p75^{NTR}$ to regulate NF-κB signaling in the latter. HCNs might lack components necessary for a stable interaction between RIP2 and $p75^{NTR}$ or, alternatively, the receptor might engage other effector proteins that prevent efficient recruitment of RIP2. Nevertheless, the ability of IL-1b to activate $p65^{NFkB}$ and counteract the pro-apoptotic effects of NGF in HCNs suggests that the NF-κB pathway is capable of inducing a pro-survival response in these neurons. In contrast to HCNs, $p75^{NTR}$ could not induce cell death in CGNs, but it stimulated NF-κB nuclear translocation and expression of the pro-survival NF-κB target genes NIAP and Livin in a ligand-dependent manner in these neurons. Interestingly, both genetic (i.e. $p75^{NFkB}$ mutant) and pharmacological (i.e. DAPT) manipulations allowed $p75^{NTR}$ to induce cell death in CGNs, indicating that the receptor is indeed capable of coupling to cell death pathways in these cells. The fact that this apoptotic response could be blocked by JNK-IN-8 indicates that JNK activity contributes to CGN apoptosis, as previously shown for HCNs (Friedman, 2000). We suggest that the ability of $p75^{NTR}$ to induce cell death in CGNs in response to NGF is normally suppressed or masked by concurrent activation of NF-κB signaling, which has pro-survival effects in these neurons. As NGF was able to induce JNK phosphorylation in CGNs, NF-κB signaling might intercept the pro-apoptotic pathway downstream of JNK in these neurons.

Following the discovery of the proteolytic processing of $p75^{NTR}$ (Jung et al., 2003; Kanning et al., 2003), subsequent studies have attempted to elucidate its functional significance and regulation. It is now widely accepted that both endogenous as well as overexpressed $p75^{NTR}$ undergoes proteolytic cleavage by α- and γ-secretases constitutively in cells, indicating that this process is part of the normal lifecycle of the receptor. Proteolytic cleavage is greatly enhanced by PMA, which activates α-secretases through PKC (Jung et al., 2003; Kanning et al., 2003). There is less consensus, however, as to whether proteolytic cleavage can also be regulated by ligand binding and other agents. The majority of studies have reported that neurotrophin binding to $p75^{NTR}$ does not have a direct effect on the proteolytic processing of the receptor (Ceni et al., 2010; Jung et al., 2003; Kanning et al., 2003; Sykes et al., 2012; Urra et al., 2007). In contrast, Carter and colleagues reported induction of $p75^{NTR}$ cleavage by brain-derived neurotrophic factor (BDNF) in sympathetic neurons isolated from the rat superior cervical ganglion (Kenchappa et al., 2006; Kenchappa et al., 2010). In our own experiments, we have not been able to detect significant changes in the proteolytic cleavage of $p75^{NTR}$ in either HCNs or CGNs following NGF treatment (data not shown). Other studies have indicated that several other stimuli can influence the proteolytic processing of $p75^{NTR}$, including interaction with sortilin (Skeldal et al., 2012), ubiquitylation and palmitoylation (Powell et al., 2009; Underwood et al., 2008), activation of Trk receptors (Ceni et al., 2010; Kanning et al., 2003; Urra et al., 2007), myelin-derived ligands (Domeniconi et al., 2005), β-amyloid peptide (Sotthibundhu et al., 2008) and oxidative stress (Kraemer et al., 2014; Le Moan et al., 2011). Aside from its regulation, most studies are in agreement that proteolytic cleavage can affect $p75^{NTR}$-mediated signaling and biological activities. Based primarily on pharmacological inhibition, these studies have, by and large, reported detrimental effects on $p75^{NTR}$ function following blockade of cleavage, either by α- or γ-secretases. In most cases, interference with either α- or γ-cleavage was reported to have similar consequences on receptor activity, which has been taken as evidence for the functional importance of the ICD fragment.

In our present study, we find that proteolytic processing of $p75^{NTR}$ is not per se required for activation of cell death or NF-κB pathways in HCNs and CGNs following stimulation with NGF. This conclusion was based on both pharmacological and genetic inhibition of $p75^{NTR}$ cleavage. Although TAPI caused some attenuation of NGF-induced death of HCNs, these cells remained responsive to NGF. A similar attenuating effect, without complete inhibition, was also reported by Friedman and colleagues (Volosin et al., 2008). This could be due to non-specific effects of TAPI on other cellular targets, as we could not detect any decrease in the activity of the N-glyco mutant, which does not undergo proteolytic processing. In CGNs, we found that DAPT blocked activation of NF-κB in response to NGF. However, this was not a reflection of an overall requirement for receptor cleavage because TAPI, which interrupts both α- and γ-cleavages, had no effect. Based on these findings, we propose that proteolytic cleavage of $p75^{NTR}$ by α- and γ-secretases leading to release of its ICD is not an obligatory step for its bioactivity in either HCNs or CGNs, at least as far as cell death and NF-κB pathways are concerned. It remains possible that in some circumstances, or in other cell types, receptor cleavage and release of the ICD might play ancillary roles by, for example, amplifying or modulating certain signaling pathways.

Our studies in CGNs revealed an unexpected divergence between the significance of α- and γ-cleavage for $p75^{NTR}$ signaling. Although neither pharmacological nor genetic inhibition of α-cleavage (which also interrupts γ-cleavage) had any effect, both DAPT and $\gamma sec^{mut}$ blocked NGF-mediated activation of NF-κB signaling and, somewhat paradoxically, allowed induction of cell

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death. Importantly, NGF-mediated death of CGNs in the presence of DAPT was prevented by the N-glyco mutation, indicating that it was not due to non-specific effects of the compound on other γ-secretase targets. Taken together, these data indicate that α- and γ-cleavage of $p75^{NTR}$ are not equivalent and can have distinct functional outcomes. Moreover, our results suggest that the CTF, if left uncleaved by γ-secretase, might negatively affect the ability of the receptor to regulate NF-κB signaling. Further work will be required to elucidate the mechanisms by which the CTF of $p75^{NTR}$ affects full-length receptor function.

We found different patterns of $p75^{NTR}$ proteolytic cleavage in HCNs and CGNs, both *in vivo* and in cell culture. CGNs displayed higher levels of fully processed ICD than HCNs, and this correlated with higher levels of both full-length and proteolytically activated PS-1. In a previous study, NGF-mediated degradation of IκB was found to be compromised in mouse embryo fibroblasts (MEFs) derived from PS-1-knockout mice (Powell et al., 2009), which is in agreement with our DAPT and $γsec^{mut}$ results in CGNs. In fact, pharmacological or genetic blockade of $p75^{NTR}$ cleavage by γ-secretase in these cells phenocopied the functional profile of HCNs, suggesting that differences in proteolytic processing of $p75^{NTR}$ might, in part, underlie the divergent signaling activities of this receptor in the two neuronal types. We propose that cell-type-specific differences in intracellular processes, such proteolytic intramembrane cleavage, can skew the activity profiles of $p75^{NTR}$ and other multifunctional receptors to allow different subsets of signaling and biological activities in different cell types.

## MATERIALS AND METHODS

### Plasmids, antibodies, proteins and chemicals

Full-length rat $p75^{NTR}$ was expressed from a pCDNA3 vector backbone (Invitrogen). The $p75^{NFkB}$ and $p75^{JNK}$ mutant constructs have been described previously (Charalampopoulos et al., 2012), and correspond to the triple mutant D355A/H359A/E363A and the double mutant D392A/S393A, respectively. N-glyco (V243N) and $γsec^{mut}$ (V264A) mutations were introduced using QuikChange (Stratagene) and verified by DNA sequencing. Cell surface expression of the mutant constructs was verified by whole-cell biotinylation and immunoprecipitation experiments. EGFP plasmid was from Clontech. The origins of antibodies were as follows: 9992 anti-$p75^{NTR}$ (for immunoblotting) from Promega; anti-cleaved caspase-3, anti-IκB and anti-phospho-JNK from Cell Signaling; anti-$p65^{NFkB}$ and anti-actin from Santa Cruz Biotechnology; anti-β-III-tubulin and anti-MAP2 from Sigma or Abcam; anti-PS-1 from Chemicon; anti-RIP2 from BD Biosciences. Fluorophore-conjugated secondary antibodies were from Molecular Probes. NGF was purchased from Alomone Labs and IL-1 (beta) from R&D. Both were used at 100 ng/ml. The NF-κB inhibitor JSH-23 was from Millipore, JNK inhibitor JNK-IN-8 was from Millipore, epoxomicin, PMA and DAPT were from Sigma and TAPI-2 was from Enzo Life Sciences.

### Tissue culture and cell transfection

Primary neurons were prepared from wild-type and $p75^{NTR}$-knockout mice (Jackson Labs). Hippocampal neurons (HCNs) were isolated from embryonic day 17.5 mouse embryos. HCNs were maintained in Neurobasal medium supplemented with 2% B27 (Invitrogen), 1 mM glutamine and 50 µg/ml gentamycin (Gibco). Cerebellar granule neurons (CGNs) were isolated from postnatal day 7 (P7) mouse pups. CGNs were maintained in basal medium Eagle (BME) supplemented with 10% fetal calf serum, 25 mM KCl, 1 mM glutamine and 2 mg/ml gentamicin. Transfection was performed after 3 days (HCNs) or 5 days (CGNs) *in vitro* with Lipofectamine LTX (Invitrogen).

### $p75^{NTR}$ cleavage and immunoblotting

PMA was used at 200 nM for 1 h. Epoxomicin was used at 1 µM, TAPI-2 at 20 µM and DAPT at 2 µM. These compounds have previously been reported to effectively disrupt cleavage of $p75^{NTR}$ in sympathetic neurons (Kenchappa et al., 2006; Kraemer et al., 2014), CGNs (Ceni et al., 2010) and HCNs (Volosin et al., 2008) at concentrations equal to or lower than those used in this study. They were applied 1.5 h prior to PMA as indicated. Immunoblots were developed using the ECL Advance Western blotting detection kit (Life Technologies) and exposed to Kodak X-Omat AR films. Image analysis and quantification of band intensities was done with ImageQuant (GE Healthcare).

### Proximity ligation assay

After 5 days *in vitro*, CGN cultures were switched to serum-free medium and 12.5 mM KCl for 2 h prior to treatment. HCNs and CGNs were treated with NGF (100 ng/ml) for 15 or 30 min. Cells were fixed for 20 min in 4% paraformaldehyde (PFA)/4% sucrose, permeabilized for 5 min in ice-cold methanol, and blocked in 10% normal donkey serum in PBS. Fixed cells were incubated at 4℃ overnight with anti-p75 (1:300), anti-RIP2 (1:100) and anti-MAP2 (1:4000) antibodies in PBS supplemented with 3% BSA. The Duolink *In Situ* Proximity Ligation kit (Sigma) was used as per the manufacturer's instructions with fluorophore-conjugated secondary antibody to recognize MAP2 (Molecular Probes, 1:2000) included during the amplification step. MAP2-positive cells were imaged with an LSM Imager Z2 confocal microscope (Zeiss) to detect PLA puncta, puncta on MAP2-positive neurons were counted and the number of puncta was normalized to the area of MAP2-positive cells using ImageJ software.

### $p65^{NFkB}$ nuclear translocation

After 5 days *in vitro*, CGN cultures were switched to serum-free medium and 12.5 mM KCl for 2 h prior to addition of NGF (100 ng/ml). After 30 min with NGF, cells were fixed for 20 min in 4% PFA, permeabilized for 5 min in ice-cold methanol and blocked in 10% normal donkey serum. JSH-23 inhibitor was applied for 24 h. Fixed cells were incubated at 4℃ overnight with anti-$p65^{NFkB}$ (1:100) and monoclonal anti-MAP2 (1:4000) antibodies in PBS supplemented with 3% BSA, followed by incubation with fluorophore-conjugated secondary antibodies (1:1500). GFP/MAP2-positive cells were analyzed with a LSM Imager Z2 confocal microscope (Zeiss) to detect nuclear $p65^{NFkB}$ staining, and normalized to the total number of GFP/MAP2-positive neurons to estimate the extent of $p65^{NFkB}$ nuclear translocation among transfected neurons.

### Quantitative PCR

Total cellular RNA was isolated by using the RNAeasy kit (Qiagen) according to the manufacturer's instructions. Isolated RNA samples were digested with DNase prior to reverse transcription with Superscript II (Invitrogen). Quantitative (Q)-PCR of cDNA samples was performed on a ABI StepOne Plus instrument (Applied Biosystems) using SYBR Green master mix (Applied Biosystems) and primers as follows: NIAP forward, 5′- GGTGAAGAGTCCAGAGAAGATG-3′; NIAP reverse, 5′-CTGA-GAGTACACGTGGTGATG-3′; Livin forward, 5′-ACTAATGAGAA-GGGTCCCAAAG-3′; Livin reverse, 5′-TGTGCACCTGGAAAGGAT-AG-3′. The annealing temperature was 60℃ for both templates. Q-PCR data were normalized using the expression of ribosomal 18 S as indicated previously (Guo et al., 2014).

### Cell death assays

NGF (100 ng/ml) was added to cultures after 5 days (HCNs) or 6 days (CGNs) *in vitro* and kept for an additional 24 h. During this period, CGNs were switched to lower (12.5 mM) KCl and serum-free medium. TUNEL was assessed using a kit from Roche following the manufacturer's instructions. Activated caspase-3 was detected by immunocytochemistry. Briefly, cells were washed once with PBS, fixed for 20 min at room temperature with 4% PFA, permeabilized for 10 min at room temperature with PBS plus 0.2% Triton X-100, and blocked in PBS plus 10% normal donkey serum and 0.2% Triton X-100. Fixed cells were incubated at 4℃ overnight with anti-cleaved-caspase-3 (1:400) and monoclonal anti-MAP2 (1:4000) antibodies, followed by incubation with fluorophore-conjugated secondary antibodies (Molecular Probes, 1:2000). GFP/MAP2-positive cells were analyzed with a LSM Imager Z2 confocal microscope (Zeiss) to detect cleaved caspase-3, and

the data were normalized to the total number of GFP/MAP2-positive neurons to estimate the extent of cell death among transfected neurons. The results were typically plotted relative to cell death levels under control conditions (e.g. no treatment). Levels of cell death (by either TUNEL assay or caspase-3 activation) under these conditions varied between 2 and 10% depending on the experiment.

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### Competing interests
The authors declare no competing or financial interests.

### Author contributions
A.V., L.K., J.T. and C.K. performed experiments. C.I. designed the research and wrote the paper.

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