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Paraxanthine, the Primary Metabolite of Caffeine, Provides Protection against Dopaminergic Cell Death via Stimulation of Ryanodine Receptor Channels

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ABSTRACT

Epidemiological evidence suggests that caffeine or its metabolites reduce the risk of developing Parkinson's disease, possibly by protecting dopaminergic neurons, but the underlying mechanism is not clearly understood. Here, we show that the primary metabolite of caffeine, paraxanthine (PX; 1,7-dimethylxanthine), was strongly protective against neurodegeneration and loss of synaptic function in a culture system of selective dopaminergic cell death. In contrast, caffeine itself afforded only marginal protection. The survival effect of PX was highly specific to dopaminergic neurons and independent of glial cell line-derived neurotrophic factor (GDNF). Nevertheless, PX had the potential to rescue dopaminergic neurons that had matured initially with and were then deprived of GDNF. The protective effect of PX was not mediated by blockade of adenosine receptors or by elevation of intracellular cAMP levels, two pharmacological effects typical of methylxanthine derivatives. Instead, it was attributable to a moderate increase in free cytosolic calcium via the activation of reticulum endoplasmic ryanodine receptor (RyR) channels. Consistent with these observations, PX and also ryanodine, the preferential agonist of RyRs, were protective in an unrelated paradigm of mitochondrial toxin-induced dopaminergic cell death. In conclusion, our data suggest that PX has a neuroprotective potential for diseased dopaminergic neurons.

Nigrostriatal dopaminergic (DA) neurons are critically involved in the control of voluntary movements (Grillner and Mercuri, 2002). As a result, their death in Parkinson's disease (PD) leads to profoundly disabling motor symptoms (Agid, 1991). The identification and characterization of signals and factors that control the survival and function of these neurons are therefore of interest because they may not only provide key insights into the pathogenetic mechanisms of the disease but also may help to develop new neuroprotective or neurorestorative strategies.

Results of case-control and prospective studies indicate that consumption of caffeine in coffee, tea, and caffeinated beverages may significantly reduce the risk of developing PD, after accounting for smoking, and other potentially confounding factors such as estrogen replacement therapy (Ross et al., 2000; Ascherio et al., 2001; Hancock et al., 2007; Sääksjärvi et al., 2008). As a possible explanation for the inverse association of PD and caffeine consumption, some investigators proposed a preclinical aversion hypothesis in which the apparent protective effects of caffeine may reflect a preclinical stage of the disease in which caffeine intake may become progressively less rewarding (Louis et al., 2003). Arguing against this possibility, the inverse association persisted when the years preceding onset of the disease were excluded (Hancock et al., 2007). Moreover, experimental studies have demonstrated that caffeine and/or possibly its metabolites are neuroprotective in mouse models of PD (Xu et al.,

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ABBREVIATIONS: DA, dopamine (dopaminergic); PD, Parkinson's disease; PX, paraxanthine; GDNF, glial cell line-derived neurotrophic factor; Ca^{2+}_{cyt}, free cytosolic calcium; RyR, ryanodine receptor; MPP^+, 1-methyl-4-phenylpyridinium; MX, methylxanthine; PSB36, 1-butyl-8-(hexahydro-2,5-methanopentalen-3*a*(1*H*)-yl)-3,7-dihydro-3-(3-hydroxy-propyl)-1*H*-purine-2,6-dione; SCH 58261, 5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo[4,3-epsilon]-1,2,4-triazolo[1,5-*c*]pyrimidine; MRS1334, 1,4-dihydro-2-methyl-6-phenyl-4-(phenylethynyl)-3,5-pyridinedicarboxylic acid 3-ethyl-5-[(3-nitrophenyl)-methyl]ester; NMDA, *N*-methyl-D-aspartate; MK-801, 5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine (dizocilpine maleate); TH, tyrosine hydroxylase; DIV, day in vitro; PBS, phosphate-buffered saline; MAP, microtubule-associated protein; GBR-12909, 1-{2-[bis-(4-fluorophenyl)methoxy]ethyl}-4-(3-phenylpropyl)piperazine.

2002a,b, 2006; Aguiar et al., 2006). The protective effects of caffeine were attributed, in part, to the antagonism of A_{2A} adenosine receptors (Jacobson and Gao, 2006), but other mechanisms were not excluded (Xu et al., 2002).

About 80% of caffeine is *N*3-demethylated to form paraxanthine (PX), through the catalytic action of cytochrome P450 subtypes 1A2 and 2E1 (Kennedy et al., 1987; Lelo et al., 1989; Magkos and Kavouras, 2005). However, the biological effects of PX, in particular its neuroprotective activity, have received little attention in the literature. To investigate the potential neuroprotective effects of PX, we used a model system of dissociated midbrain DA neurons that presents several interesting features: 1) DA neurons are maintained in a tightly controlled environment in the context of their physiological neighbors; 2) they degenerate specifically and progressively as a function of time (Michel and Agid, 1996); and 3) they become dependent for their survival on glial cell line-derived neurotrophic factor (GDNF), a trophic peptide reported to exert beneficial effects when injected directly in the putamen of PD patients (Love et al., 2005).

We show here that PX has a robust protective effect on DA neurons in our preparation, whereas its parent compound caffeine has a more limited impact. The protective effect of PX was not related to adenosine receptor blockade or cAMP elevation but resulted from a moderate increase in intracellular cytosolic free calcium (Ca^{2+}_{cyt}) through the activation of ryanodine receptor (RyR) calcium release channels. Interestingly, PX also exerted substantial neuroprotection against DA cell death triggered by withdrawal of GDNF in mature cultures or by exposure to the mitochondrial toxin 1-methyl-4-phenylpyridinium (MPP^+).

Materials and Methods

Pharmacological Agents. PX was synthesized and purified by the Institut de Recherche Pierre Fabre (Labège, France). Other xanthine derivatives, including xanthine itself, 1-methylxanthine (MX), 3-MX, 7-MX, and caffeine (1,3,7-trimethylxanthine) as well as ryanodine, were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). Xanthines were first solubilized with 1 N NaOH, and stock solutions were then prepared by adjusting the concentration to 25 mM and the pH to 7.4. For treatments, stock solutions were dissolved initially at 10 mM in culture medium before being added to the cultures at their final concentrations. The adenosine receptor antagonists PSB36, SCH 58261, and MRS 1334 were from Tocris Cookson Inc. (Bristol, UK), except for chlorostyryl-caffeine and alloxazine, which were obtained from Sigma-Aldrich. The mitochondrial toxin MPP^+ and the *N*-methyl-D-aspartate receptor/channel (NMDA) antagonist dizocilpine (MK-801) were also from Sigma-Aldrich. GDNF was purchased from AbCys (Paris, France), and the anti-GDNF neutralizing antibody (AB-212-NA) was from R&D Systems Europe Ltd (Lille, France). Tritiated neurotransmitters and tritiated methyl-thymidine were obtained from GE Healthcare (Chalfont St. Giles, UK).

Cell Culture. Animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996), European Directive 86/609, and the guidelines of the local institutional animal care and use committee. Cultures were prepared from the ventral mesencephalon of Wistar rat embryos, at gestational age 15.5 (Janvier Breeding Center, Le Genest St Isle, France) using N5 medium and 1 mg/ml polyethylenimine as coating as described previously (Michel and Agid, 1996). To assess DA cell survival and function, mesencephalic cultures were established on 24-well multiwell culture plates (Costar, Corning Life Sciences, Acton, MA), but for calcium measurements, they were grown on Lab-Tek glass chamber slides (Nalge Nunc International, Rochester, NY) to optimize fluorescent signal detection with the calcium probe. Note that these cultures contain tyrosine hydroxylase $(TH)^+$ neurons that are exclusively dopaminergic (Traver et al., 2006).

Treatment Paradigms. When using the spontaneous DA cell death model, treatment paradigms are described in the text for each given experiment. Treatments with MPP^+ were performed in cultures where the spontaneous death process was prevented by long-term exposure to 30 mM K^+, as described previously (Douhou et al., 2001). Treatments with MPP^+ and potential neuroprotective molecules were carried out between 5 and 7 days in vitro (DIV), and the cultures were left to recover until 10 DIV in the presence of control medium. GDNF deprivation was carried out in cultures maintained initially with the trophic peptide at 20 ng/ml for 10 DIV. Substitutive treatments with PX were performed between 11 and 16 DIV. Glutamate-mediated excitotoxic stress was induced at 8 DIV. After 24 h of treatment, glutamate was removed, and the cultures were allowed to recover in fresh medium until 10 DIV.

Quantification of Neuronal Survival. The survival of DA neurons was quantified by counting the number of cells labeled with an antibody against TH, as described previously (Traver et al., 2006). In brief, the cultures were fixed with 4% formaldehyde in Dulbecco's phosphate-buffered saline (PBS) for 12 min. Cells were washed three times with PBS and then incubated for 24 h at 4°C with a monoclonal anti-TH antibody (Diasorin, Stillwater, MN) diluted 1:5000 in PBS containing 0.2% Triton X-100. The TH antibody was then revealed with an anti-mouse IgG cyanin-3 conjugate (1:500; Sigma-Aldrich) for 2 h at room temperature. Illustrations describing TH^+ neuron survival are presented as inverted images. All neurons, regardless of their neurotransmitter phenotype, were identified by labeling microtubule-associated protein (MAP)-2 with a monoclonal antibody (AP-20; Sigma-Aldrich) diluted 1:50 in PBS and revealed with an anti-mouse Alexa Fluor 488 conjugate.

Measurement of Neurotransmitter Uptake and Release. The functional integrity and synaptic function of DA neurons were evaluated by their ability to accumulate and release DA (Michel et al., 1999; Rougé-Pont et al., 1999). The uptake was initiated by addition of 50 nM [^{3}H]DA (40 Ci/mmol) in cultures maintained at 37°C in 500 μl of PBS containing 5 mM glucose and 100 μM ascorbic acid, and it was terminated after 15 min by two rapid washes with ice-cold PBS. Cultured cells were then scraped off the culture wells, and the radioactivity was counted by liquid scintillation spectrometry. Blank values were obtained in the presence of 0.5 μM GBR-12909 (Sigma-Aldrich), a selective inhibitor of the DA transporter. The release of DA was carried out in DA neurons that were allowed to initially accumulate 50 nM [^{3}H]DA for 30 min. The uptake process was then terminated by two rapid washes, and the fraction of DA released spontaneously or after 40 mM K^+-evoked depolarization was quantified over the next 20 min using the same incubation buffer as for the uptake. Blank values for release experiments were obtained from cultures exposed to GBR-12909 during the phase of the uptake. GABA uptake used as an indicator of GABAergic function was measured using 50 nM [^{3}H]GABA (88 Ci/mmol), as described previously (Douhou et al., 2001), using an incubation time of 4 min.

Quantification and Identification of Proliferating Cells. [Methyl-^{3}H]thymidine, a marker of DNA synthesis, was used to label and quantify proliferating cells, as described previously (Douhou et al., 2001; Mourlevat et al., 2003). Mesencephalic cultures maintained for up to 7 DIV in the presence of test treatments were exposed to 1 μCi of [methyl-^{3}H]thymidine (40 Ci/mmol; GE Healthcare) for 2 h at 37°C in serum-free N5 medium supplemented with 5 mM glucose. After three rapid washes, the cells were allowed to recover for 1 h in the same culture medium to remove unincorporated radioactivity. The cultures were fixed in 4% formaldehyde for 15 min, and, when necessary, they were subsequently processed for the immunofluorescence detection of TH. The cultures were then dehydrated in ethanol

and exposed to Hypercoat LM-1 emulsion (GE Healthcare) for 4 days at 4°C to detect the tritiated label in cell nuclei.

Quantification of Intracellular Free Calcium Levels. Cytoplasmic free calcium levels were measured in individual neurons using Calcium Green-1-acetoxymethyl ester (Molecular Probes, Carlsbad, CA), as described previously (Douhou et al., 2001; Salthun-Lassalle et al., 2004). In brief, cultures grown for 7 to 8 days were incubated with 10 μM Calcium Green-1-acetoxymethyl for 30 min at 37°C, washed twice with serum-free glucose-supplemented N5 medium to remove excess indicator, and then left to recover for 30 min before assessment. The test treatments were then applied, and the fluorescent signal (excitation, 480 nm; emission, 510 nm) was quantified using the Simple-PCI software from C-Imaging Systems and a Nikon (Tokyo, Japan) TE-300 inverted microscope equipped with an ORCA-ER digital camera from Hamamatsu (Bridgewater, NJ). Fluorescent images of randomly chosen fields (six to 10 in each culture condition) were acquired with a 63× fluorescent objective. The average pixel intensity over the surface of each cell body was determined under the different experimental conditions. Background fluorescence was subtracted from raw data, and the results were expressed as a percentage of mean fluorescence intensity per cell under control conditions. A minimum of 150 cells were analyzed under each test condition.

Determination of cAMP Levels. Cyclic AMP levels were determined using a protocol established initially for measuring cGMP levels (Michel and Agid, 1995). After termination of the treatments by aspiration of the culture medium, the cells were scraped off the wells with 200 μl of a 4 mM ice-cold EDTA solution, pH 7.5, to prevent enzymatic degradation of cAMP, sonicated for 5 s, and heated at 100°C for 3 min to coagulate proteins. Samples were then centrifuged for 2 min at 13,000*g*, and cAMP was assayed in the supernatant using a radioimmunoassay kit (GE Healthcare).

Statistical Analysis. Simple comparisons between two groups were performed with Student's *t* test. Multiple comparisons against a single reference group were made by one-way analysis of variance followed by Dunnett's test. When all pairwise comparisons were made, the Student-Newman-Keuls test was used. S.E.M. values were derived from at least three independent experiments.

Results

Long-Term Exposure to PX Promotes the Survival of Mesencephalic DA Neurons. In the culture model of mesencephalic cultures we have developed, DA neurons degenerate spontaneously and progressively, whereas other types of neurons are little affected (Michel and Agid, 1996). In the present study, we found that ~35% of TH$^+$ neurons had disappeared at 6 DIV and more than 84% at 16 DIV (Fig. 1A). When PX was applied to the cultures for a prolonged period, the number of TH$^+$ neurons was augmented in a dose-dependent manner (Fig. 1, B and C). The effect of PX, already significant at 100 μM, increased gradually and remained optimal between 800 and 1000 μM, at 10 DIV. Counts of TH$^+$ neurons performed at different stages of maturation of the cultures indicate that PX most likely prevented DA cell loss (Fig. 1A). Consistent with this view, we failed to detect the

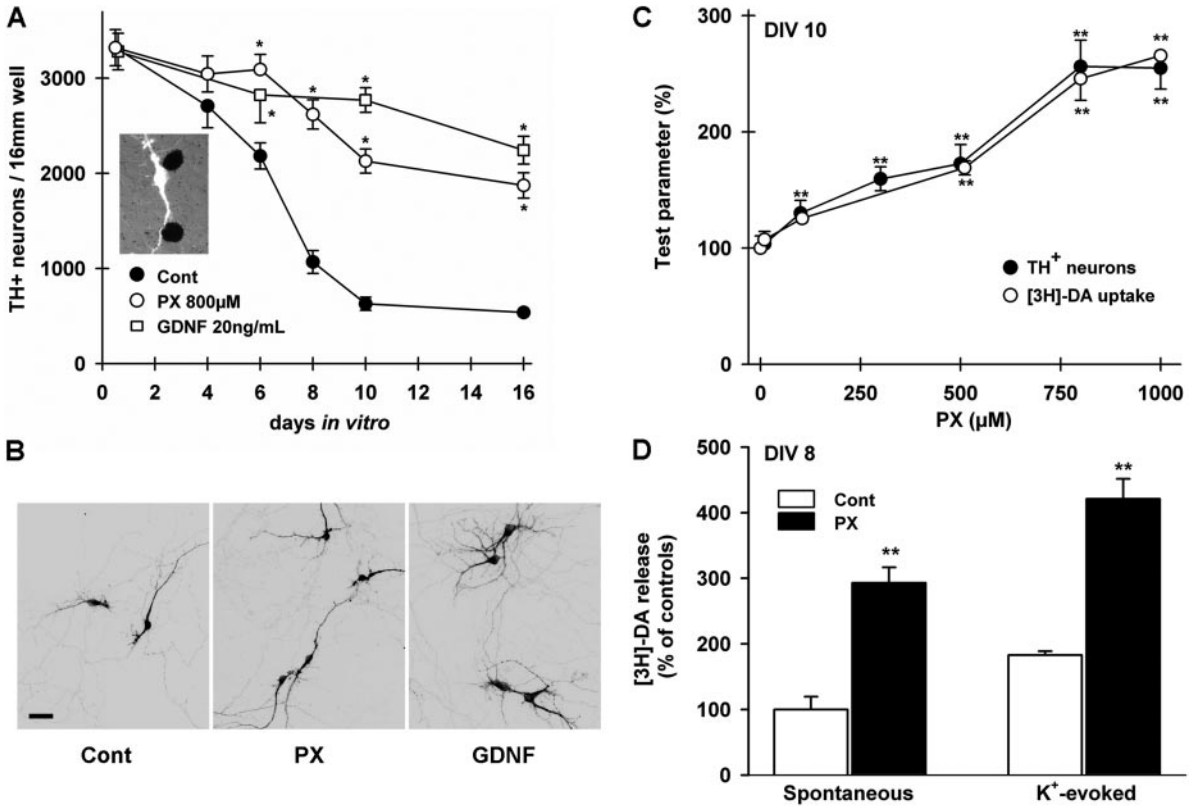

Fig. 1. Protective effects of PX for DA neurons in mesencephalic cultures. A, number of TH$^+$ neurons in 800 μM PX-, 20 ng/ml GDNF-treated, or control (Cont) cultures as a function of the days in vitro. Inset, illustration showing that tritiated thymidine$^+$ nuclei (dark aggregates of silver grains) do not colocalize with TH-immunopositive cells in PX-treated cultures. B, TH$^+$ neurons at 10 DIV in cultures treated with 800 μM PX and 20 ng/ml GDNF. Scale bar, 40 μm. C, number of TH$^+$ neurons and quantification of [^{3}H]DA uptake as a function of the concentration of PX (0–1000 μM), at 10 DIV. The number of TH$^+$ neurons and the uptake of [^{3}H]DA in control cultures, expressed per 16-mm well, were estimated to be 720 and 20.1 fmol/min, respectively. D, spontaneous and 40 mM K$^+$-evoked release of [^{3}H]DA in control and 800 μM PX-treated 8 DIV cultures. The spontaneous release of [^{3}H]DA in control cultures was estimated to be 1.6 fmol/min/16 mm well. $*$, $p < 0.05$, different from corresponding age-matched control cultures. $**$, $p < 0.05$, different from corresponding control cultures.

presence of newborn TH^+ neurons that may have originated from precursor cells in PX-treated cultures (Fig. 1A, inset). GDNF, a prototypical trophic factor for DA neurons (Lin et al., 1993), was only slightly more effective than 800 μM PX in rescuing DA neurons after 10 and 16 DIV when used at an optimal concentration of 20 ng/ml.

Note that the DA neurons exposed to PX were also functional, because they efficiently accumulated [^{3}H]DA via active transport (Fig. 1C). The rate of DA uptake per TH^+ neuron, however, was similar in control ($27.9 \times 10^{-3} \pm 0.3$ fmol/min) and PX (800 μM)-treated cultures ($26.7 \times 10^{-3} \pm 5.0$ fmol/min). Given that DA uptake sites are located essentially on neuritic extensions, this latter finding suggests that PX had a strong impact on survival but did not stimulate morphological differentiation.

To further evaluate the integrity and function of DA neurons undergoing long-term treatment with PX, we also measured their efficacy to release DA (Fig. 1D). Our data show that both basal and K^+-evoked DA release were increased substantially in mesencephalic cultures exposed to PX (8 DIV) over a prolonged term, indicating that the protective treatment also preserved the synaptic function of the rescued DA neurons.

Caffeine Is Less Potent Than Its Primary Metabolite PX in Protecting DA Neurons. About 80% of caffeine is *N*3-demethylated to form PX, through the catalytic action of cytochrome P450 subtypes 1A2 and 2E1 (Kennedy et al., 1987; Lelo et al., 1989; Magkos and Kavouras, 2005). Unlike PX, caffeine was poorly effective in protecting DA neurons from death (Fig. 2). For example, at a concentration of 800 μM, caffeine produced only a modest 40% increase in the number of TH^+ cells at 10 DIV, whereas the same concentration of PX optimally promoted DA cell survival (169% increase). Furthermore, 1-MX and 7-MX, the demethylated metabolites of PX (Lelo et al., 1989; Magkos and Kavouras, 2005), afforded moderate and no protection for DA neurons, respectively. 3-MX, a metabolite produced through a minor metabolic pathway of caffeine (Magkos and Kavouras, 2005), and xanthine itself both failed to improve DA cell survival in our model system.

Survival Promotion of DA Neurons by PX Depends on the Duration of Treatment. When the treatment with an optimal concentration of PX was postponed after plating, the number of rescued TH^+ neurons diminished as a function of increasing time delays before treatment (Fig. 3). Cultures treated continuously for 10 days (0–10 DIV) with 800 μM PX had 194% more TH^+ neurons than untreated cultures, whereas cultures treated only on days 5 to 10 and 7 to 10 had only 142 and 69% more, respectively. Conversely, the protective effect of PX was rapidly reversible if the treatment was stopped prematurely and the cultures were maintained in control medium up to 10 DIV. If PX was withdrawn at day 5 or even day 7, virtually no TH^+ neurons were saved 5 or 3 days later (Fig. 3).

Trophic Effects of PX Are Selective for DA Neurons in Mesencephalic Cultures. TH^+ cells represent only a small percentage of the neurons in mesencephalic cultures (Michel et al., 1999). To determine whether PX also affected the survival of non-DA neurons, which are predominantly GABAergic (Lannuzel et al., 2002), we labeled the entire population of neuronal cells with an antibody against MAP-2. Different from what we observed with DA neurons, the number of MAP-2^+ neuronal cells remained stable during the maturation process of mesencephalic cultures (Fig. 4, A and B). Furthermore, the treatment with PX had no significant impact on the density of MAP-2^+ neurons in these cultures (Fig. 4, A–C). The uptake of tritiated GABA used to assess the function of GABAergic neurons was also unaffected by the treatment with PX at 10 DIV (Fig. 4D).

Proliferating Glial Cells Are Not the Targets of PX. We know from previous studies that DA neurons can be rescued efficiently in this culture model by halting the pro-

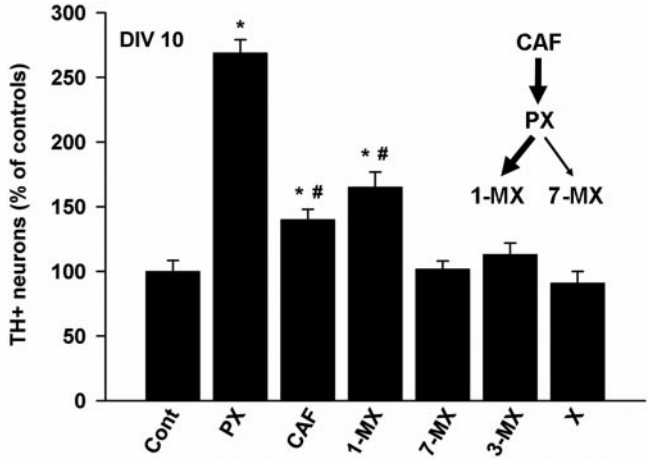

Fig. 2. Neuroprotective activity of methylxanthine derivatives involved in the major metabolic pathway of caffeine in humans. Number of DA neurons in 10 DIV mesencephalic cultures exposed to PX, its parent compound caffeine, or its metabolites 1-MX and 7-MX. Other test molecules included 3-MX produced through a minor metabolic pathway of caffeine and xanthine itself. All test compounds were added at 800 μM. *, $p < 0.05$, higher than corresponding control cultures; #, $p < 0.05$, protective effect smaller than in PX-treated cultures. Inset, methylxanthines involved in the major metabolic pathway of caffeine. Bold and normal arrows symbolize major and intermediate metabolic pathways, respectively (Magkos and Kavouras, 2005).

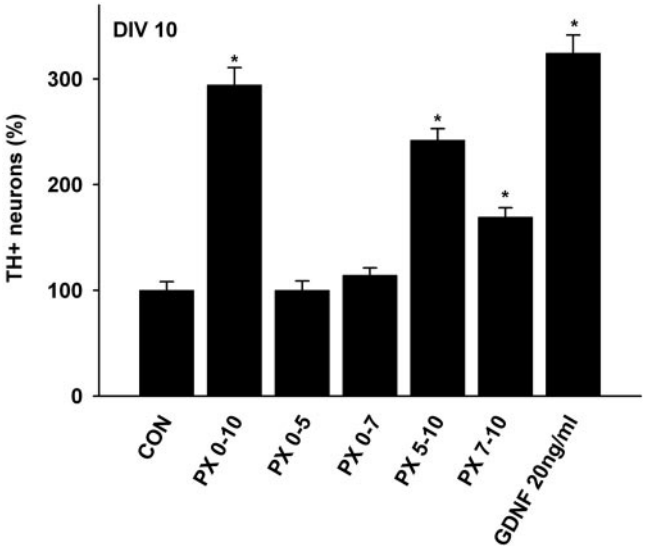

Fig. 3. The effect of PX on DA neurons depends on the duration of the treatment and the time of its initiation. Mesencephalic cultures exposed to 800 μM PX for various times between 0 and 10 DIV were assessed by TH immunocytochemistry at 10 DIV. PX still increased DA cell survival when applied after a delay. The effects of PX were rapidly reversed when the treatment with PX was stopped prematurely. *, $p < 0.05$, different from corresponding control cultures.

liferation of astrocytes or their precursor cells with synthetic deoxynucleosides (Michel et al., 1997) or purine derivatives acting as antimitotic agents (Michel et al., 1999; Mourlevat et al., 2003). Thus, the question arose as to whether PX, which has a purine-like chemical structure, protected TH^+ neurons indirectly by repressing a glia-dependent mechanism. Arguing against this possibility, 800 μM PX did not modify the proliferation rate of dividing glial cells at 6 DIV (Fig. 5, A and B), i.e., at a stage when the degenerative process is partial but already ongoing. Note that GDNF, which strongly protected DA neurons in this model system, also had no impact on glial cell proliferation. Similar results were obtained at 10 DIV, i.e., at a more advanced stage of the degenerative process (data not shown).

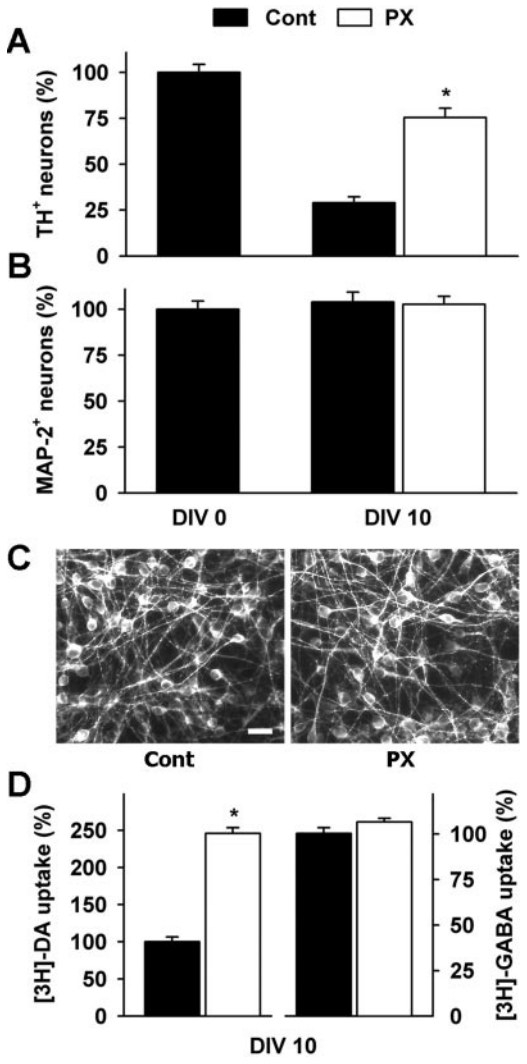

Fig. 4. Protective effects of PX are specific to DA neurons. A, treatment of the cultures with 800 μM PX between 0 and 10 DIV promoted the survival of TH^+ neuronal cells. B, the same treatment had no effect on the number of MAP-2^+ neurons. Numbers of TH^+ and MAP-2^+ neurons per well in control cultures at 0 DIV were estimated at 3300 and 423,000, respectively. C, illustration showing that PX did not influence the density of MAP-2^+ neuronal cells in mesencephalic cultures. Scale bar, 40 μm. D, treatment with PX strongly promoted the accumulation of [^{3}H]DA but had no effect on the uptake of [^{3}H]GABA. Uptake of [^{3}H]DA, and [^{3}H]GABA was 20.2 fmol/min/well and 1.43 pmol/min/well in control cultures at 10 DIV, respectively. *, $p < 0.05$, different from corresponding control cultures, at 10 DIV.

The Effect of PX Does Not Depend on Adenosine Receptor Blockade or on cAMP-Dependent Signaling Events. Methylxanthine derivatives are known to act via a variety of mechanisms, one mechanism of which implicates the blockade of A_1 and A_{2A} adenosine receptors (Jacobson and Gao, 2006). This led us to evaluate the survival of DA neurons in cultures exposed to selective antagonists of A_1 (2 μM PSB36) or A_{2A} (5 μM SCH 58261 or 10 μM chlorostyrylcaffeine) adenosine receptors. Neither of these compounds increased DA cell survival (Fig. 6A). Compounds that block A_{2B} and A_3 receptor sites also failed to prevent DA cell demise, indicating that adenosine receptor blockade was not responsible for the protective action of PX in our model system (Fig. 6A).

Many methylxanthine derivatives are known to exert their effect by stimulating intracellular cAMP production through the blockade of phosphodiesterases (Daly, 2007). Interestingly, cAMP has been proposed to play a key role in DA cell survival (Michel and Agid, 1996). However, PX did not stimulate intracellular cAMP production at a concentration of 800 μM that was optimally neuroprotective, whereas forskolin, which activates the adenylate cyclase, caused a robust increase in the levels of the cyclic nucleotide at a concentration of 10 μM that was approximately as potent as PX in terms of survival (Fig. 6, B and C). This led us to conclude that PX-mediated neuroprotection was probably not mediated by a cAMP-dependent mechanism.

Protective Effect of PX Results from a Moderate Rise in Cytosolic Ca^{2+} Caused by RyR Channel Activation. RyR channels, which are calcium release channels located in the endoplasmic reticulum, participate actively in the control of cytoplasmic free calcium levels (Fill and Copello, 2002; Verkhratsky, 2005). We wanted to address the possible role of RyR channels in the survival-promoting effect of PX (Fig. 7, A and B). Treatment of the cultures with the selective RyR agonist ryanodine at 10 μM mimicked the neuroprotective effect of 800 μM PX, whereas 30 μM dantrolene, a selective

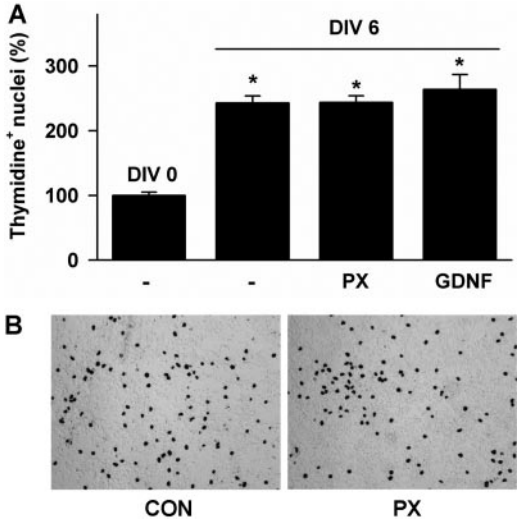

Fig. 5. Treatment with PX has no effect on glial cell proliferation in mesencephalic cultures. A, number of thymidine$^+$ nuclei in 0 DIV or 6 DIV mesencephalic cultures maintained or not in the presence of 800 μM PX or 20 ng/ml GDNF. The number of thymidine$^+$ nuclei in control cultures was estimated to be 14,400, at 0 DIV. B, illustration showing that PX does not modify the number of thymidine$^+$ nuclei in mesencephalic cultures.

blocker of this receptor (Fruen et al., 1997; Ma et al., 2004), prevented the survival-promoting effects of both PX and ryanodine. In line with these findings, 1) cytosolic calcium levels that were moderately increased in the presence of 800 μM PX or 10 μM ryanodine returned to control values when dantrolene was concurrently added to the cultures, and 2) the calcium elevation elicited by PX remained detectable when extracellular calcium was chelated by 3 mM EGTA. Interestingly, within the range of PX concentrations that were neuroprotective, i.e., 100 to 800 μM, Ca^{2+} elevations were correlated to TH^+ cell numbers (Fig. 7C), confirming that calcium was crucial for DA cell survival. Note that when optimal concentrations of PX were applied to the cultures, the presence of 10 μM ryanodine did not further increase survival promotion nor intracellular free calcium levels.

Next, we wanted to evaluate the influence of caffeine and other methylxanthines on cytoplasmic Ca^{2+} levels (Fig. 7D). Similar to PX, caffeine and 1-MX produced elevations in cytoplasmic calcium that were prevented by dantrolene (data not shown), indicating the contribution of RyRs to this effect. Interestingly, the increase in $[Ca^{2+}]_{cyt}$ produced by optimal concentrations of either caffeine or 1-MX was of lower amplitude than that evoked by an optimal concentration of PX, which may probably account for the fact that caffeine and 1-MX were also less protective for DA neurons than PX in our model system. Not surprisingly, Ca^{2+} levels remained unchanged in the presence of 3-MX and 7-MX, two methylxanthines that convey no protective effect for DA neurons.

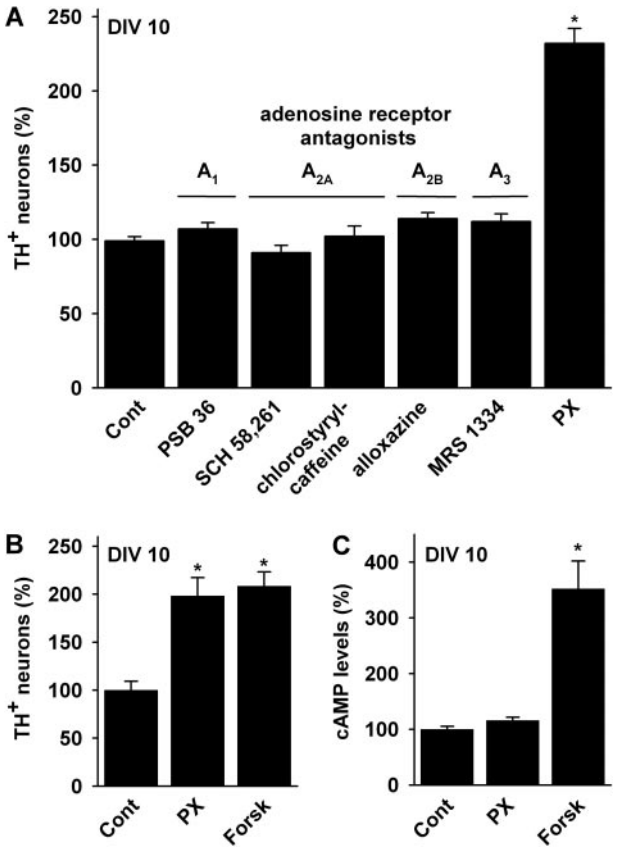

Fig. 6. Neuroprotection by PX requires neither the blockade of adenosine receptors nor the elevation of cAMP levels. A, survival of DA neurons in 10 DIV mesencephalic cultures exposed to PX or selective blockers of A_1 (2 μM PBS36), A_{2A} (5 μM SCH 58261; 10 μM chlorostyrylcaffeine), A_{2B} (20 μM alloxazine), or A_3 (20 μM MRS 1334) adenosine receptors. B, counts of TH^+ neurons in cultures exposed to 800 μM PX or 10 μM forskolin. C, measurement of cAMP levels after short-term exposure (15 min) to the same treatments as in B. $*$, $p < 0.05$, higher than corresponding control cultures.

To determine whether the activation of the RyR-dependent mechanism was also protective in a paradigm of toxin-induced DA cell death, we exposed our cultures to MPP^+, the active metabolite of the DA neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. Our data show both 800 μM PX and 10 μM ryanodine were substantially protective against 0.5 and 3 μM MPP^+ (Fig. 7E).

Given that Ca^{2+} can become deleterious as in the case of glutamate-mediated excitotoxic stress, we also wanted to compare the elevations in Ca^{2+}_{cyt} produced by glutamate and PX, at toxic and neuroprotective concentrations, respectively. Our results show that the elevation in Ca^{2+}_{cyt} elicited by glutamate had to exceed 100% of control values to trigger DA cell death by a mechanism that requires activation of NMDA receptors. In comparison, a moderate increase of ~15% resulting from the activation of RyRs was sufficient for optimal neuroprotection by PX (Fig. 7F).

The Effect of PX Is Independent of GDNF. To address the possibility that the calcium rise produced by PX served to stimulate the production and subsequent release of GDNF into the culture medium, we tested the effect of PX in the presence of an anti-GDNF antibody (AB-212-NA; 10 μg/ml) that neutralizes the biological activity of the neurotrophic peptide (Fig. 8). Whereas the antibody was sufficient to prevent the increase in DA cell survival resulting from a treatment with GDNF, it failed to reduce neuronal survival in the presence of 800 μM PX, indicating that the effect of PX did not result from the secretion of GDNF in the culture medium. The effect of GDNF, unlike that of PX, was resistant to dantrolene, suggesting that mobilization of intracellular calcium via RyRs was not involved in the effect of the trophic peptide. It should be noted that GDNF was used at a suboptimal concentration of 10 ng/ml when we wanted to completely inactivate its effects with the neutralizing antibody, but it was used at an optimal concentration of 20 ng/ml to evaluate the potential antagonistic effect of dantrolene (Fig. 8).

Mature DA Neurons Deprived of GDNF Can Be Rescued Efficiently by PX. To verify that the effect of PX was not restricted to a short developmental period after plating, we used cultures where the spontaneous death process was prevented by long-term application of 20 ng/ml GDNF. Ablation of GDNF from these cultures at 11 DIV, led to a massive loss of TH^+ neurons within 6 days. Interestingly, ~80% of these neurons survived when GDNF was replaced by 800 μM PX (Fig. 9, A and B).

A schematic representation of the mechanism by which PX may prevent DA cell demise in mesencephalic cultures is shown in Fig. 10.

Discussion

The present study demonstrates that the primary metabolite of caffeine, PX, provides neuroprotection in different model systems of selective DA cell death. Survival promotion by PX was unrelated to adenosine receptor blockade but resulted from a moderate elevation of Ca^{2+}_{cyt} through the activation of RyR channels.

PX Is a True Neuroprotective Factor for DA Neurons. The increase in the number of TH^+ neurons resulting from long-term exposure to PX may have several explanations. PX may possibly stimulate the proliferation of putative DA precursor cells, but this is unlikely because we failed to detect the presence of [^{3}H]thymidine, a marker of DNA synthesis, in the nuclei of TH^+ cells in PX-treated cultures. Alternatively, PX might reactivate TH synthesis in DA neurons that no longer express the enzyme because they have entered a premorbid (but reversible) state of degeneration. This is also unlikely, however, because PX failed to resuscitate the fraction of TH^+ neurons that was already lost when the treatment was applied after a delay. Counts of TH^+ neurons obtained at different stages of maturation of the cultures indicate that PX acted most likely by preventing DA cell loss. This conclusion is also supported by previous data showing that DA neurons die progressively by apoptosis in this model system (Michel and Agid, 1996; Salthun-Lassalle et al., 2004). It is noteworthy that DA neurons rescued by PX were efficient in accumulating and releasing DA, which indicates that the protective treatment also preserved the synaptic function of these neurons.

Caffeine Itself Is Less Potent Than PX in Protecting DA Neurons. PX, chemically referred to as 1,7-dimethylxanthine, results from the bioconversion of caffeine in vivo by *N*3-demethylation (Lelo et al., 1989). Because the chemical structures of the two xanthines are closely related, we wanted to compare their neuroprotective activities. Unlike PX, caffeine provided only limited neuroprotection to DA neurons in our culture system of spontaneous DA cell death. This may indicate that neuroprotection afforded by caffeine in vivo (Xu et al., 2002) might be in large part due to PX. Consistent with this view, 1-MX and 7-MX, the N7- and N1-demethylated metabolites of PX, were either less potent than PX itself or totally inactive, respectively. Incidentally, these results and the observation that 3-MX and xanthine itself failed to protect DA neurons, also indicate that *N*-substitution at position 1 was required for survival promotion by the test methylxanthine derivatives and that this effect was largely modulated by the presence of other *N*-substituents.

Neuroprotection by PX Does Not Require Blockade of Adenosine Receptors or Activation of a cAMP-Dependent Mechanism. Methylxanthine derivatives are known to have several pharmacological effects, one of which implicates adenosine receptor blockade (Jacobson and Gao, 2006). Interestingly, several studies have shown that blocking A_{2A} receptors is neuroprotective in models of PD (Jacobson and Gao, 2006). This led us to evaluate the survival of DA neurons in cultures exposed to selective antagonists of A_{2A} adenosine receptors SCH 58261 and chlorostyrylcaffeine. Neither compound reproduced the increase in DA cell sur-

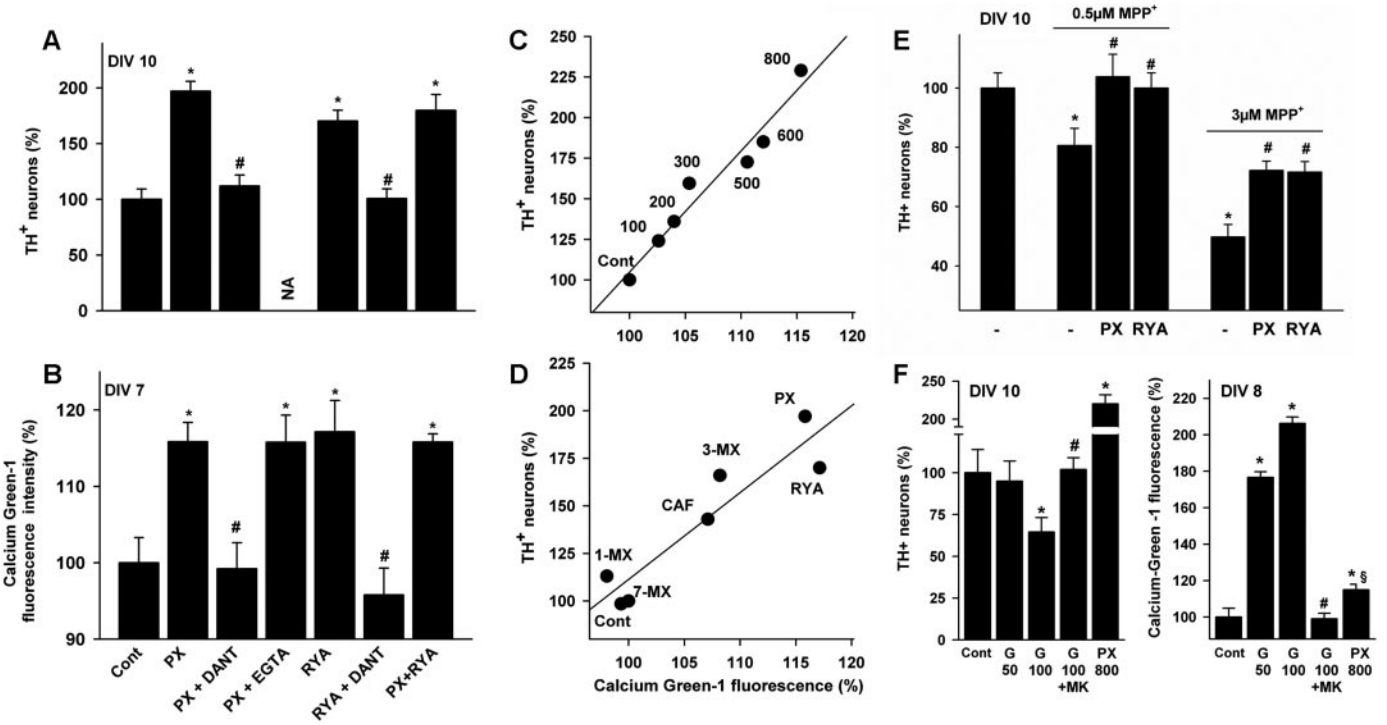

Fig. 7. Role of RyR calcium release channels in the neuroprotective effect of PX on DA neurons. A, number of TH^+ neurons at 10 DIV, in cultures exposed for a prolonged period to PX or ryanodine in the presence or absence of dantrolene, a specific antagonist of RyR channels. B, calcium-green-1 fluorescence levels in 7 DIV cultures exposed for a short time to the same treatments as mentioned above. Some measures were performed in the presence of 3 mM EGTA to chelate extracellular calcium. NA, nonapplicable, because widespread degeneration occurs when low-calcium conditions are used for prolonged periods in our culture model. C, survival of DA neurons in the presence of 100 to 800 μM PX plotted against Calcium-Green-1 fluorescence levels. D, survival of DA neurons in the presence of various structural analogs of PX (all at 800 μM) plotted against Calcium-Green-1 fluorescence levels. The solid line shows the best fit, with 95% confidence. For various calcium measurements, a minimum of 150 neurons was analyzed in each test condition. E, protective effects of PX and ryanodine against 0.5 and 3 μM MPP^+-induced DA cell death. F, DA cell survival and measurement of intracellular cytosolic calcium levels after exposure to 50 and 100 μM glutamate and PX. The NMDA receptor antagonist MK-801 used to block the effects of glutamate was applied at 2 μM. All methylxanthines including PX were added at 800 μM, ryanodine was added at 10 μM, and dantrolene at 30 μM. *, $p < 0.05$, different from corresponding control cultures; #, $p < 0.05$, significant inhibition of the effect of the test compound; and §, $p < 0.05$, lower than glutamate-treated cultures.

vival observed with PX. Furthermore, blockers of other adenosine receptors were also inactive against DA cell demise, indicating that adenosine receptor blockade was not likely to be a mechanism of action of PX.

Several methylxanthines exert their effects by increasing cAMP levels through the blockade of phosphodiesterases (Daly, 2007). For example, inhibition of phosphodiesterases seems to account for some of the effects of theophylline (1,3-dimethylxanthine), a bronchodilator used in the management of severe asthma (Barnes, 2006). Interestingly, cAMP-dependent mechanisms have been found to be effective in protecting DA neurons (Michel and Agid, 1996; Troadec et al., 2002). More specifically, forskolin, which activates the adenylate cyclase, caused a pronounced increase in the levels of the cyclic nucleotide, at a concentration that was as potent as PX in terms of survival. However, PX itself failed to stimulate cAMP production, leading us to conclude that PX-mediated neuroprotection occurred via a mechanism unrelated to cAMP production. This conclusion is supported indirectly by the observation that PX did not prevent the proliferation of dividing glial cells, a classic effect of cAMP-elevating agents in brain culture models (Bayatti and Engele, 2002; Mourlevat et al., 2003).

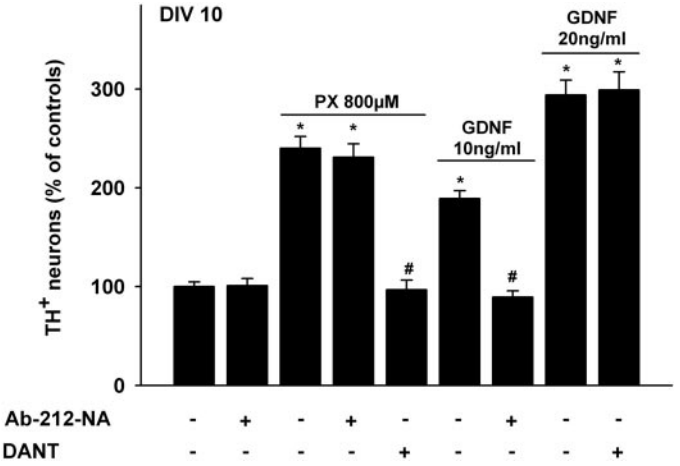

Fig. 8. PX and GDNF operate via distinct mechanisms. A, number of TH^+ neurons in mesencephalic cultures treated with PX or GDNF in the presence or absence of a neutralizing anti-GDNF antibody (AB-212-NA) or the RyR channel blocker dantrolene. PX, 800 μM; dantrolene, 30 μM; and AB-212NA, 10 μg/ml. To obtain complete inactivation of the effects of GDNF with the neutralizing antibody, we used a suboptimal concentration of 10 ng/ml of the trophic peptide. GDNF, however, was used at an optimal concentration of 20 ng/ml GDNF to evaluate the potential antagonistic effect of dantrolene. *, $p < 0.05$, higher compared with controls; #, $p < 0.05$, lower than corresponding cultures treated with PX or GDNF.

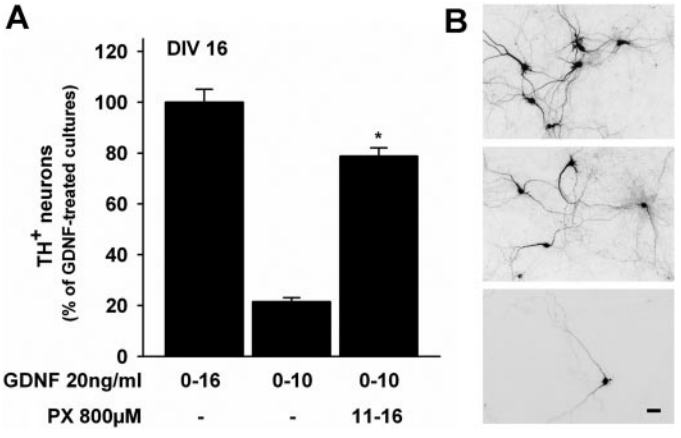

Fig. 9. Mature DA neurons deprived of GDNF can be rescued efficiently by PX. A, rescuing effect of 800 μM PX in cultures exposed to 20 ng/ml GDNF for 10 DIV and then deprived of trophic peptide between 11 and 16 DIV. Comparison to cultures maintained continuously with GDNF up to 16 DIV. *, $p < 0.05$, significant protection of GDNF-deprived TH^+ neurons. B, illustration of the protective effects of 800 μM PX at 16 DIV in the paradigm of GDNF deprivation. Scale bar, 35 μm.

Effect of PX Results from the Activation of RyR Channels. RyR channels are calcium release channels in the endoplasmic reticulum that actively participate in the control of $[Ca^{2+}]_{cyt}$ (Fill and Copello, 2002; Verkhratsky, 2005). Several arguments suggest that PX could act on DA neurons through the mobilization of intracellular calcium stores via RyR channels: 1) several methylxanthines, and in particular PX, can act as activators of RyR channels (Hawke et al., 2000; Zalk et al., 2007); 2) dantrolene, a selective blocker of these receptor channels (Bidasee and Besch, 1998), prevented the survival effect of PX in our preparation; 3) ryanodine, the preferential agonist of RyR channels, caused a substantial increase in the survival of DA neurons, an effect that was also prevented by dantrolene; and 4) the increase in $[Ca^{2+}]_{cyt}$ induced by PX or ryanodine was also abolished by dantrolene. The rise in Ca^{2+}_{cyt} elicited by PX was small compared with that produced by excitotoxic concentrations of glutamate, reinforcing the idea that Ca^{2+}_{cyt} has to be maintained just slightly above control levels for optimal survival of DA neurons (Douhou et al., 2001; Salthun-Lassalle et al., 2004). Confirming the key role of cytoplasmic calcium in the control of DA cell survival, we also found that within the range of PX concentrations that were neuroprotective, $[Ca^{2+}]_{cyt}$ elevations and survival promotion were closely correlated.

Results obtained with structural congeners of PX are also consistent with these observations: 1) caffeine and 1-MX, which were less protective than PX for DA neurons, were also less effective to elevate Ca^{2+}_{cyt}; and 2) 3-MX and 7-MX, two methylxanthines that were not protective for DA neurons, were also unable to evoke calcium responses in our prepara-

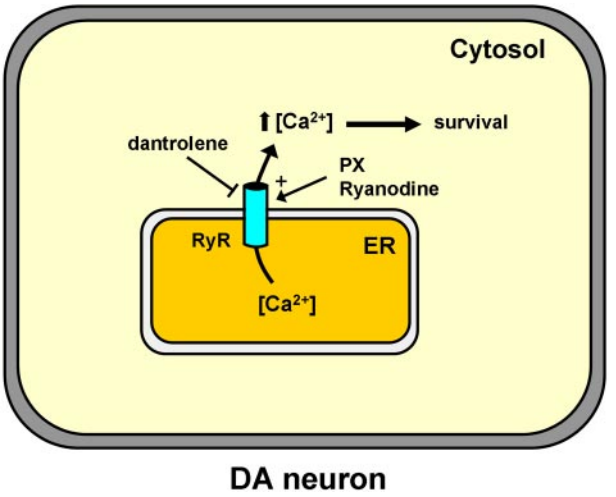

Fig. 10. Schematic representation of the mechanism by which PX may prevent DA cell demise in mesencephalic cultures. PX stimulates the mobilization of intracellular Ca^{2+} stores by activation of RyR channels. This leads to a moderate increase in cytoplasmic free Ca^{2+} levels, which prevents DA cell demise by a mechanism that remains elusive. Ryanodine, the preferential agonist of RyR channels, mimics the effects of PX. Dantrolene, a selective blocker for RyRs, prevents both the elevation in cytoplasmic free Ca^{2+} and the increase in survival caused by a treatment with either PX or ryanodine.

tion. The mechanisms by which the rise in Ca^{2+}_{cyt} prevented DA cell death, however, remain to be established. Of interest, both PX and ryanodine also provided substantial protection against MPP^+-induced DA cell death, suggesting that activation of the RyR-dependent mechanism was also effective against mitochondrial dysfunction. Finally, it is somewhat surprising to see that only DA neurons responded to PX despite the ubiquitous nature of its target receptor. A likely explanation may be, however, that it is essentially the population of DA neurons that shows a progressive loss over time, because other populations of neurons are not dependent on elevated Ca^{2+}_{cyt} for their survival (Michel et al., 2007).

Do PX and GDNF Operate via Common Mechanisms? GDNF is a potent trophic peptide for developing (Lin et al., 1993) and ageing DA neurons (Love et al., 2005; Kramer et al., 2007). In our culture model, GDNF was slightly more effective than PX in rescuing DA neurons from spontaneous death. Therefore, we wondered whether PX acted indirectly by stimulating the secretion of GDNF into the culture medium. Arguing against this possibility, an antibody that neutralized the biological activity of GDNF failed to prevent the effect of PX. Furthermore, excluding the possibility that PX and GDNF could operate by activation of a common downstream cellular target, the rescuing effect of GDNF was resistant to blockade of RyR receptors by dantrolene. Nevertheless, we found that PX was highly effective to reduce the death of DA neurons exposed initially to GDNF and then induced to die by withdrawal of the trophic peptide, suggesting that the protective effect of PX was not restricted to a short developmental period after plating.

Are the Effects of PX in Cell Culture Physiologically Relevant to Brain Neurons in Vivo? Our work provides a probable explanation for the protective effect of PX on DA neurons in vitro. However, the concentrations of PX needed for these effects are probably severalfold above optimal concentrations that can be reached in the brain, in vivo. Despite the relatively high concentrations of PX required to confer an optimal protective effect in our cellular model system, our findings may nevertheless be relevant for neurodegenerative events occurring in a pathological brain, because a variety of endogenous compounds, such fatty acyl-CoA esters, cyclic ADP-ribose, and ATP have been reported to sensitize RyRs to exogenous agonists in vivo (Magkos and Kavouras, 2005). This suggests that the effects of PX may manifest themselves in vivo at lower concentrations than those determined from in vitro studies. Consistent with this view, PX was reported to increase $[Ca^{2+}]_{cyt}$ in intact skeletal muscle preparations at 10 μM, a concentration that is physiologically relevant after systemic administration of caffeine (Ferré et al., 1990; Hawke et al., 2000), whereas concentrations higher than 500 μM were required to produce the same effect on single isolated muscle fibers (Hawke et al., 2000).

In conclusion, we propose that PX, the primary metabolite of caffeine, could operate as a survival factor for diseased DA neurons, possibly via a mechanism that requires activation of RyR channels.

Acknowledgments

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