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Induction of Neuronal Cell Death by Paraneoplastic Ma1 Antigen

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Abstract

Paraneoplastic Ma1 (PNMA1) is a member of a family of proteins involved in an autoimmune disorder called paraneoplastic neurological syndrome. Although it is widely expressed in brain, nothing is known about the function of PNMA1 in neurons. We find that PNMA1 expression is highest in the perinatal brain, a period during which developmentally regulated neuronal death occurs. PNMA1 expression increases in cerebellar granule neurons (CGNs) induced to die by low potassium (LK) and in cortical neurons following homocysteic acid (HCA) treament. Elevated PNMA1 expression is also observed in the degenerating striatum in two separate mouse models of Huntington's disease, the R6/2 transgenic model and the 3-nitropropionic acid-induced chemical model. Suppression of endogenous PNMA1 expression inhibits LK-induced neuronal apoptosis. Ectopic expression of PNMA1 promotes apoptosis even in medium containing high potassium, a condition that normally ensures survival of CGNs. Deletion of the N-terminal half of the PNMA1 protein abrogates its apoptotic activity, whereas deletion of the C-terminal half renders the protein more toxic. Within the N-terminal half, the ability to induce neuronal death depends on the presence of a BH3-like domain. In addition to being necessary for apoptosis, the BH3-like domain is necessary for self-association of PNMA1. Apoptosis by PNMA1 expression is inhibited by overexpression of Bcl2, suggesting that PNMA1-induced neuronal death may depend on the binding of a proapoptotic member of the Bcl2 family to the BH3 domain. Taken together, our results suggest that PNMA1 is a proapoptotic protein in neurons, elevated expression of which may contribute to neurodegenerative disorders.

Keywords

paraneoplastic neurological syndrome; paraneoplastic Ma1 antigen; PNMA1; apoptosis; cerebellar granule neurons; cortical neurons

During development of the nervous system, large numbers of newly produced neurons are eliminated through apoptosis. This process plays a critical role in ensuring proper connectivity of neurons and in matching the number of neurons to their target size. Abnormal loss of neurons can occur in the adult brain and is the cause of neurodegenerative disorders, including Alzheimer's disease, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis. Identifying molecules that promote or inhibit neuronal apoptosis will not only increase our understanding of normal neurodevelopment but also

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shed insight into how neurons die in various neurodegenerative diseases. Such molecules can be targeted in the development of novel approaches to treat neurodegenerative diseases.

As part of an effort to identify genes whose expression is regulated by a neuroprotective compound called *GW5074* (Chin et al., 2004), we performed a GeneChip microarray analysis. Among the genes picked up in this screen was paraneoplastic Ma1 antigen (PNMA1). PNMA1 is a member of a family of five proteins, PNMA1, PNMA2, PNMA3, PNMA4 (more commonly referred to as *MOAP1*), and PNMA5 (Dalmau et al., 1999; Schuller et al., 2005). A sixth member of this family, designated as PNMA6A (Schuller et al., 2005), has been proposed, although this protein has yet to be characterized. PNMA proteins were first discovered because of their involvement in paraneoplastic neurological syndromes, a group of rare disorders triggered by an auto-immune response against specific antigens expressed in the brain as a consequence of tumors in other parts of the body (Albert and Darnell, 2004). Neurological dysfunction caused by abnormalities such as cerebellar degeneration, limbic or brainstem encephalitis, neuromyotonia, and sensory neuropathy characterizes paraneoplastic neurological syndromes (Voltz, 2002). Despite their association with disease, the PNMA proteins are poorly studied. It is known that these proteins are expressed at high levels in the brain, with low or no expression in other organs and tissues with the exception of the testis (Dalmau et al., 1999; Takaji et al., 2009). The best studied of the PNMA proteins is MOAP1 (PNMA4; Tan et al., 2001, 2005; Baksh et al., 2005; Vos et al., 2006; Fu et al., 2007; Foley et al., 2008). Work performed in nonneuronal cell lines has revealed that MOAP1 has apoptosis-inducing activity. It promotes apoptosis by serving as an adaptor, bringing together RASSF1A, a tumor suppressor protein, and Bax, a proapoptotic member of the Bcl2 family of proteins (Baksh et al., 2005; Vos et al., 2006; Foley et al., 2008). This results in the activation of Bax and, consequently, the release of cytochrome c from mitochondria (Tan et al., 2005).

Here we report that PNMA1 expression is increased in neurons induced to undergo apoptosis. Over-expression of PNMA1 induces death in otherwise healthy neurons, whereas the suppression of PNMA1 expression has a protective effect. Although the mechanism by which PNMA1 promotes cell death is not clear, we provide evidence that its self-association, possibly as a dimer, is necessary. Moreover, the apoptosis-promoting activity depends on the presence of a BH3-like motif in PNMA1 and can be blocked by expression of Bcl2.

MATERIALS AND METHODS

Antibodies and Reagents

Unless otherwise mentioned, all supplies were purchased from Sigma-Aldrich (St. Louis, MO). Tissue culture medium and reagents were purchased from Gibco (Invitrogen, Carlsbad, CA). The sources of antibodies used in the study are as follows: mouse anti-Flag-M2, mouse anti-Myc, and mouse anti-α-tubulin antibodies were purchased from Sigma; rabbit anti-HA and goat anti-PNMA1 were from Santa Cruz Biotechnology (Santa Cruz, CA); and Texas red-conjugated goat anti-mouse IgG secondary antibodies were from were from Jackson Immunoresearch (West Grove, PA). The QuikChange II XL Site-Directed

Mutagenesis kit was from Stratagene (La Jolla, CA). Z-VAD-FMK was purchased from ENZO Life Sciences.

Expression Vectors

Mouse cDNAs encoding PNMA1, $PNMA1_{1–187}$, $PNMA1_{188–354}$ were generated by PCR amplification and subcloned into p3xFlag-CMV7.1, an expression vector containing three Flag tags in tandem. Rat PNMA1 cDNA with Myc tag was generated by RT-PCR of rat brain mRNA and subcloned in pTarget (Promega, Madison, WI). p3F-PNMA1-ΔBH3 were subcloned from p3F-PNMA1 by removing the mouse PNMA1 open reading frame of 349–381 bp. Deletion constructs were made with the QuikChange Site-Directed Mutagenesis kit, following the manufacturer's protocols. All constructs generated in house were fully sequenced to ensure that no error was introduced. HA-RASSF1A was a kind gift from Joseph Avruch (Ortiz-Vega et al., 2002), and PNMA1 short hairpin RNA vectors shPN1 (112523) and shPN2 (112524) were obtained from Sigma.

Analysis of Expression by RT-PCR

Total RNA was extracted with Trizol (Invitrogen) and subjected to reverse transcription using the SuperScript First-Strand Synthesis System kit from Invitrogen for RT-PCR. The primers used for PCR amplification were as follows: PNMA1 forward, 5′-gttctggagggaagaaaatg-3′; PNMA1 reverse, 5′-tcccaaacacctgctcaag-3′. PNMA2 forward, 5′-ggttcccagtgagg tgc-3′; PNMA2 reverse, 5′-gaagggttgtctgcttgtac-3′; PNMA3 forward, 5′-gcaaggcttatcaggaac-3′; PNMA3 reverse, 5′-ctgccaa agtctggaaca-3′; PNMA4 forward, 5′-aatgtagccttggttggg-3′; PNMA4 reverse, 5′-acgggttgtttatcttgagg-3′; PNMA5 forward, 5′-gctctgccctgtcaatca-3′; PNMA5 reverse, 5′-ttctgctccttccc tacc-3′; RASSF1A forward, 5′-aagttcacctgccattaccga-3′; RASSF1A reverse, 5′-ctgctcctcatctgacagtttcc-3′; β-actin forward, 5′-aggactcctatgtgggtgacga-3′; β-actin reverse, 5′-cgttgc caatagtgatgacctg-3′; 18S forward, 5′-gctaccacatccaaggaagg-3′; and18S reverse, 5′-ggcctcgaaagagtcctgta-3′.

Transfection of Cell Lines

HEK293T and HT-22 transfection was performed with Lipofectamine 2000 (Invitrogen). In each transfection, DNA was added at a DNA:Lipofectamine ratio of 1 μg:2 μl. Cells were maintained in transfection medium throughout the experiment.

Culturing and Transfection of Neurons

Cerebellar granule neurons (CGNs) were cultured from 7–8-day-old Wistar rats and plated on poly-L-lysine-coated dishes as described previously (D'Mello et al., 1993). Cytosine arabinofuranoside (10 μM) was added to the medium 18–22 hr after plating to prevent replication of nonneuronal cells. Treatments were performed 6–7 days after plating by replacing the medium with low-potassium (LK) medium (serum-free BME medium, 5 mM KCl) or high-potassium (HK) medium (serum-free medium, 25 mM KCl). Cortical neurons were cultured from the cerebral cortex of E17 Wistar rats as described previously (Murphy et al., 1990). Cortical neurons that were either transfected (for viability assay) or untransfected (for Western blot or RT-PCR) were subjected to 1 mM homocysteic acid (HCA) treatment for 18 hr or as indicated. CGNs and cortical neurons were transfected by

calcium-phosphate DNA precipitation as previously described (Chen et al., 2008; Majdzadeh et al., 2008). Immunocytochemical analysis of transfected cultures and 4′6′-diamidino-2-phenylindole hydrochloride (DAPI) staining was performed as previously described (Chen et al., 2008; Majdzadeh et al., 2008).

Quantification of Neuronal Viability

Viability was quantified as previously described (Koulich et al., 2001; Yalcin et al., 2003). In short, transfected cells identified by immunocytochemistry displaying condensed or fragmented nuclei (as visualized by DAPI staining) were scored as dead. Each condition was carried out in duplicate coverslips; 80–150 transfected cells were counted from each coverslip, and all experiments were repeated at least three times. Thus, more than 500 cells were counted for each condition (three experiments) unless otherwise stated. Standard error of the mean was used to determine error bars for experiments. Results were evaluated by Student's *t*-test (two-tailed), unless otherwise stated. Results obtained by DAPI staining were confirmed via TUNEL staining performed using the DeadEnd Fluorometric TUNEL System (Promega).

Western Blot and Coimmunoprecipitation Analysis

Western blot analysis was performed as previously described (Chen et al., 2008; Majdzadeh et al., 2008). After antibody probing, the chemiluminescene signal was examined either by ECL or by ECL Plus (GE Healthcare, Piscataway, NJ). For analysis of protein interaction by coimmunoprecipitation, HEK293-transfected cultures were lysed with cell lysis buffer (Cell Signaling, Beverly, MA). The lysates were incubated with 1 μg antibody and 30 μl protein A/G plus agarose beads overnight. Immunoprecipitates were collected after centrifugation, washed twice with cell lysis buffer, and then subjected to Western blot to evaluate protein interactions.

Huntington's Disease Mouse Models

Breeding pairs of R6/2 mice made up of an ovarian transplant hemizygote female and a B6CBAF1/J male, both on a C57BL/6J background strain, were purchased from Jackson Laboratories (Bar Harbor, ME). Genotyping of litters was performed 10–14 days after birth by PCR of tail-tip DNA. At 6 weeks after birth, mice heterozygous for the transgene (R6/ 2) and wild-type (WT) littermates were sacrificed by carbon dioxide inhalation. The brains were dissected, and the striatum was separated. The rest of the brain tissue was termed *extrastriatal tissue*.

3-Nitropropionic acid (3-NP) administration was performed as previously described (Chin et al., 2004; Chen et al., 2008). Briefly, 12-week-old C57BL/6J mice were injected intraperitoneally with 3-NP at 50 mg/kg twice per day for 5 days. Mice displayed severe locomotor dysfunction at this time. After euthanasia, the brains were removed, and striatal/ extrastriatal tissue was collected for RNA purification.

RESULTS

Expression of PNMA1 in the Brain

As a first step toward gaining some insight into the role of PNMA1 in the brain, we analyzed its expression in the developing and mature brain. As shown in Figure 1A, PNMA1 mRNA is clearly detectable until postnatal day 7, after which expression decreases. At 12 months, expression is barely detectable. We also analyzed the expression in various brain parts at postnatal day 7. As shown in Figure 1B, PNMA1 is expressed widely in the perinatal brain, with the lowest expression in the olfactory bulb and highest expression in the striatum.

PNMA1 Expression Is Increased in Neurons Primed To Undergo Apoptosis

Cerebellar granule neurons (CGNs) undergo apoptosis when switched from a medium containing depolarizing levels of potassium (HK) to medium containing low potassium (LK; D'Mello et al., 1993). Although cell death begins only after about 16 hr, previous work has shown that commitment to death occurs 4–6 hr after LK treatment. As shown in Figure 2A, PNMA1 mRNA expression is elevated by 3 hr (Fig. 2B), suggesting that PNMA1 might contribute to triggering neuronal death. This result was confirmed by Western blot analysis (Fig. 2C), which showed significantly increased expression of PNMA1 at 6 hr (Fig. 2D). A previous paper has reported that MOAP1 is localized to mitochondria (Tan et al., 2005). As shown in Figure 2E, in contrast to MOAP1, ectopically expressed PNMA1 localizes predominantly to the cytoplasm. The predominantly cytoplasmic localization of PNMA1 is supported by results from experiments using mitotracker to stain mitochondria (Fig. 2E, Supp. Info. Fig. 1A), as well as immunostaining with an antibody against Cox4, a mitochondrial protein (Supp. Info. Fig. 1B).

We extended our studies to primary cultures of cortical neurons. Treatment of cortical neurons with homocysteic acid (HCA) causes oxidative stress resulting in cell death (Chen et al., 2008; Majdzadeh et al., 2008). A small but discernible increase in PNMA1 mRNA (Fig. 3A,B) and protein expression (Fig. 3C,D) is observed starting at 6 hr after HCA treatment.

Expression of Other PNMA Family Members

We examined whether the other members of the PNMA family respond to apoptotic stimuli like PNMA1. As shown in Figure 4A, the expression of PNMA2, PNMA3, and MOAP1 showed no change in CGNs treated with LK. In contrast, PNMA5 expression was robustly increased. In cortical neurons, however, PNMA5 expression could not be detected either before or after treatment with HCA (Fig. 4B). In these neurons, MOAP1 expression was increased slightly by HCA treatment. Therefore, PNMA1 is the only member of the family that displays increased expression in both models of neuronal death.

PNMA1 Promotes Neuronal Apoptosis

The correlation between increased PNMA1 expression and apoptosis suggested that PNMA1 promotes neuronal death. To examine this issue, we expressed Flag-tagged PNMA1 in CGNs. As shown in Figure 5A, ectopic expression of PNMA1 induced death in

otherwise healthy neurons (maintained in HK medium) and increased the extent of cell death in LK. A similar loss of neuronal viability was observed when PNMA1 was expressed in cortical neurons that were either untreated or treated with HCA (Fig. 5B). Besides nuclear condensation and fragmentation (visuialized by DAPI staining), PNMA1-transfected neurons displayed positive TUNEL staining, confirming (Fig. 5C) that death was due to apoptosis. Interestingly, and in contrast to its apoptotic effect in nonneuronal cell types (Tan et al., 2001, 2005; Vos et al., 2006; Fu et al., 2007; Foley et al., 2008), the overexpression of MOAP1 did not reduce viability of either HK- or LK-treated CGNs to a significant extent (Supp. Info. Fig. 2).

To confirm that PNMA1 contributes to the promotion of neuronal death, we suppressed expression of endogenous PNMA1 using shRNA. A control shRNA (pLKO.1) and two shRNA-expressing constructs were tested for their ability to suppress PNMA1 expression in the HEK293T cell line (Fig. 5D). While PNMA1 expression was suppressed by one of the two shRNAs (shPN1), the other shRNA (shPN2) had no discernible effect (Fig. 5D). As shown in Figure 5E, expression of shPN1 in CGNs inhibited LK-induced neuronal death. In contrast, shPN2 and the control shRNA had no effect. Not unexpectedly, the reduction of PNMA1 expression had no effect on survival in HK.

The BH3-Like Motif Is Necessary for PNMA1-Induced Neuronal Death

Previous work performed in nonneuronal cell lines has shown that overexpression of MOAP1 induces apoptosis (Tan et al., 2001). In the case of MOAP1, this apoptosis-promoting activity was found to depend on a Bcl2 homology 3 (BH3)-like motif (Tan et al., 2001), a sequence most commonly found in members of the Bcl2 family of proteins. Members of the Bcl2 family of proteins that have a BH3 domain, but lack other BH domains (commonly referred to as *BH3-only proteins*), promote activation of proapoptotic Bcl2 proteins, leading to pore formation in mitochondria. In MOAP1, the mutation or deletion of the BH3-like motif was found to abrogate its ability to induce apoptosis (Tan et al., 2001). Although not identical in sequence to the MOAP1 BH3-like motif or to the consensus sequence for the BH3 domain that many Bcl2 proteins possess, PNMA1 does have a motif resembling the BH3 consensus sequence (Fig. 6A). We generated and expressed a PNMA1 construct, PNMA1-ΔBH3, lacking a stretch of 11 amino acids containing the BH3-like sequence (Fig. 6B). The expression of PNMA1-ΔBH3 in HK reduced the extent of cell death observed with wild-type PNMA1 to a small extent (Fig. 6C). Furthermore, the PNMA1-ΔBH3 abolished the higher level of death induced by wild-type PNMA1 in LK (Fig, 6C), suggesting that the BH3-like domain was necessary for the death-promoting activity of PNMA1. We also examined the effect of expressing two other mutant constructs: $PNMA1_{1–187}$ and $PNMA1_{188–354}$. Expression of $PNMA1_{188–354}$ had an insignificant effect on neuronal viability. In contrast, $PNMA1_{1–187}$, a construct that contains the BH3-like sequence, displayed a higher level of toxicity compared with full-length PNMA1 in HK. The different PNMA1 deletion constructs displayed a pattern of subcellular localization that was similar to that of wild-type PNMA1 (Fig. 6D), ruling out the possibility that the differences in their effect on neuronal viability were due to difference in subcellular localization.

PNMA1-Induced Apoptosis Occurs Through a Mechanism Different From the Mechanism Utilized by MOAP1

MOAP1 promotes apoptosis by interaction with RASSF1A, a tumor suppressor protein (Tan et al., 2001; Baksh et al., 2005; Vos et al., 2006; Foley et al., 2008). It has been proposed that this interaction changes the conformation of MOAP1, permitting Bax to associate with the BH3-like motif in MOAP1. To examine whether the apoptotic action of PNMA1 also involved interaction with RASSF1A, we performed a coimmunoprecipitation experiment in which both proteins were expressed in HEK293T cells. As shown in Figure 7, although association between MOAP1 and RASSF1A is readily observed, we have not been able to detect any interaction between PNMA1 and RASSF1A. We also were unable to detect interaction between PNMA1 and Bax (data not shown). These results suggest that the molecular mechanisms underlying the apoptotic activity of PNMA1 are different from those utilized by MOAP1.

Neurotoxic Effect of PNMA1 Is Blocked by Bcl2 and by Caspase Inhibition

Although interaction between Bax and PNMA1 could not be detected, the finding that PNMA1 does possess a BH3-like sequence, the deletion of which reduces the level of neurotoxicity, raises the possibility that another member of the Bcl2 family interacts with the BH3-like sequence in PNMA1 and promotes neuronal death. It is well-established that, under normal circumstances of cell survival, the activity of proapoptotic Bcl2 proteins is kept in check by antiapoptotic Bcl2 proteins, the best characterized of which is Bcl2. If a proa-poptotic Bcl2 protein is involved in the neurotoxic action of PNMA1, overexpression of Bcl2 would be expected to inhibit toxicity. As shown in Figure 8A, the overexpression of Bcl2 completely inhibits the neurotoxic effect of PNMA1 in both HK and LK medium. Proapoptotic Bcl2 proteins promote apoptosis by inducing the release of mitochondrial cytochrome c into the cytoplasm, leading to caspase activation. Not surprisingly, the pharmacological inhibition of caspases using Z-VAD-FMK, a pancaspase inhibitor, inhibits the neurotoxic effect of PNMA1 (Fig. 8B).

PNMA1 Self-Associates Through the BH3-Like Domain

Because Bcl2 family members control cell death by forming homo- or heterodimers among anti-apoptotic or proapoptotic members of this family through BH domains, we examined the possibility of whether PNMA1 self-associates through its BH3-like domain. A Flag-tagged form of PNMA1 was coexpressed with a Myc-tagged form of the protein. As shown in Figure 9A, coimmunoprecipitation experiments performed using either Flag or Myc antibody revealed that PNMA1 does self-associate. In contrast, PNMA1-ΔBH3 did not interact with the full form of PNMA1 (Fig. 9A,B). This suggests that self-association requires the BH3-like domain. In support of this conclusion is the finding that $PNMA1_{1–187}$ (which contains the BH3-like sequence) associates with the full form of the PNMA1, whereas $PNMA1_{188–354}$ does not. The lower amount of $PNMA1_{1–187}$ (see Fig. 9B) pulled down with wild-type myc-PNMA1 correlates with the lower amount of input from whole-cell lysate. Taken together, this suggests that the reduced pull-down of PNMA1 by $PNMA_{1-187}$ is not due to reduced interaction per se, but rather due to reduced expression of the truncated $PNMA_{1-187}$ protein likely due to its reduced stability. To investigate this issue

further, we compared the stability of wild-type PNMA1 with that of $PNMA1_{1–187}$. Although detailed quantification has not been performed, the half-life of wild-type PNMA1 is ~4.5 hr, whereas that of $PNMA1_{1–187}$ is ~ 15 min (Fig. 9C).

PNMA1 Expression Is Increased in Mouse Models of Huntington's Disease

One of the most widely utilized models of neuro-degenerative disease is the R6/2 transgenic mouse model of HD (Li et al., 2005). R6/2 mice display selective striatal atrophy and neurological deficits within 6–8 weeks after birth. We examined the expression pattern of PNMA1 in striatal and nonstriatal brain tissue from R6/2 transgenic mice (R6/2) and wild-type (WT) litter-mates. As shown in Figure 10A, PNMA1 expression can be detected in both striatal and nonstriatal brain tissue. Although expression of PNMA1 was comparable in nonstriatal brain tissue, expression was discernibly higher in the R6/2 striatum compared with wild-type controls. To verify this finding we used mice administered with 3-nitropropionic acid (3-NP). 3-NP is an irreversible inhibitor of succinate dehydrogenase, which, when administered to rodents, produces a Huntington's disease-like pathology, including selective striatal neurodegeneration and movement deficits (Beal et al., 1993; Dautry et al., 1999). As shown in Figure 10B, PNMA1 expression is increased in the striatum of mice administered 3-NP.

DISCUSSION

Our study focuses on PNMA1, a poorly studied protein whose cellular function is unknown. PNMA1 was first identified as an antigen in the sera of patients with paraneoplastic neurological disorders (Dalmau et al., 1999). A previous study has shown that PNMA1 is expressed at a low level in the brain and testis of mice but at a much higher level in the monkey brain, in which it is widely expressed (Takaji et al., 2009). With the use of a more sensitive method of analysis than was previously used, we report that PNMA1 is expressed widely in the rat brain as well (Fig. 1B). Expression of PNMA1 is highest in the embryonic brain through the week after birth, but then expression decreases and is barely detectable in the brains of 1-year-old rats (Fig. 1A).

The expression of PNMA1 is increased in CGNs induced to die by LK treatment (Fig. 2). Forced expression of PNMA1 induces death in otherwise healthy neurons (maintained in HK) and increases the extent of neuronal death in LK (Fig. 5A). Suppression of endogenous PNMA1 expression protects neurons, suggesting that PNMA1 contributes to LK-mediated neuronal death (Fig. 5E). An increase in PNMA1 expression is also seen in cortical neurons that are induced to die by treatment with HCA (Fig. 3), suggesting that the role of PNMA1 in promoting cell death extends to other neuronal populations. Indeed, forced expression of PNMA1 in cortical neurons promotes their demise (Fig. 5B). Although capable of inducing apoptosis in primary neurons, the overexpression of PNMA1 has no effect on the viability of the hippocampally derived HT22 neuroblastoma cell line or on the human embryonic kidney HEK293T cell line. This raises the possibility that the apoptosis-inducing activity of PNMA1 can be inhibited by a factor present in transformed cell types. It is also possible that the apoptotic activity of PNMA1 is restricted to neurons, perhaps depending on the presence of another protein.

In mammals, developmentally regulated death of neurons is highest around the time of birth. This is also the time at which PNMA1 expression is highest. Further work is needed to determine whether PNMA1 is involved in promoting developmentally-regulated neuronal death. We have observed that PNMA1 expression is increased in two separate mouse models of HD: the R6/ 2 genetic model and the 3-NP chemically induced model (Fig. 10). Interestingly, in both of these models, the increased expression was observed in the striatum but not in extrastriatal brain tissue. This observation raises the possibility that increased PNMA1 expression could contribute to the pathological loss of neurons in HD and perhaps other neurodegenerative diseases.

PNMA1 is a member of a family of five proteins that includes MOAP1. Previous studies have shown that MOAP1 also promotes apoptosis when overexpressed in cell lines (Tan et al., 2001, 2005; Vos et al., 2006; Fu et al., 2007). MOAP1 was found to connect RASSF1A to Bax physically (Baksh et al., 2005; Vos et al., 2006; Foley et al., 2008), leading to the activation of Bax and cytochrome c release (Tan et al., 2005). In contrast to the case with MOAP1, we have not been able to detect interaction between PNMA1 and either Bax or RASSF1A (Fig. 7). The mechanism by which PNMA1 kills neurons is, thus, not identical to the one utilized by MOAP1. Indeed, whereas MOAP1 has been reported to localize to mitochondria (Tan et al., 2005), PNMA1 is localized abundantly in the cytoplasm (Fig. 2E, Supp. Info. Fig. 1). It is possible that, instead of Bax, another member of the Bcl2 family of proteins associates with PNMA1 to promote neuronal death. In support of such a mechanism is that, like MOAP1, PNMA1 possesses a BH3-like sequence (Fig. 6A). Deletion of this sequence reduces the apoptosis-inducing activity of PNMA1 (Fig. 6C). Proapoptotic Bcl2 proteins generally function to facilitate release of cytochrome c from mitochondria, an event leading to activation of caspases. We find that the pharmacological inhibition of caspases blocks PNMA1-induced neurotoxicity (Fig. 8B), supporting the idea that PNMA1 acts in conjunction with proapoptotic Bcl2 proteins. We also find that overexpression of Bcl2 inhibits the ability of PNMA1 to induce apoptosis (Fig. 8A). We have not been able to detect interaction between Bcl2 and PNMA1 using coimmunoprecipitations, suggesting that Bcl2 inhibits PNMA1-induced neurotoxicity indirectly, possibly by sequestering the proapoptotic member of the family that associates with the BH3 domain of PNMA1. As with MOAP1 (Tan et al., 2001), we find that PNMA1 self-associates (Fig. 9), although it is not clear whether this association results in PNMA1 dimers or more complex multimers. Deletion of the C-terminus half of PNMA makes the protein more toxic in HK medium, suggesting that this half of the protein negatively regulates apoptotic activity (Fig. 6C). A similar observation was reported for MOAP1 (Baksh et al., 2005). In the case of MOAP1, the C-terminus has been proposed to interact electrostatically with the N-terminus end, keeping it in an inactive state. Apoptosis-inducing effectors such as RASSF1A bind to the N-terminus of MOAP1, promoting the open and active form of MOAP1 (Baksh et al., 2005). It is possible that the C-terminus of PNMA1 negatively regulates its apoptotic activity through a similar mechanism involving intramolecular interaction. In contrast to the C-terminus, the deletion of the N-terminus region involved in self-association also eliminates the ability of PNMA1 to promote cell death.

An analysis of the expression of the other members of the PNMA family revealed that the expression of PNMA5 is also induced in neurons primed to die (Fig. 4). In contrast, the

expression of PNMA2, PNMA3, and MOAP1 is not discernibly altered. Takaji et al. (2009) reported that, in the monkey brain, PNMA5 is strongly expressed in association areas, such as the prefrontal and temporal cortex, and in the limbic regions, but not in primary sensory areas. These scientists could not detect PNMA5 expression in the mouse brain by in situ hybridization and Northern blot analysis. Although it was suggested that PNMA5 might play a role in the association functions of the neocortex, the up-regulation of its expression in neurons primed to die raises the possibility that, like PNMA1, under certain circumstances PNMA5 might participate in inducing neuronal death. The lack of up-regulation of MOAP1 is somewhat surprising given its established role in promoting apoptosis in certain cell lines. Interestingly, overexpression of MOAP1 does not induce apoptosis in primary neurons (Supp. Info. Fig. 2) or in SH-SY5Y and the Neuro2a neuroblastoma cell lines (Fu et al., 2007; Takaji et al., 2009). We have observed that PNMA1 overexpression does not affect viability of HEK293T cells. Taken together, these observations suggest that these two members of the PNMA family induce death in a cell-specific manner.

In summary, our studies show for the first time that PNMA1 functions to promote neuronal death. We show that PNMA1-mediated apoptosis can be prevented by Bcl2. Although the detailed mechanism remains to be delineated, it is likely to involve association of proteins with PNMA1 through its BH3-like sequence.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

Albert ML, Darnell RB. Paraneoplastic neurological degenerations: keys to tumour immunity. Nat Rev Cancer. 2004; 4:36–44. [PubMed: 14708025]

Baksh S, Tommasi S, Fenton S, Yu VC, Martins LM, Pfeifer GP, Latif F, Downward J, Neel BG. The tumor suppressor RASSF1A and MAP-1 link death receptor signaling to Bax conformational change and cell death. Mol Cell. 2005; 18:637–650. [PubMed: 15949439]

Beal MF, Brouillet E, Jenkins BG, Ferrante RJ, Kowall NW, Miller JM, Storey E, Srivastava R, Rosen BR, Hyman BT. Neurochemical and histologic characterization of striatal excitotoxic lesions produced by the mitochondrial toxin 3-nitropropionic acid. J Neurosci. 1993; 13:4181–4192. [PubMed: 7692009]

Chen HM, Wang L, D'Mello SR. Inhibition of ATF-3 expression by B-Raf mediates the neuroprotective action of GW5074. J Neurochem. 2008; 105:1300–1312. [PubMed: 18194435]

Chin PC, Liu L, Morrison BE, Siddiq A, Ratan RR, Bottiglieri T, D'Mello SR. The c-Raf inhibitor GW5074 provides neuroprotection in vitro and in an animal model of neurodegeneration through a MEK-ERK and Akt-independent mechanism. J Neurochem. 2004; 90:595–608. [PubMed: 15255937]

Dalmau J, Gultekin SH, Voltz R, Hoard R, DesChamps T, Balmaceda C, Batchelor T, Gerstner E, Eichen J, Frennier J, Posner JB, Rosenfeld MR. Ma1, a novel neuron- and testis-specific protein, is recognized by the serum of patients with paraneoplastic neurological disorders. Brain. 1999; 122:27–39. [PubMed: 10050892]

Dautry C, Conde F, Brouillet E, Mittoux V, Beal MF, Bloch G, Hantraye P. Serial ^{1}H-NMR spectroscopy study of metabolic impairment in primates chronically treated with the succinate dehydrogenase inhibitor 3-nitropropionic acid. Neurobiol Dis. 1999; 6:259–268. [PubMed: 10448053]

D'Mello SR, Galli C, Ciotti T, Calissano P. Induction of apoptosis in cerebellar granule neurons by low potassium: inhibition of death by insulin-like growth factor I and cAMP. Proc Natl Acad Sci U S A. 1993; 90:10989–10993. [PubMed: 8248201]

Foley CJ, Freedman H, Choo SL, Onyskiw C, Fu NY, Yu VC, Tuszynski J, Pratt JC, Baksh S. Dynamics of RASSF1A/MOAP-1 association with death receptors. Mol Cell Biol. 2008; 28:4520–4535. [PubMed: 18474619]

Fu NY, Sukumaran SK, Yu VC. Inhibition of ubiquitin-mediated degradation of MOAP-1 by apoptotic stimuli promotes Bax function in mitochondria. Proc Natl Acad Sci U S A. 2007; 104:10051–10056. [PubMed: 17535899]

Koulich E, Nguyen T, Johnson K, Giardina C, D'Mello S. NF-kappaB is involved in the survival of cerebellar granule neurons: association of IkappaBbeta [correction of Ikappabeta] phosphorylation with cell survival. J Neurochem. 2001; 76:1188–1198. [PubMed: 11181838]

Li JY, Popovic N, Brundin P. The use of the R6 transgenic mouse models of Huntington's disease in attempts to develop novel therapeutic strategies. NeuroRx. 2005; 2:447–464. [PubMed: 16389308]

Majdzadeh N, Wang L, Morrison BE, Bassel-Duby R, Olson EN, D'Mello SR. HDAC4 inhibits cell-cycle progression and protects neurons from cell death. Dev Neurobiol. 2008; 68:1076–1092. [PubMed: 18498087]

Murphy TH, Schnaar RL, Coyle JT. Immature cortical neurons are uniquely sensitive to glutamate toxicity by inhibition of cystine uptake. FASEB J. 1990; 4:1624–1633. [PubMed: 2180770]

Ortiz-Vega S, Khokhlatchev A, Nedwidek M, Zhang XF, Dammann R, Pfeifer GP, Avruch J. The putative tumor suppressor RASSF1A homodimerizes and heterodimerizes with the Ras-GTP binding protein Nore1. Oncogene. 2002; 21:1381–1390. [PubMed: 11857081]

Schuller M, Jenne D, Voltz R. The human PNMA family: novel neuronal proteins implicated in paraneoplastic neurological disease. J Neuroimmunol. 2005; 169:172–176. [PubMed: 16214224]

Takaji M, Komatsu Y, Watakabe A, Hashikawa T, Yamamori T. Paraneoplastic antigen-like 5 gene (PNMA5) is preferentially expressed in the association areas in a primate specific manner. Cereb Cortex. 2009; 19:2865–2879. [PubMed: 19366867]

Tan KO, Tan KM, Chan SL, Yee KS, Bevort M, Ang KC, Yu VC. MAP-1, a novel proapoptotic protein containing a BH3-like motif that associates with Bax through its Bcl-2 homology domains. J Biol Chem. 2001; 276:2802–2807. [PubMed: 11060313]

Tan KO, Fu NY, Sukumaran SK, Chan SL, Kang JH, Poon KL, Chen BS, Yu VC. MAP-1 is a mitochondrial effector of Bax. Proc Natl Acad Sci U S A. 2005; 102:14623–14628. [PubMed: 16199525]

Voltz R. Paraneoplastic neurological syndromes: an update on diagnosis, pathogenesis, and therapy. Lancet Neurol. 2002; 1:294–305. [PubMed: 12849427]

Vos MD, Dallol A, Eckfeld K, Allen NP, Donninger H, Hesson LB, Calvisi D, Latif F, Clark GJ. The RASSF1A tumor suppressor activates Bax via MOAP-1. J Biol Chem. 2006; 281:4557–4563. [PubMed: 16344548]

Yalcin A, Koulich E, Mohamed S, Liu L, D'Mello SR. Apoptosis in cerebellar granule neurons is associated with reduced interaction between CREB-binding protein and NF-kappaB. J Neurochem. 2003; 84:397–408. [PubMed: 12559002]

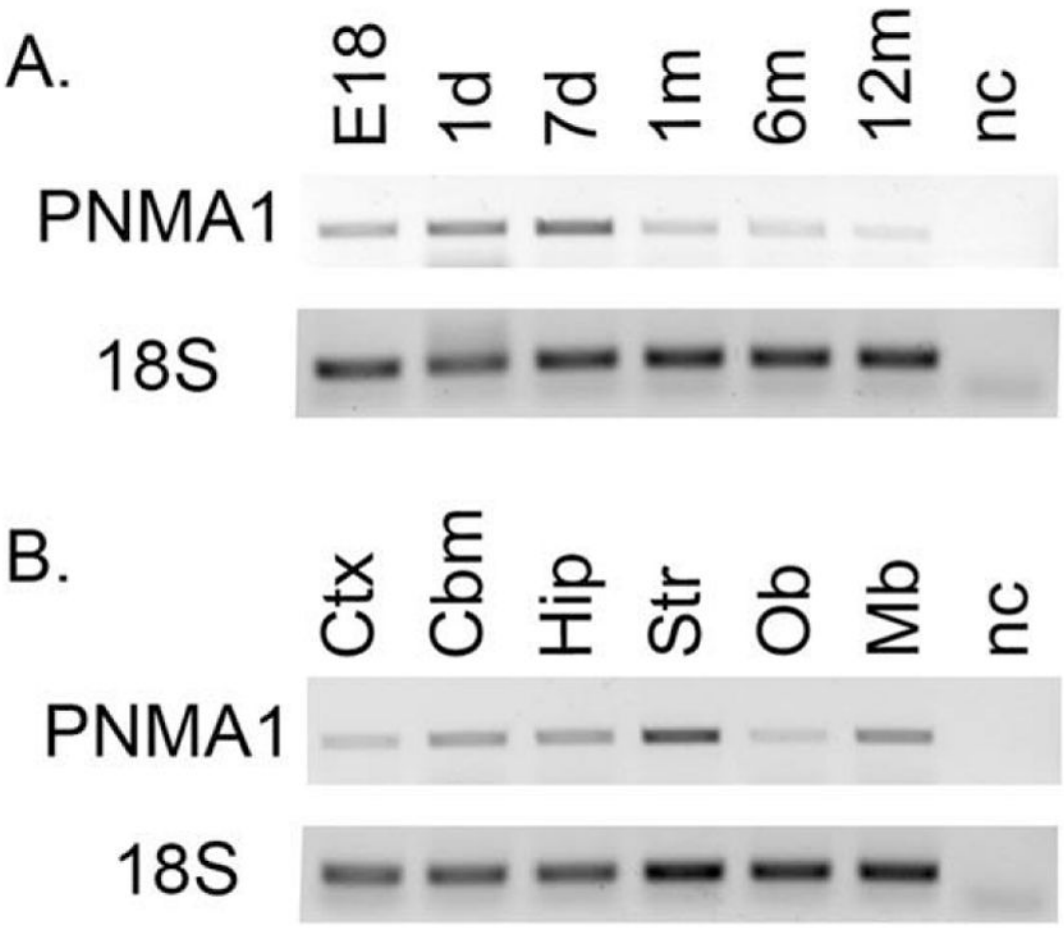

Fig. 1.

Expression of PNMA1 during brain development and in different brain regions. **A:** RNA isolated from whole-brain samples collected from rats (Wistar strain) of different ages ranging from embryonic day 18 to postnatal day 1, day 7, 1 month, 6 month, and 12 month (E18, 1d, 7d, 1m, 6m, 12m) were subjected to RT-PCR analysis. Amplification of 18S RNA was used to demonstrate that similar quantities of template were used in RT-PCR. nc, Negative control. **B:** Brains from postnatal day 7 rats were collected, and different brain regions including cortex (Ctx), cerebellum (Cbm), hippocampus (Hip), striatum (Str), olfactory bulb (Ob), and midbrain (Mb) were dissected out. RNA was prepared from the brain parts and used in RT-PCR with PNMA1 primers. Amplification of 18S RNA was used as a loading control.

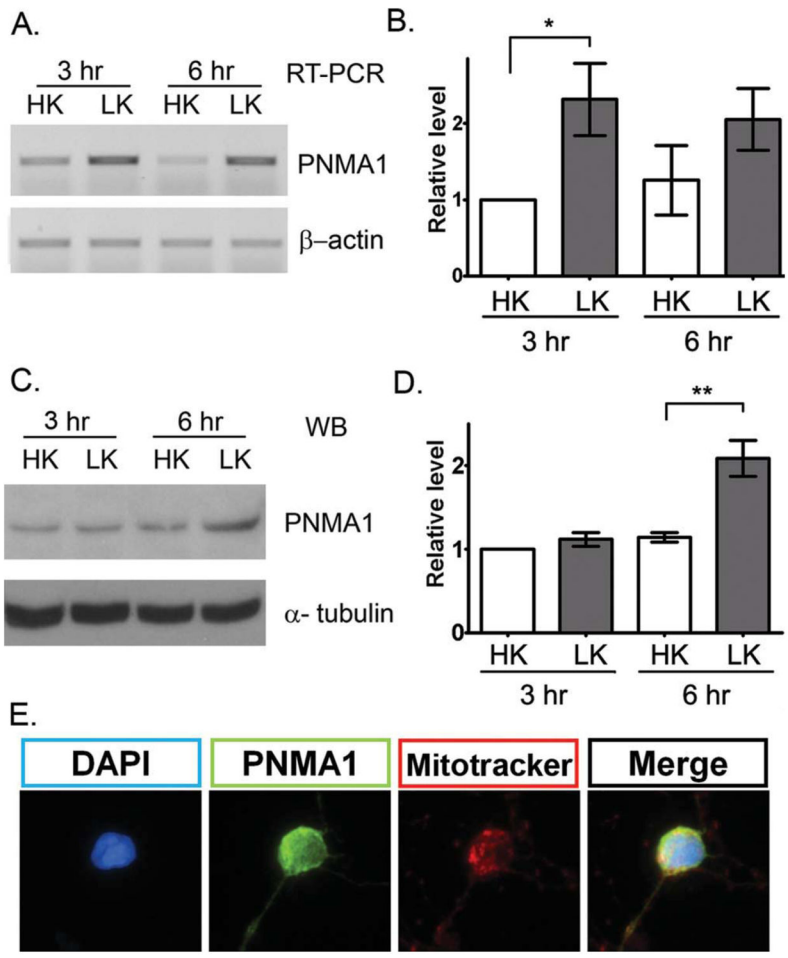

Fig. 2.

PNMA1 expression increases during LK-induced CGNs apoptosis. **A:** Cultured CGNs were treated with HK or LK for 3 or 6 hr. Relative levels of PNMA1 mRNA expression were determined by RT-PCR. The level of β-actin mRNA serves as a loading control. **B:** Quantification of PNMA1 mRNA expression in CGNs. Densitometric analysis of RT-PCR was normalized with respect to β-actin. Relative levels were compared with 3 hr HK. ★P < 0.05, two-tail paired t-test; n = 3. **C:** Lysates were obtained from CGNs treated with HK or LK for 3 or 6 hr and subjected to Western blot analysis using a PNMA1 antibody. The same membrane was reprobed with an antibody against α-tubulin to confirm equal loading. **D:** Quantification of PNMA1 protein levels in CGNs. Densitometric analysis of Western blots was normalized with respect to α-tubulin. Relative levels were compared with 3 hr HK. ★★P < 0.01, two-tail paired t-test; n = 3. **E:** CGNs were transfected with a Flag-tagged PNMA1 expressing plasmid and expressed for 24 hr. Cells were then stained with anti-Flag (green) and mitotracker (red). PNMA1 is found predominantly in the cytoplasm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

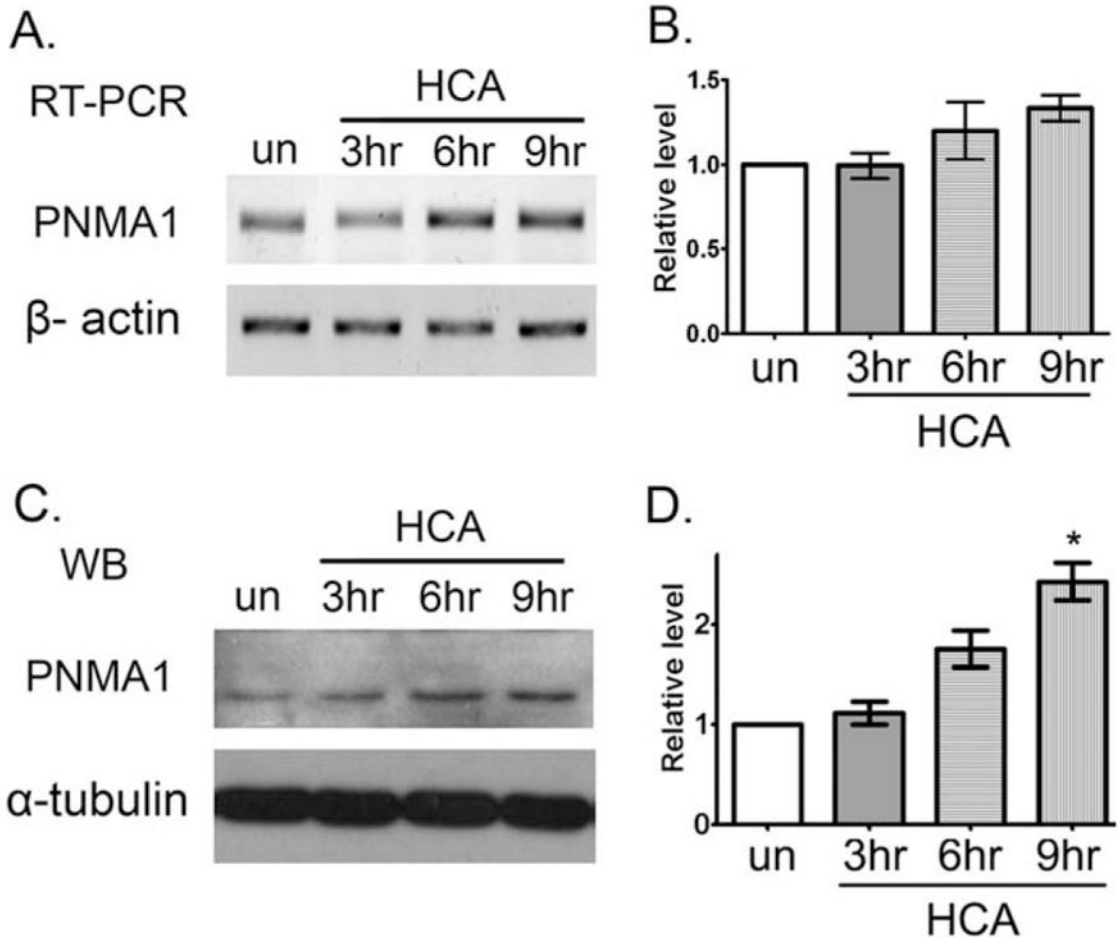

Fig. 3.

PNMA1 level increases during HCA-induced apoptosis of cortical neurons. **A:** RNA isolated from untreated (un) cortical neuron cultures and cultures treated with 1 mM HCA for 3, 6, and 9 hr were subjected to RT-PCR. β-actin serves as a loading control. **B:** Quantification of alterations of PNMA1 mRNA in HCA-treated cortical neurons. Graph shows data derived from densitometric scans of RT-PCR. PNMA1 bands were normalized with respect to β-actin bands. Relative levels were compared with untreated group; n = 3. **C:** Lysates of cortical neurons either untreated or treated with 1 mM HCA for 3, 6, and 9 hr were subjected to Western blot analysis using a PNMA1 antibody. The blot was reprobed with α-tubulin to demonstrate equal sample loading. **D:** Quantification of PNMA1 protein in HCA-treated cortical neurons. Graph shows data derived from densitometric scans of Western blots. PNMA bands were normalized to expression of α-tubulin. Relative levels were compared with the untreated group. ★$P < 0.01$ vs. untreated cortical neurons, two-tail paired *t*-test; n = 3.

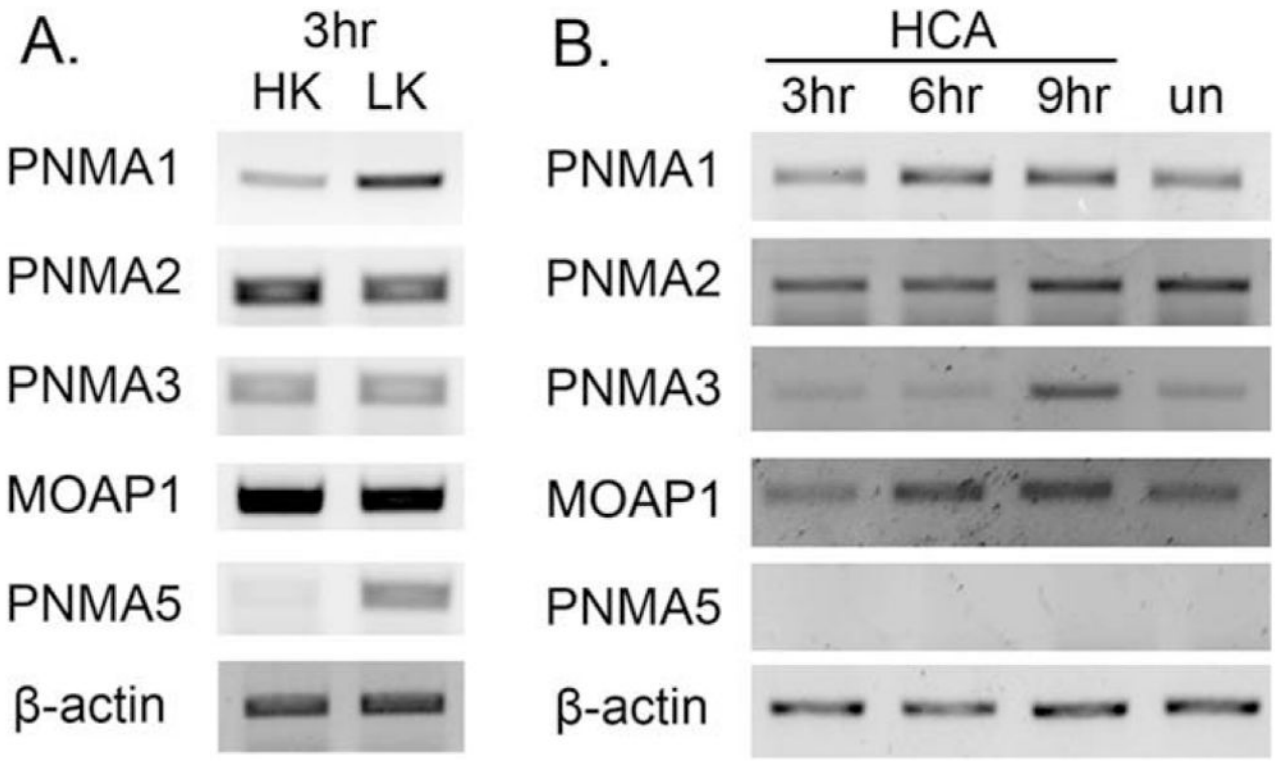

Fig. 4.

Expression of PNMA family members in CGNs and cortical neurons. **A:** Total RNA from CGNs was collected after 3 hr of HK or LK treatment. Expression of each PNMA member was determined by RT-PCR. β-actin serves as loading control. **B:** Cortical neurons were treated with 1 mM HCA for 3, 6, and 9 hr. Untreated (un) cells serve as a control group. mRNA levels of PNMA family members were assessed by RT-PCR.

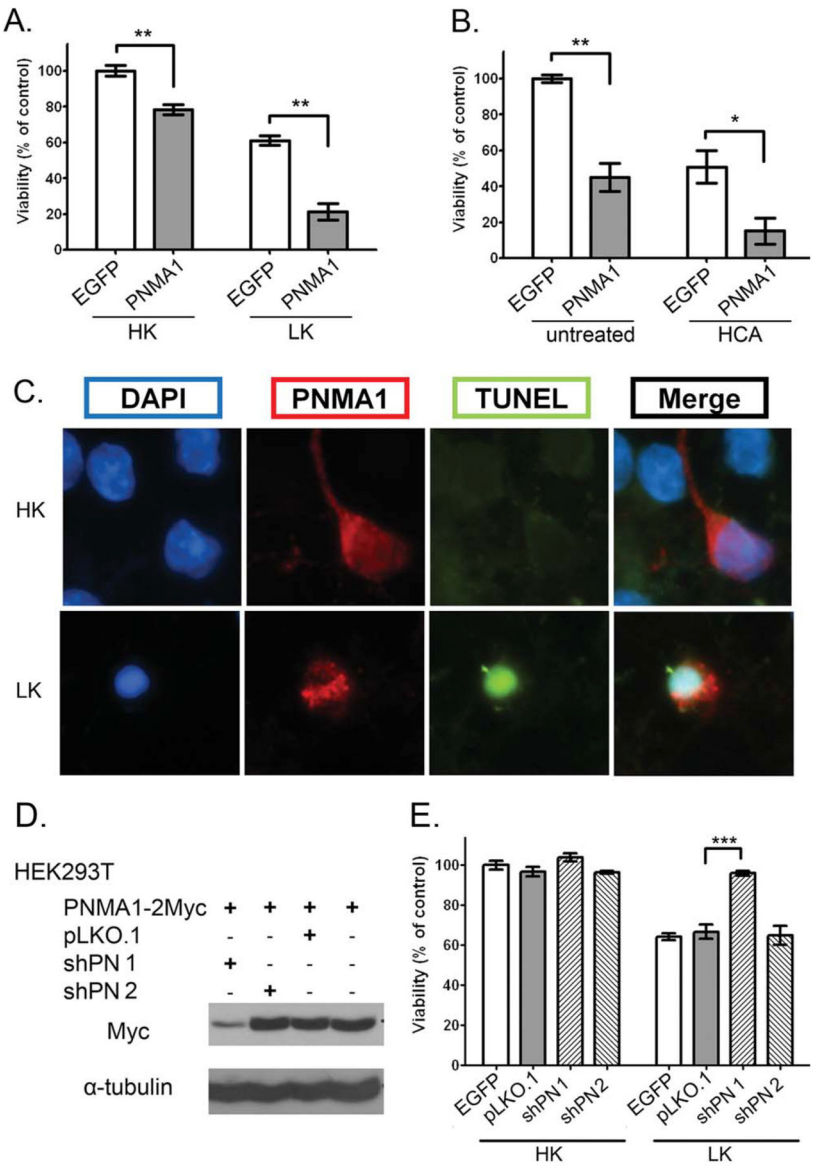

Fig. 5.

Ectopic expression of PNMA1 promotes apoptosis in CGNs and cortical neurons. **A:** CGNs transfected with Flag-tagged PNMA1 or EGFP were switched to HK or LK for 24 hr. Transfected cells were visualized by immunocytochemistry with Flag antibody. Viability of transfected neurons was quantified using DAPI staining and compared with control cultures (transfected with EGFP). Each experiment was performed in duplicate and repeated four times. The graph shows mean values ± sem; ★★$P < 0.01$ by two-tailed *t*-test. **B:** Cortical neurons were transfected with Flag-tagged PNMA1 or EGFP expression plas-mids and then treated with 1 mM HCA. Viability of transfected cells was examined 24 hr after HCA treatment by immunocytochemistry and DAPI staining. Each experiment was performed four times in duplicate. The graphs shows mean values ± sem; ★$P < 0.05$, ★★$P < 0.01$ by two-tailed *t*-test. **C:** TUNEL staining of PNMA1 transfected CGNs. Twenty-four hours after HK or LK treatment, PNMA1-transfected CGNs were visualized by immunocytochemistry

using a Flag antibody (Texas red), and apoptotic cells were identified by TUNEL staining (green) or DAPI staining (blue) under a fluorescence microscope. **D:** Knockdown of PNMA1 expression by shRNA. HEK293T cells were cotransfected with a plasmid expressing Myc-tagged rat PNMA1 and plasmids expressing either a scrambled control shRNA (pLKO.1) or two shRNAs designed to target mouse PNMA1 mRNA (shPN1 and shPN2). Although shPN1 is completely complementary to rat PNMA1, shPN2 has two mismatches against the rat PNMA1 mRNA sequence. Seventy-two hours after transfection, total cell lysates were collected, and the extent of knockdown was evaluated by Western blotting using an anti-Myc antibody. **E:** Knockdown of PNMA1 expression by shRNA protects CGNs from LK-induced apoptosis. CGNs were transfected with a plasmid expressing EGFP alone or cotransfected with plasmids expressing EGFP (for visualization of trans-fected cells) and pLKO.1, shPN1, or shPN2. The cultures were then treated with HK or LK medium for 24 hr. Ratio of EGFP to reported plasmid was 1:7. Each experiment was performed in duplicate and repeated four times. Graph shows mean viability ± sem; ★★★$P < 0.001$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

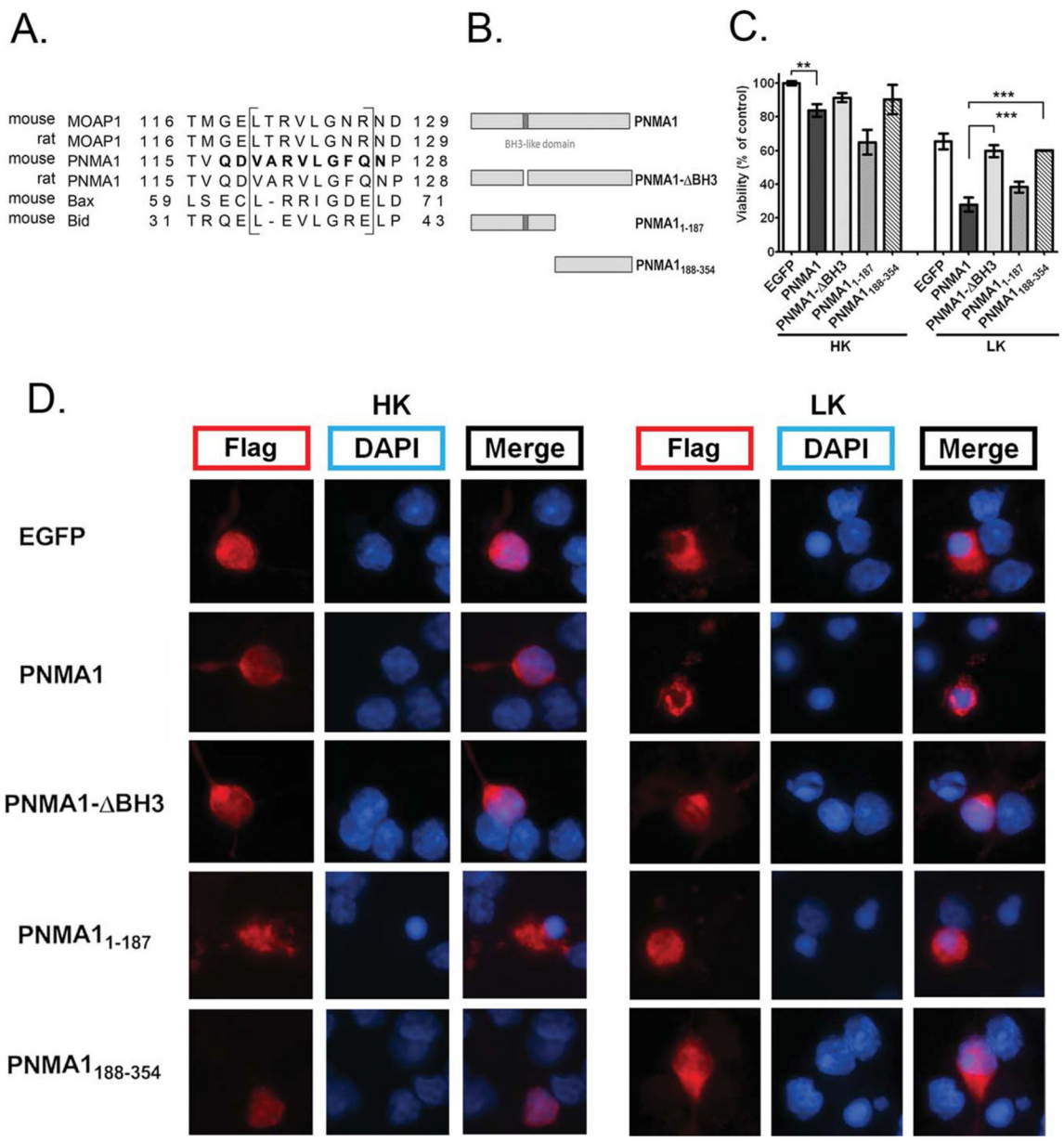

Fig. 6.

PNMA1 toxicity requires the BH3-like domain. **A:** Sequence of the BH3-like domain of mouse and rat MOAP1 is compared with the corresponding sequence in mouse and rat PNMA1. The BH3-like domain is boxed. The PNMA1-ΔBH3 deletion region is in boldface. Although the sequence is not identical, key residues of the BH3 motif are conserved in PNMA1. **B:** Schematic diagram of PNMA1 and mutant forms used in the study. Shown are constructs $PNMA1_{1–187}$, $PNMA1_{188–354}$, and PNMA1-ΔBH3 (lacking 11 amino acids containing the BH3-like sequence). **C:** CGNs trans-fected with plasmids expressing EGFP (control), PNMA1, or the mutant constructs PNMA1-ΔBH3, $PNMA1_{1–187}$, and $PNMA1_{188–354}$ were switched to HK or LK medium. Viability of transfected neurons was quantified 24 hr later by DAPI staining. Each experiment was performed four times in

duplicate. Graphs show mean viability compared with control ± sem; ★★$P < 0.01$, ★★★$P < 0.001$. Significance was evaluated by Student's *t*-test (two-tailed) compared with full-length PNMA1. **D:** Subcellular localization of EGFP, PNMA1, PNMA1-ΔBH3, $PNMA1_{1–187}$, and $PNMA1_{188–354}$. Indicated plasmids were transfected into CGNs and treated with HK or LK as described previously. All constructs were Flag-tagged. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary. com.]

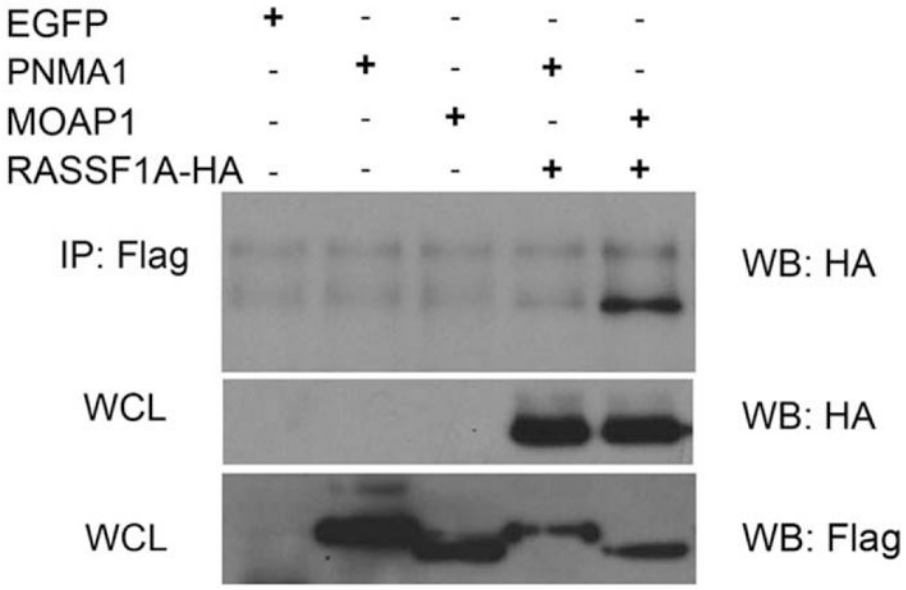

Fig. 7.

PNMA1 induces apoptosis through a mechanism different from MOAP1. HEK293T cells were transfected with plasmids expressing Flag-PNMA1, Flag-MOAP-1, and RASSF1A-HA either by themselves or in combination as indicated. EGFP was used as a negative control. Twenty-four hours after transfection, the cells were lysed and immunoprecipitated using a Flag antibody. Interaction with RASSF1A was evaluated by Western blotting of the immunoprecipitate using an HA antibody. An aliquot of the whole-cell lysate (WCL) taken prior to immunoprecipitation was also included in the Western blot.

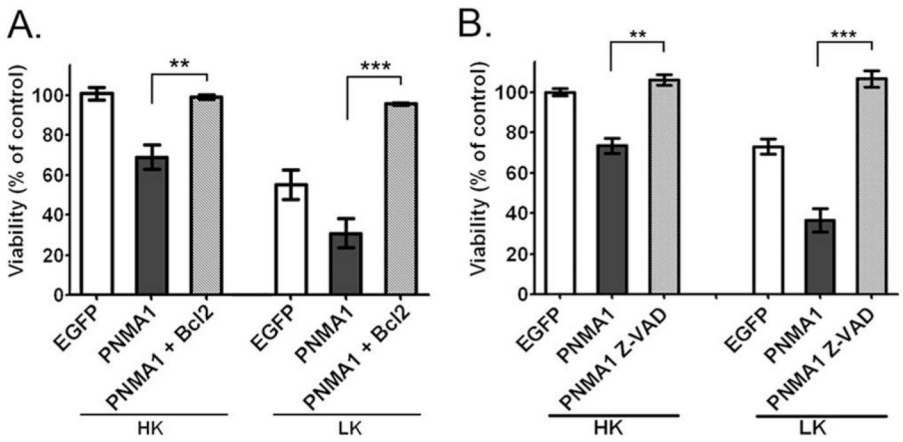

Fig. 8.

The neurotoxic effect of PNMA1 is blocked by expression of Bcl2 and by caspase inhibition. **A:** CGNs were transfected with equal amounts of Bcl2 and PNMA1 expression plasmids and then treated with HK or LK for 24 hr. Viability of transfected neurons was analyzed by DAPI staining. Each experiment was performed in duplicate and repeated three times. Graph shows mean ± sem; ★★$P < 0.01$, ★★★$P < 0.001$, two-tailed *t*-test. **B:** Z-VAD inhibits PNMA1-mediated neuron apoptosis. CGNs transfected with PNMA1 were switched to HK or LK medium with or without 50 μM Z-VAD-FMK as indicated. Viability of transfected neurons was analyzed 24 hr later by DAPI staining. Each experiment was performed three times in duplicate. Graph shows mean ± sem; ★★$P < 0.01$, ★★★$P < 0.001$, two-tailed *t*-test.

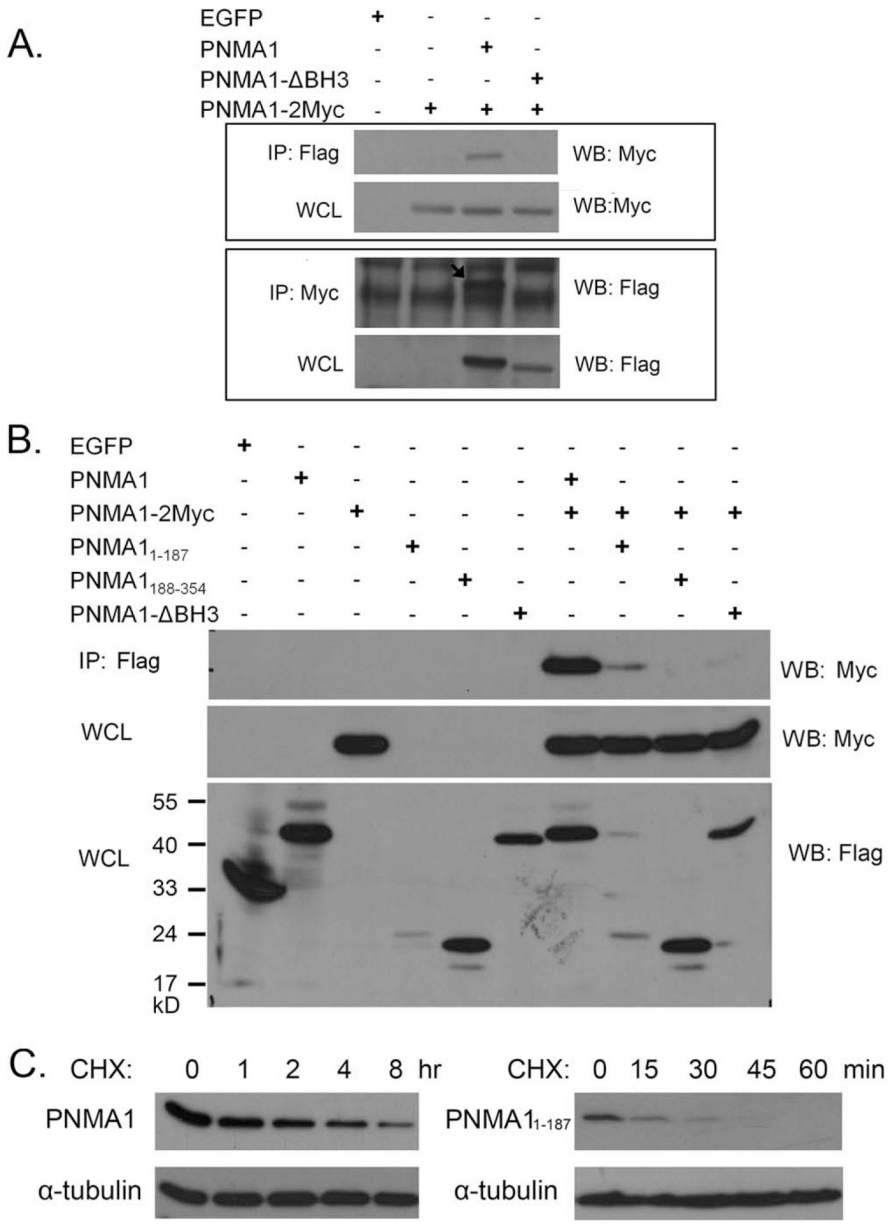

Fig. 9.

PNMA1 self-associates through its BH3-like domain. **A:** HEK293T cells were transfected with Myc-tagged PNMA1 (PNMA1-2Myc) by itself, along with Flag tagged-PNMA1 (PNMA1), or along with Flag tagged- PNMA1-ΔBH3. Forty-eight hours after transfection, lysates were immunoprecipitated with a Flag antibody, and the immunoprecipitate was subjected to Western blotting using an anti-Myc antibody. Separately, an anti-Myc antibody was used for immunoprecipitation, and an anti-Flag antibody was used for Western blotting. **B:** Coimmunoprecipitation shows that Myc-tagged PNMA1 can interact with full-length PNMA1 (Flag-tagged) and $PNMA1_{1–187}$ (Flag-tagged), but does not associate with $PNMA1_{188–354}$ (Flag-tagged) or PNMA1-ΔBH3 (Flag-tagged). **C:** Full-length PNMA1 or $PNMA1_{1–187}$ was transfected in HEK293T 16 hr before treating with 1 μM cyclohexamide

(CHX). Lysates were prepared 1, 2, 4, and 8 hr later. With PNMA1, a half-life of about 4.5 hr was observed (left panel). Because a significant reduction in $PNMA1_{1–187}$ was observed within 1 hr, separate analysis were conducted using lysates prepared at 15, 30, 45, and 60 min after CHX treatment.

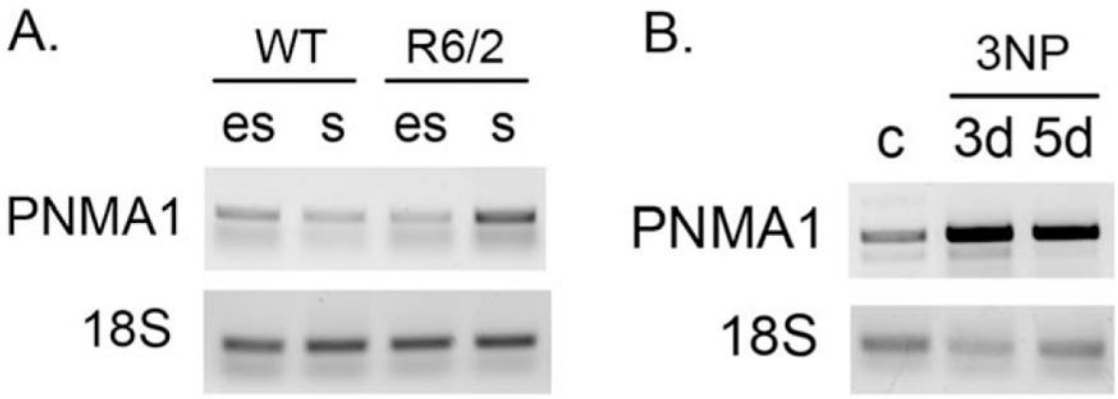

Fig. 10.

Expression of PNMA1 in mouse Huntington's disease models. **A:** Striatum and extrastriatal brain tissue (brain minus striatum) from 6-week-old wild-type control (WT) or R6/2 transgenic mice (R6/2) were collected, and RNA was extracted. PNMA1 mRNA expression in extrastriatal brain tissue (es) or striatum (s) was examined. Amplification of 18S RNA was used as a loading control. **B:** Mice injected with saline or with 3-NP for 3 days or 5 days. RNA was prepared from the striatum and used in RT-PCR analysis to measure PNMA1 expression.