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The Iron-Binding Protein Lactoferrin Protects Vulnerable Dopamine Neurons from Degeneration by Preserving Mitochondrial Calcium Homeostasis

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Running title: Lactoferrin-mediated neuroprotection

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Abbreviations: aTf, human apotransferrin; ARA-C, cytosine arabinoside; Ca^{2+}_{cyt}, cytosolic free calcium; Ca^{2+}_{mit}, mitochondrial calcium; DA, dopamine; DEF, deferoxamine; DIV, day in vitro; ECM, extracellular matrix; ER, endoplasmic reticulum; FAK, focal adhesion kinase; GDNF, Glial cell line-derived neurotrophic factor; Hep, heparinase I; hLf, recombinant human holo-lactoferrin; hTf, human holo-transferrin; IP3, inositol-1,4,5-triphosphate; IP3R, IP3 receptor; Lf, recombinant human lactoferrin; LY, LY294002; LY294002, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one; MAP-2, microtubule-associated protein-2; MCU, mitochondrial calcium uniporter; mLf, human milk-derived lactoferrin; MPP^+, 1-methyl-4-phenylpyridinium; PBS, phosphate-buffered saline; PD, Parkinson disease; PI3K, phosphoinositide 3-kinase; ROS, reactive oxygen species; Ru360, ruthenium 360; TH, tyrosine hydroxylase; VER, veratridine; XEC, xestospongin-C.

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ABSTRACT

Previous studies on postmortem human brain tissue have shown that the iron-binding glycoprotein lactoferrin is up-regulated in dopamine (DA) neurons resistant to degeneration in Parkinson disease (PD). To study how this could possibly relate to disease progression, we used midbrain cultures and experimental settings that model the progressive loss of DA neurons in this disorder. Human lactoferrin of either recombinant or natural origin provided robust protection to vulnerable DA neurons in a culture paradigm where these neurons die spontaneously and selectively as they mature. The efficacy of lactoferrin was comparable to that of GDNF, a prototypical neurotrophic factor for DA neurons. Neuroprotection by lactoferrin was attributable to its binding to heparan sulfate proteoglycans onto the cell surface of DA neurons and subsequently to partial inactivation of focal adhesion kinase (FAK), a major effector kinase of integrins. We established that FAK inactivation served to unmask a prosurvival phosphoinositide 3-kinase (PI3K)/AKT-dependent signaling pathway that stimulates calcium shuttling from endoplasmic reticulum to mitochondria. DA neurons exposed to the mitochondrial toxin 1-methyl-4-phenylpyridinium were also partially protected by lactoferrin, comforting the view that mitochondria may represent a downstream target for lactoferrin protective actions. Finally, we found that the iron binding capability of lactoferrin intervened in DA cell rescue, only when neurodegeneration was consecutive to iron-catalyzed oxidative stress. Overall, our data suggest that the accumulation of lactoferrin in PD brains might be evidence of an attempt by the brain to minimize the consequences of neurodegeneration.

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INTRODUCTION

Lactoferrin is an 80-kDa glycosylated protein of ~700 amino acids, related in structure to the serum iron transport protein transferrin (Brock, 2002; Metz-Boutigue et al., 1984). Lactoferrin consists of a simple polypeptide chain folded into two symmetrical lobes that are highly homologous with one another. Each lobe can bind one ferric (Fe^{3+}) ion in synergy with the carbonate ion CO_3^{2-}. Because of its ability to reversibly bind Fe^{3+}, lactoferrin can exist free of Fe^{3+} or associated with Fe^{3+}, and it has a different three-dimensional conformation depending on whether or not it is bound to iron (Jameson et al., 1998; Wally and Buchanan, 2007). Unlike transferrin, only low concentrations of lactoferrin are normally present in blood serum. Lactoferrin is abundant instead in exocrine fluids such as breast milk and colostrum, in mucosal secretions and in secondary granules of neutrophils (Garcia-Montoya et al., 2012; Levay and Viljoen, 1995).

Because of its wide distribution in various tissues, lactoferrin is a highly multifunctional protein. Indeed, it is involved in many physiological functions, including regulation of iron absorption and immune responses. It also exhibits antioxidant activities and has both anti-carcinogenic and anti-inflammatory properties (Garcia-Montoya et al., 2012). While iron chelation is directly responsible for some of the biological functions of lactoferrin, other activities require interactions of lactoferrin with cell-specific receptors located on target cells (Pierce et al., 2009; Suzuki et al., 2005) or with molecular and cellular components of both hosts and pathogens, including heparan sulfate proteoglycans (HSPG) (Lang et al., 2011; Thorne et al., 2008) and bacterial lipopolysaccharides (Drago-Serrano et al., 2012).

Parkinson disease (PD) is a neurodegenerative disorder of ageing characterized by invalidating motor symptoms resulting from the progressive loss of substantia nigra (SN) dopamine (DA) neurons (Dauer and Przedborski, 2003). Despite substantial advances in the understanding of the pathomechanism in both inherited and sporadic forms of the disease (Hirsch et al., 2013), our knowledge of the cascade of events leading to DA cell demise remains incomplete. There are some lines of evidence that lactoferrin could possibly modulate PD progression: (i) the analysis of

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postmortem brain tissue sections of PD patients revealed that the immunolabeling of lactoferrin and lactoferrin receptors was augmented in DA neurons resistant to the disease process (Faucheux et al., 1995; Leveugle et al., 1996); (ii) striatal lactoferrin mRNA transcripts were increased in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD (Fillebeen et al., 2001).

In this study, we sought to determine whether lactoferrin was able to modify the progression of the degenerative process using midbrain cultures and different experimental settings that model the loss of DA neurons observed in PD (Toulorge et al., 2011). We found that lactoferrin had the potential to interfere with pathomechanisms likely to contribute to PD-mediated neurodegeneration. This suggested that the accumulation of lactoferrin in PD patients might be evidence of an attempt by the brain to combat ongoing neuronal insults.

MATERIALS AND METHODS

Pharmacological agents

Recombinant human lactoferrin (Lf, L4040) and human milk-derived lactoferrin (mLf, L4894) (both being partially iron-saturated), recombinant human holo (iron-saturated)-lactoferrin (hLf, L1294), human apo- (aTf, T1147) and holo- (hTf, T0665) transferrins, deferoxamine mesylate salt (DEF), cytosine arabinoside (ARA-C), the phosphatidylinositol-3-kinase (PI3K) inhibitor LY294002 (LY), dibutyryl adenosine cyclic monophosphate (dbcAMP), the voltage-gated Na^+ channel opener veratridine (VER) and the mitochondrial neurotoxin MPP^+ (MPP^+) were all from Sigma-Aldrich (Saint-Quentin-Fallavier, France). The inhibitor of inositol (1,4,5) triphosphate (IP3)-mediated Ca^{2+} release Xestospongin C (XEC) and the selective inhibitor of the focal adhesion kinase (FAK) PF573228 (PF) were purchased from RD Systems Europe (Lille, France). The anti-lactoferrin rabbit polyclonal antibody was from Abcam (#Ab15811; Cambridge, MA, USA). The inhibitor of the mitochondrial Ca^{2+} uniporter Ruthenium 360 (Ru360) was obtained from Merck Calbiochem (Darmstadt, Germany). Glial cell line-derived neurotrophic factor (GDNF) was from AbCys (Paris, France).

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Midbrain cell cultures

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 midbrain of gestational age 15.5 days Wistar rat embryos (Janvier LABS, Le Genest-St-Isle, France). Dissociated cells in suspension obtained by mechanical trituration of midbrain tissue pieces were seeded at a density of 1.2–1.5 10^5 cells/cm^2 onto Nunc 48-well multi-dish plates or, when appropriate, onto Nunc Lab-Tek glass chamber slides (Thermo Scientific, Villebon-sur-Yvette, France) pre-coated with 1 mg/mL polyethylenimine diluted in borate buffer pH 8.3 as previously described (Toulorge et al., 2011). The cultures were generally maintained in N5 medium supplemented with 5 mM glucose, 5% horse serum, and 0.5% fetal calf serum, except for the first 3 days in vitro (DIV), when the concentration of fetal calf serum was set at 2.5% to favor initial maturation of the cultures (Guerreiro et al., 2008). To study the antioxidant potential of lactoferrin the cultures were seeded in a chemically defined serum-free medium consisting of equal volumes of Dulbecco's minimal essential medium and Ham's F12 nutrient mixture (Life Technologies-Invitrogen, St Aubin, France), supplemented with 10 μg/ml insulin, 30 mM glucose and 100 U/ml penicillin and streptomycin (Toulorge et al., 2011). DA neurons detected by tyrosine hydroxylase (TH) immunofluorescence staining, represented approximately 2–3% of the total number of neuronal cells present in these cultures, after plating.

Protein detection by immunofluorescence

The cultures, fixed for 12 min using 4% formaldehyde in Dulbecco's phosphate-buffered saline (PBS), were washed twice with PBS before an incubation step at 4°C for 24–72 h with primary antibodies. A monoclonal anti-TH antibody diluted 1/5000 (ImmunoStar Inc, WI, USA) or a polyclonal anti-TH antibody diluted 1/1000 (US Biologicals, Salem, MA, USA) were used to assess the survival of DA neurons whereas all neurons, regardless of their neurotransmitter phenotype,

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were identified on the basis of their content in microtubule-associated protein (MAP-2) using a monoclonal antibody diluted 1/50 (clone AP-20, Sigma-Aldrich). Microglial cells were characterized using a mouse anti-CD11b antibody (1/50; clone MRC OX-42; BD Pharmingen, Le Pont-de-Claix, France). All antibodies were diluted in PBS containing 0.2% Triton X-100 except the mouse anti-CD11b and the polyclonal anti-lactoferrin antibodies, which were diluted in PBS only. Detection of primary antibodies was performed with an Alexa Fluor-488 conjugate of an anti-mouse IgG antibody (TH, MAP-2, CD11b; 1/500, Sigma-Aldrich) or with an Alexa Fluor-555 conjugate of an anti-rabbit antibody (1/500; TH, lactoferrin; Invitrogen, Carlsbad, CA). Cell counting was performed at 200x magnification using a 20x objective matched with a 10x ocular. The number of TH^+ neurons in each culture well was estimated after counting 20 visual fields distributed along the X- and Y-axes. Counts of $MAP\text{-}2^+$ neurons and microglial cells were performed in ten visual fields randomly distributed in the whole surface area of each culture well.

Measurement of DA uptake

The functional integrity and synaptic function of DA neurons were evaluated by their ability to accumulate [^{3}H]-DA (50 nM; 40 Ci/mmol; PerkinElmer, Courtaboeuf, France), as previously described (Guerreiro et al., 2008).

Treatment paradigms for the assessment of DA neuron survival

When using the spontaneous DA cell death model, treatment paradigms are described directly in the text when required. The paradigm of deprivation was initiated in 10 DIV midbrain cultures that had been exposed to a chronic treatment with 20 ng/ml GDNF. After GDNF withdrawal, the effects of lactoferrin were assessed between 11 and 15 DIV. Treatments with the mitochondrial toxin MPP^+ were performed in cultures where the spontaneous death process was prevented by supplementing the culture medium with a depolarizing concentration of K^+ (30 mM) in the presence of 1 µM MK-801, as previously described (Salthun-Lassalle et al., 2004). This treatment did not

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interfere with the toxic effects of MPP^+. MPP^+ was applied to midbrain cultures between 5 and 7 DIV whereas protective treatments were added after 2 DIV until fixation at 10 DIV. Finally, oxidative stress-mediated DA cell death was achieved by placing the cultures in a defined serum-free medium that contained trace amounts of Fe^{3+} ion (Guerreiro et al., 2009) and was supplemented with ARA-C (3 µM).

Quantification of cytosolic and mitochondrial calcium levels

Changes in free cytosolic (Ca^{2+}_{cyt}) and mitochondrial (Ca^{2+}_{mit}) calcium levels were monitored in individual neurons using fluo-8 and rhod-4 no-wash calcium assays (Abcam, Cambridge, MA, USA), respectively. Pre-loading times were 20 min for both fluorescent probes. The effects of lactoferrin and other test compounds on calcium levels were generally evaluated in cultures that had been chronically exposed to the test treatments until 7 DIV, except when noted. Data acquisition was performed using HCI software (Hamamatsu Corp., Bridgewater, NJ, USA) and a Nikon TE-300 inverted microscope (Nikon, Tokyo, Japan) equipped with an ORCA-ER digital camera (Hamamatsu). Fluorescent images of randomly chosen fields (8–10 in each culture condition) were acquired with a 20x fluorescent objective. Fractional fluorescence (F/F_0) was evaluated by dividing the average fluorescence intensity of the neurons in each collected image of treated cultures by the average fluorescence levels of corresponding controls (Salthun-Lassalle et al., 2004; Toulorge et al., 2011). A minimum of 300 cells was analyzed for each test condition. Note that because of the technical constraints imposed by our model system, we extrapolated calcium data from the whole population of neuronal cells to the few DA neurons present in midbrain cultures. This extrapolation was justified by experimental data showing that the effects of Lf occurred through cellular mechanisms that are not specific to DA neurons.

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Quantification of reactive oxygen species

DHR-123 (20 µM; Invitrogen) was used as a fluorescent probe for reactive oxygen species (ROS) level measurement (Guerreiro et al., 2009). The quantification was carried out at a stage where ROS production is at its peak in control conditions, i.e., after 4–5 DIV. ROS production was widespread and not specific to DA neurons in the culture setting used for this study. Nevertheless, ROS could be visualized more specifically in DA neurons after post hoc TH staining and field relocation as described (Lotharius et al, 1999) using 35-mm-diameter µ-dishes with imprinted grids (Ibidi, Biovalley, Marne la Vallée, France).

Detection and quantification of P-FAK and P-AKT in DA neurons

After blocking non-specific binding sites with 5% bovine serum albumin in PBS for 2 days, the cultures were incubated for the next 3 days with anti-rabbit P-AKT (Ser-473; #4060; 1/250; Cell Signaling, Danvers, MA, USA) or anti-rabbit P-FAK (Y-397; #Ab4803; 1/100; Abcam, Cambridge, MA, USA) antibodies to detect the activated (phosphorylated) forms of AKT and FAK, respectively. An Alexa Fluor-555 conjugate of an anti-rabbit antibody was used to reveal the phospho-antibodies. Variations in fluorescence intensities were analyzed in regions of interest, i.e., the cell bodies of TH^+ neurons, using the HCI software from Hamamatsu (Bridgewater, NJ, USA). Fluorescent images of randomly chosen fields (10–15 in each culture condition) were acquired with a 20x fluorescent objective. Specific fluorescence intensities for P-FAK and P-AKT were corrected by subtracting local background intensities in TH^+ neurons after omission of the phosphorylated antibodies. Results were expressed in arbitrary units (A.U.).

Stripping of heparan sulfate proteoglycans (HSPG) and lactoferrin detection

The presence of lactoferrin was assessed before and after removal of membrane-associated heparan sulfate proteoglycans (HSPGs) in 10 DIV midbrain cultures treated chronically or not with Lf. Briefly, the cultures were exposed for 1 h at 37°C to 10 IU/ml heparinase I (Hep) from

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flavobacterium heparinum (Sigma-Aldrich) as described previously (Lang et al., 2011) using a pH 7.5 buffer solution containing 20 mM Tris–HCl, 50 mM NaCl, 4 mM $CaCl_2$, 0.01% BSA. Cultures were then washed 3 times with PBS and fixed with formaldehyde for 12 min or scraped off with a RIPA lysis buffer (Pierce) for cellular detection and western immunoblotting analysis, respectively. Protein extracts (40 µg of total protein per lane) were resolved on NuPAGE® Novex pre-cast 10% Bis-Tris gels and then transferred onto 0.2-µm nitrocellulose membranes. The same rabbit polyclonal lactoferrin antibody (1/500; #Ab15811, Abcam) was used for both cellular and immunoblotting detection. Specific fluorescence intensities for lactoferrin were analyzed in TH^+ neurons as described for the phospho-proteins.

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 required, the Student–Newman–Keuls test was used. SEM values were derived from at least five independent experiments.

RESULTS

Lactoferrin prevents spontaneous and progressive DA cell death in midbrain cultures

We report, here, that Lf, a partially iron-saturated recombinant lactoferrin, produced a concentration-dependent increase in the number of DA (TH^+) neurons when applied chronically for 10 DIV to rat midbrain cultures (Fig. 1A). The effect of Lf plateaued between 10–30 µM. In this range of concentrations, the increase in TH^+ cell numbers was two-fold. mLf, a partially saturated form of lactoferrin purified from human milk, had the same efficacy as Lf. The concentrations of Lf and mLf required to produce half maximal increases in TH^+ cell numbers (EC_{50s}) were also comparable; graphical extrapolation of corresponding EC_{50s} gave 3 and 5 µM, respectively, suggesting the two proteins possess similar potencies to protect DA neurons in this experimental

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setting (Fig. 1A). Note that except when stated otherwise, we used Lf in the following experimental protocols.

When monitoring DA cell numbers as a function of the culture age, we found that more than half of DA neurons were lost at 6 DIV and more than 70% by 10 DIV (Fig. 1B). At each stage of the degenerative process, the number of TH^+ neurons was always higher in Lf-treated cultures compared to control cultures (Fig. 1B) without exceeding, however, the number of TH^+ neurons estimated at the time of plating. This indicated that Lf operated most likely by reducing DA cell demise and not by inducing TH in a subpopulation of neurons that did not express originally detectable levels of the enzyme, as reported in other model systems (He et al., 2011; Traver et al., 2006). The effect of Lf was comparable in intensity to that elicited by the dopaminotrophic factor GDNF (20 ng/ml). The effects of Lf and GDNF are illustrated by microphotographs shown in Fig. 1C. Note that the effect of Lf was highly specific to DA neurons, as other populations of midbrain neurons characterized by microtubule-associated protein-2 (MAP-2) immunofluorescence staining remained unaffected in the same experimental setting (Fig. 1D). Of interest, the increase in TH^+ cell numbers was associated with a proportional increase in [^{3}H]-DA uptake, a biochemical parameter taken as an index of the survival and function of DA neurons (Fig. 1E).

We also wished to determine whether Lf retained its ability to rescue DA neurons when the degenerative process was already ongoing in midbrain cultures. To test this possibility, we estimated DA cell survival in 10 DIV cultures when treatments with Lf (10 µM) had been delayed until 5 or 7 DIV. Results showed that delayed treatments with Lf protected DA neurons but with decreasing efficacy as degeneration progressed (Fig. 1F).

Lactoferrin remains protective for DA neurons that degenerate after GDNF deprivation

To determine whether the protective effect of lactoferrin remained observable in more mature DA neurons, we used midbrain cultures where the spontaneous death process was postponed for 10 days by chronic application of GDNF (20 ng/ml). Ablation of GDNF from these cultures at 11 DIV,

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led to a massive and selective loss of TH^+ neurons within 5 days in agreement with previous data (Guerreiro et al, 2008). Interestingly, a large population of these neurons were saved from death if Lf (10 μM) was added to the culture medium in replacement of GDNF (Fig. 2).

Iron-binding capability of lactoferrin and DA cell rescue

The functionality of lactoferrin being frequently related to its iron-binding capacity (Okazaki et al., 2012; Ward and Conneely, 2004), we wished to determine whether the iron saturation level of lactoferrin had an impact on its protective potential for spontaneously dying DA neurons. This was apparently not the case as hLf, an iron-saturated form of lactoferrin (10 μM), was as effective as Lf (10 μM), which is only partially saturated (Fig. 3A). In addition, we tested whether other molecules with iron chelating properties were able to mimic the protective effects of Lf in the same experimental context. Transferrin, an iron binding protein quite similar in structure and in sequence to lactoferrin, failed to provide protection to vulnerable DA neurons when used under its iron-free (apo) form (10 μM) and DEF (10 μM), a synthetic iron chelator was ineffective as well (Fig. 3A). These different results clearly indicate that lactoferrin-mediated protection occurred in this model system independently of iron chelation. Note that the iron-saturated form of hTf provided also no rescue to DA neurons.

Interestingly, Lf (10 μM) retained its protective potential for DA neurons in a situation where low-level oxidative stress generated by catalytically active iron was causative for degeneration (Guerreiro et al., 2009) (Fig. 3B). As expected in this experimental context, aTf (10 μM) and DEF (10 μM) reproduced the protection of Lf for DA neurons whereas hLf and hTf were totally ineffective. Use of the fluorescent probe DHR-123 (20 μM) revealed that the effects of Lf, aTf and DEF resulted from a drastic reduction in intracellular ROS production in all neuronal cells (Fig. 3C, D). The evaluation of imaged cultures by post-hoc TH staining and field relocation (Lotharius et al, 1999) confirmed that Lf caused a strong reduction of ROS produced by DA neurons (Fig 3E).

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Impact of lactoferrin treatments on glial cell populations

Astrocytes that divide from immature precursor cells have been suggested to intervene actively in DA cell demise in this culture paradigm (Mourlevat et al., 2003) suggesting that the protective effect of lactoferrin against the spontaneous loss of DA neurons may possibly result from an indirect effect on dividing glial cells. Also in support of this possibility, lactoferrin was reported to inhibit the growth of some cancer cells (Tung et al., 2013; Zemann et al., 2010) and to improve the therapeutic effects of anticancer therapies (Sun et al., 2012). Hence, we wished to determine whether the proliferation rate of astrocytes or their precursor cells (both cell populations represent more than 95% of dividing cells at this stage of the cultures) was affected by Lf in midbrain cultures. More precisely, we compared the effect of Lf (10 µM) to that of ARA-C (1 µM), an anti-mitotic molecule known to provide protection in this experimental setting through its inhibitory action on astrocytes (Mourlevat et al., 2003). While Lf and ARA-C had the same efficacy in preventing DA cell death (Fig. 4A), only ARA-C reduced glial cell proliferation, as estimated through [^{3}H]-thymidine incorporation (Fig. 4B). Thus, the protective action of Lf could not be explained by an effect on astrocytes or their precursor cells. This signified that Lf probably acted downstream of the level at which the toxic mechanism of astroglial origin intervenes.

Microglial ($CD11b^+$) cells have the potential to mediate inflammatory processes and also to exert neuroprotective functions in the brain, depending on the challenging stimuli and the situational context (Hirsch and Hunot, 2009; Kettenmann et al., 2011). These cells account only for a minor fraction of dividing glial cells in these cultures (<5 %) but they were highly responsive to a treatment with Lf; their number rose more than 2.5-fold in the presence of 10 µM Lf (Fig. 4C, D). Yet, reducing microglial cell numbers by mean of a transient application (0–1 DIV) of dbcAMP (1 mM) (Mourlevat et al., 2003), a lipophilic analog of cAMP, failed to reduce the survival-promoting action of Lf for DA neurons at 10 DIV (Fig. 4E), indicating that the proliferative effect of Lf on microglial cells did not intervene in neuroprotection.

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The protective effect of lactoferrin for spontaneously dying DA neurons results from a moderate rise in Ca^{2+}_{mit}

The maintenance of Ca^{2+}_{cyt} within a narrow range of concentrations has been reported to be crucial for the survival of DA neurons (Salthun-Lassalle et al., 2004). Therefore, we wished to determine whether Lf was protective through a mechanism that modulates Ca^{2+}_{cyt}. Neither acute (not shown) nor chronic applications of Lf (10 µM) had an impact on Ca^{2+}_{cyt} levels measured with the fluorescent dye fluo-8 in 7 DIV cultures (Fig. 5A). As expected, however, VER, a Na^{+} channel opener that provides robust protection to DA neurons in this culture paradigm, (Salthun-Lassalle et al., 2004), led to a sustained increase in basal Ca^{2+}_{cyt} when applied to midbrain cultures, thus confirming the validity of the detection protocol.

In 7 DIV cultures that were treated chronically with Lf (10 µM), we observed, however, a 1.5-fold increase in basal Ca^{2+}_{mit} levels measured with the fluorescent probe rhod-4 (Fig. 5B). This increase was reduced in a situation where Lf was withdrawn at 5 DIV and the cultures left to recover until 7 DIV (Fig. 5B), suggesting that Ca^{2+}_{mit} remained at an optimal level only if Lf was present continuously in the culture medium. Interestingly, in cultures where Lf was withdrawn at 5 DIV, the rescue of DA neurons by Lf was almost completely lost at 10 DIV (Fig. 5C). The elevation in Ca^{2+}_{mit} produced by Lf was also significantly reduced by XEC (2.5 µM), a selective and membrane-permeable inhibitor of endoplasmic reticulum (ER) IP3 receptors (IP3Rs) and by Ru360 (0.6 µM), an inhibitor of the mitochondrial uniporter (MCU). Interestingly, both XEC and Ru360 also greatly diminished Lf-mediated protection, confirming that the rise in Ca^{2+}_{mit} elicited by Lf was instrumental in its survival-promoting action. This observation was also an indication that Lf operated by stimulating a direct transfer of Ca^{2+} from the ER to the mitochondria via a mechanism involving sequentially IP3Rs and the MCU. It is worth noting that dantrolene (30 µM), which blocks selectively ER RyRs, failed to affect Lf-mediated protection, confirming that ER calcium mobilization occurred, here, through IP3Rs.

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The rise in Ca^{2+}_{mit} produced by lactoferrin is controlled by a mechanism involving PI3K

To further characterize the mechanism that underlies Lf-mediated protection, we investigated whether AKT, a protein kinase substrate of PI3K, was activated (phosphorylated) in Lf-treated cells (Fig. 6A). Consistent with our hypothesis, the immunofluorescent signal for P-AKT was substantially increased in TH^+ neurons exposed chronically to Lf (10 µM), suggesting that the treatment with Lf promoted AKT phosphorylation. As expected, blockade of PI3K by LY reduced AKT phosphorylation in DA neurons from Lf-treated cultures (Fig. 6B). LY also curtailed the rescue of DA neurons by Lf and inhibited the concurrent increase in Ca^{2+}_{mit} induced by the protein (Fig. 6C, D), indicating that Lf-mediated PI3K activation served to stimulate ER calcium mobilization.

Neuroprotection of DA neurons results from the attachment of lactoferrin to specific binding sites

Classical immunodetection studies allowed us to determine that Lf was retained on specific binding sites (Fig. 7A), in particular on DA neurons and/or in their proximal environment (Fig. 7B). Western immunoblotting analysis confirmed that Lf was retained on whole-cell protein extracts (Fig. 7C). Both the cellular and biochemical signals were decreased when the cells were exposed for 1 h at 37°C to 10 IU/ml Hep just before termination of the cultures (Fig. 7A, B, C), leading us to assume that Lf was bound essentially to HSPGs, as described previously in other systems (Sakamoto et al., 2006). Figure 7D depicts variations in Lf fluorescence intensities in TH^+ neurons from Lf-treated cultures exposed or not acutely to Hep.

Neuroprotection of DA neurons by lactoferrin requires FAK inhibition

HSPGs being key components of cell surfaces and extracellular matrix (ECM) components (Sarrazin et al., 2011), we hypothesized that the action of Lf was to interfere with integrin-dependent cell attachment. Because integrins lead to activation of the downstream effector kinase

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FAK (Hehlgans et al., 2007), we estimated expression levels of P-FAK (the active form of FAK) in TH^+ neurons exposed to treatments that modulate DA cell survival. Our data show that both Lf and PF, a specific inhibitor of FAK, significantly reduced P-FAK expression in TH^+ neurons (Fig. 8A, B). Similarly to Lf, PF stimulated P-AKT expression in DA neurons (Fig. 6A, B) and produced a robust increase in DA cell survival associated with a sustained rise in Ca^{2+}_{mit} (Fig. 8C, D). Interestingly, both effects were sensitive to LY treatment suggesting that they were under the control of PI3K-dependent signaling events (Fig. 8C, D).

Lactoferrin is protective against the mitochondrial neurotoxin MPP^+

We also wished to determine whether Lf remained protective in a situation where DA cell death was caused by mitochondrial poisoning with MPP^+, the active metabolite of the dopaminergic neurotoxin MPTP. For this purpose, spontaneously occurring DA cell death was prevented by a treatment combining depolarizing concentrations of K^+ (30 mM) and MK-801 (1 µM) and the cultures were exposed to 3 µM MPP^+ between 5 and 7 DIV in order to achieve a loss of approximately 50% of DA neurons. When the cultures were exposed chronically to Lf between 2 and 10 DIV, MPP^+-induced DA cell loss was substantially reduced (Fig. 9A). Importantly, this protective effect was not due to a simple reduction in efficacy of the DA transporter (this transporter permits the accumulation of MPP^+ into DA neurons) as protective concentrations of Lf failed to reduce the uptake of [^{3}H]-DA (data not shown).

To evaluate the consequences of the treatment with MPP^+ at an early stage of the degenerative process, we performed Ca^{2+}_{mit} measurements after 6 h of incubation with the toxin. In this experimental context, a concentration of 50 µM MPP^+ was chosen to generate degenerative changes that were detectable in the whole population of midbrain neurons regardless of the neurotransmitter phenotypes. We established that basal Ca^{2+}_{mit} levels were reduced by approximately 30%, 6 h after adding MPP^+ to the cultures. Interestingly, Ca^{2+}_{mit} levels were partially restored in the presence of Lf (Fig. 9B). Similar to what was observed in the spontaneous DA cell death model, the inhibition

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of PI3K with LY curtailed, whereas the inhibition of FAK with PF mimicked, the effects of Lf against MPP^+ (Fig. 9A, B). A schematic drawing of the mechanism by which Lf might prevent spontaneous DA cell demise in midbrain cultures is given in Figure 10.

DISCUSSION

We have shown, here, that the iron-binding protein lactoferrin provided robust protection for vulnerable DA neurons in different experimental settings that model neurodegenerative events in PD. Lactoferrin neuroprotective actions were independent of glial cells. Depending on the situational context, neuroprotective effects appear to result from either the preservation of Ca^{2+}_{mit} homeostasis or the reduction of oxidative stress-mediated damage. The iron binding capability of lactoferrin was important, only for the later effect.

Role of iron chelation in the protection afforded to spontaneously dying DA neurons by lactoferrin

We found that Lf and mLf, i.e., partially iron-saturated lactoferrins of recombinant or extractive origin, respectively, provided robust protection to spontaneously dying DA neurons in midbrain cultures and most importantly preserved their function. The efficacy of lactoferrin in rescuing DA neurons from death was comparable to that of GDNF, a prototypical neurotrophic factor for DA neurons. Interestingly, DA neurons grown initially with GDNF and then deprived of it were also protected from death by Lf, indicating that neuroprotection remained effective in more mature DA neurons.

Iron chelation being one of the key features of lactoferrin (Garcia-Montoya et al., 2012), we wished to determine whether the level of iron saturation had an impact on the effect of lactoferrin in this paradigm. This was not the case as an iron-saturated form of lactoferrin hLf was as protective as Lf which is only partially saturated. Confirming that iron chelation was not involved, here, in DA cell rescue, an apo form of transferrin a plasma iron transport protein structurally related to

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lactoferrin (Garcia-Montoya et al., 2012) and a synthetic chelator of iron DEF lacked protective effects for DA neurons. These observations also suggested that the protein conformational changes that accompany metal binding and release (Wally and Buchanan, 2007) have no impact, here, on the neuroprotective potential of lactoferrin. This set of data is consistent with previous reports showing that several functions of lactoferrin are independent of its iron binding capacity (Ward and Conneely, 2004). It also confirms indirectly that, despite a high degree of homology at the amino acid sequence level between transferrin and lactoferrin, the function of lactoferrin differs profoundly from that of transferrin. Iron chelation by lactoferrin was, however, instrumental for the rescue of DA neurons in a situation where low-level oxidative stress generated by catalytically active iron is instrumental for the demise of these neurons (Guerreiro et al., 2009). This is in agreement with reports describing the free radical scavenging properties of lactoferrin (Kruzel et al., 2006; Okazaki et al., 2012) and may be of some relevance for PD, a pathological condition where DA neurons undergo oxidative stress-mediated insults (Dexter et al., 1989), partly as the result of iron accumulation within DA neurons or in their proximal environment (Hirsch et al., 1991).

The rescue of spontaneously dying DA neurons by lactoferrin occurs via an elevation in Ca^{2+}_{mit}

We previously found that the survival of DA neurons was preserved by maintaining Ca^{2+}_{cyt} within a certain range of concentrations (Michel et al., 2013). The possibility that lactoferrin could also promote DA cell survival by restoring optimal levels of Ca^{2+}_{cyt} was, however, excluded as neither acute (not shown) nor chronic treatments with Lf modified this parameter. Yet, we established that there was a significant elevation in basal Ca^{2+}_{mit} levels at 7 DIV (i.e., at a stage where DA cell death is ongoing) in cultures that were exposed chronically to neuroprotective concentrations of Lf. The rise in Ca^{2+}_{mit} was curtailed by premature withdrawal of Lf at 5 DIV, an experimental paradigm resulting in limited rescue of DA neurons at 10 DIV, leading us to assume

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that the preservation of calcium homeostasis in mitochondria was crucial for Lf-mediated DA cell protection.

In line with these observations, we established that the protection of spontaneously dying DA neurons by Lf and the concomitant elevation in Ca^{2+}_{mit} associated with it were strongly reduced when calcium accumulation in the mitochondria through the MCU was blocked with Ru360 and the release of ER calcium through IP3Rs inhibited with XEC. This suggests that the rise in Ca^{2+}_{mit} generated by Lf treatment occurred through a direct flow of calcium from the ER to the mitochondria, a mechanism probably facilitated by the close proximity of these two organelles (Kanwar and Sun, 2008; Toulorge et al., 2010).

Of note, sustained increases in Ca^{2+}_{mit} have been suggested to operate as a trigger for neurodegeneration in pathological conditions (Brookes et al., 2004; Muriel et al., 2000), which is in apparent contradiction with our results. Yet, Ca^{2+} is also known to be a key physiological regulator of mitochondrial function that acts at several levels within the organelle to stimulate ATP synthesis (Brookes et al., 2004; Duchen, 2000) a mechanism that might be impaired early in the process of DA cell death in PD (Hoglinger et al., 2003). Thus, one may assume that Lf served to reinstate Ca^{2+}_{mit} to control levels to preserve cellular bioenergetics in vulnerable midbrain DA neurons. In support of the view that mitochondria could represent a downstream target of Lf action, we found that the glycoprotein was also partially protective against MPP^+, a dopaminergic toxin that selectively interferes with the mitochondrial respiratory chain (Orth and Schapira, 2002). In this experimental context, Lf also partially restored Ca^{2+}_{mit} levels that were lowered by MPP^+ at an early stage of the neurodegenerative process. Interestingly, it was recently proposed that one of the physiological functions of α-synuclein, a synaptic protein that forms Lewy bodies aggregates in PD and is mutated in rare familial forms of the pathology, is to facilitate calcium transfer from ER to mitochondria (Cali et al., 2012).

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The elevation of Ca^{2+}_{mit} induced by lactoferrin results from the activation of a PI3K-dependent signaling pathway

IP3R-mediated calcium release is modulated through different mechanisms and in particular via PI3K/AKT-dependent signaling (Kim et al., 2009), as IP3Rs possess consensus sequences for phosphorylation by AKT (Khan et al., 2006). The possibility that Lf could activate IP3Rs through a PI3K/AKT-dependent mechanism is supported by the following observations: (i) chronic Lf treatment promoted AKT phosphorylation in DA neurons; (ii) inhibition of the immediate upstream kinase of AKT, PI3K, with LY prevented survival promotion by Lf; (iii) LY also diminished the rise in Ca^{2+}_{mit} elicited by Lf. Altogether, this set of data suggests that the consequence of Lf treatment might be to increase calcium shuttling between ER and mitochondria via a PI3K-dependent mechanism. These observations are also in line with previous observations showing AKT activation was essential for the survival of DA neurons in the post-natal and adult mouse brain (Ries et al., 2009).

Note that the phosphorylation of AKT in DA neurons declined to values that were far below controls when LY was added to Lf-treated cultures suggesting that AKT-dependent mechanisms may also play a role in the survival of DA neurons that are normally resistant to death. This was probably not the case since the number of DA neurons was not decreased below control values in cultures exposed concomitantly to Lf and LY. Consistent with this observation, LY had no influence on DA cell survival when applied alone to midbrain cultures. Note that P-AKT expression was also profoundly reduced in non-dopaminergic neurons after LY treatment with no significant impact on the viability of these neurons (not shown).

Lactoferrin is protective by reducing FAK-dependent signaling in DA neurons

The visualization of Lf in midbrain cultures by conventional immunofluorescence staining or in cell protein extracts through western immunoblotting analysis revealed that Lf was attached to specific binding sites. Enzymatic treatment of the cultures with Hep strongly reduced the Lf signal,

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suggesting that the protein was attached to a large extent to HSPGs, i.e., glycoproteins located on basement membranes and in ECM compartments (Sarrazin et al., 2011), in good agreement with the known properties of lactoferrin (Lang et al., 2011; Thorne et al., 2008). A decrease in Lf binding was also observed specifically in DA neurons, and also in their close surroundings, indicating that Lf might operate by precluding deleterious contacts occurring between DA neurons and specific ECM components secreted by immature (i.e., deleterious) astrocytes, as previously suggested (Toulorge et al., 2010). As the RGD sequence peptide that blocks integrin-ligand interactions (Moon et al., 2009) is protective for DA neurons in this model system (Toulorge et al., 2010), we might assume that these contacts required the engagement of integrins and possibly of their major downstream effector kinase, FAK (Hehlgans et al., 2007). Consistent with this, Lf significantly reduced the expression of P-FAK in DA neurons. Besides, PF a specific inhibitor of FAK also mimicked the rescuing effect of Lf for DA neurons and produced, concomitantly, a sustained rise in Ca^{2+}_{mit}. On the basis of these results, we may therefore assume that Lf operated by repressing an integrin-dependent signaling cascade that ultimately disrupts mitochondrial calcium homeostasis in spontaneously dying DA neurons. Of note, a similar mechanism appeared also involved in Lf-mediated protection against MPP^+, reinforcing the more general concept that the maintenance of calcium levels within mitochondria might be pivotal to prevent DA cell demise (Toulorge et al, 2010; Michel et al, 2013).

Overall, our present data suggest that lactoferrin is protective in experimental paradigms that model mitochondrial dysfunction and oxidative stress in DA neurons, namely two pathomechanisms relevant for PD. Increased levels of lactoferrin in DA neurons resistant to degeneration in the brain of PD patients (Faucheux et al., 1995; Leveugle et al., 1996) and in the striatum of MPTP-intoxicated mice (Fillebeen et al., 2001) might be evidence of an attempt by the brain to minimize neurodegeneration. Because plasma levels of lactoferrin were reported to be inversely correlated with disease severity (Grau et al., 2001), therapeutic strategies that elevate

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lactoferrin plasma levels might be of interest for the treatment of PD patients.

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AUTHORSHIP CONTRIBUTIONS

Participated in research design: Rousseau, Michel and Hirsch

Conducted experiments: Rousseau and Michel

Performed data analysis: Rousseau, Michel and Hirsch

Wrote or contributed to writing of the manuscript: Rousseau, Michel and Hirsch

REFERENCES

Brock, J.H. (2002). The physiology of lactoferrin. Biochem Cell Biol *80*, 1-6.

Brookes, P.S., Yoon, Y., Robotham, J.L., Anders, M.W., and Sheu, S.S. (2004). Calcium, ATP, and ROS: a mitochondrial love-hate triangle. Am J Physiol Cell Physiol *287*, C817-833.

Cali, T., Ottolini, D., Negro, A., and Brini, M. (2012). alpha-Synuclein controls mitochondrial calcium homeostasis by enhancing endoplasmic reticulum-mitochondria interactions. J Biol Chem *287*, 17914-17929.

Dauer, W., and Przedborski, S. (2003). Parkinson's disease: mechanisms and models. Neuron *39*, 889-909.

Dexter, D.T., Carter, C.J., Wells, F.R., Javoy-Agid, F., Agid, Y., Lees, A., Jenner, P., and Marsden, C.D. (1989). Basal lipid peroxidation in substantia nigra is increased in Parkinson's disease. J Neurochem *52*, 381-389.

Drago-Serrano, M.E., de la Garza-Amaya, M., Luna, J.S., and Campos-Rodriguez, R. (2012). Lactoferrin-lipopolysaccharide (LPS) binding as key to antibacterial and antiendotoxic effects. Int Immunopharmacol *12*, 1-9.

Duchen, M.R. (2000). Mitochondria and calcium: from cell signalling to cell death. J Physiol *529 Pt 1*, 57-68.

Faucheux, B.A., Nillesse, N., Damier, P., Spik, G., Mouatt-Prigent, A., Pierce, A., Leveugle, B., Kubis, N., Hauw, J.J., Agid, Y., *et al.* (1995). Expression of lactoferrin receptors is increased in the mesencephalon of patients with Parkinson disease. Proc Natl Acad Sci U S A *92*, 9603-9607.

Fillebeen, C., Ruchoux, M.M., Mitchell, V., Vincent, S., Benaissa, M., and Pierce, A. (2001). Lactoferrin is synthesized by activated microglia in the human substantia nigra and its synthesis by the human microglial CHME cell line is upregulated by tumor necrosis factor alpha or 1-methyl-4-phenylpyridinium treatment. Brain Res Mol Brain Res *96*, 103-113.

Garcia-Montoya, I.A., Cendon, T.S., Arevalo-Gallegos, S., and Rascon-Cruz, Q. (2012). Lactoferrin a multiple bioactive protein: an overview. Biochim Biophys Acta *1820*, 226-236.

Grau, A.J., Willig, V., Fogel, W., and Werle, E. (2001). Assessment of plasma lactoferrin in Parkinson's disease. Mov Disord *16*, 131-134.

Guerreiro, S., Ponceau, A., Toulorge, D., Martin, E., Alvarez-Fischer, D., Hirsch, E.C., and Michel, P.P. (2009). Protection of midbrain dopaminergic neurons by the end-product of purine metabolism uric acid: potentiation by low-level depolarization. J Neurochem *109*, 1118-1128.

Guerreiro, S., Toulorge, D., Hirsch, E., Marien, M., Sokoloff, P., and Michel, P.P. (2008). Paraxanthine, the primary metabolite of caffeine, provides protection against dopaminergic cell death via stimulation of ryanodine receptor channels. Mol Pharmacol *74*, 980-989.

He, X.B., Yi, S.H., Rhee, Y.H., Kim, H., Han, Y.M., Lee, S.H., Lee, H., Park, C.H., Lee, Y.S., Richardson, E., *et al.* (2011). Prolonged membrane depolarization enhances midbrain dopamine neuron differentiation via epigenetic histone modifications. Stem Cells *29*, 1861-1873.

Hehlgans, S., Haase, M., and Cordes, N. (2007). Signalling via integrins: implications for cell survival and anticancer strategies. Biochim Biophys Acta *1775*, 163-180.

Hirsch, E.C., Brandel, J.P., Galle, P., Javoy-Agid, F., and Agid, Y. (1991). Iron and aluminum increase in the substantia nigra of patients with Parkinson's disease: an X-ray microanalysis. J Neurochem *56*, 446-451.

Hirsch, E.C., and Hunot, S. (2009). Neuroinflammation in Parkinson's disease: a target for neuroprotection? Lancet Neurol *8*, 382-397.

Hirsch, E.C., Jenner, P., and Przedborski, S. (2013). Pathogenesis of Parkinson's disease. Mov Disord *28*, 24-30.

Hoglinger, G.U., Carrard, G., Michel, P.P., Medja, F., Lombes, A., Ruberg, M., Friguet, B., and Hirsch, E.C. (2003). Dysfunction of mitochondrial complex I and the proteasome: interactions between two biochemical deficits in a cellular model of Parkinson's disease. J Neurochem *86*, 1297-1307.

Jameson, G.B., Anderson, B.F., Norris, G.E., Thomas, D.H., and Baker, E.N. (1998). Structure of human apolactoferrin at 2.0 A resolution. Refinement and analysis of ligand-induced conformational change. Acta Crystallogr Sect D Biol Crystallogr *54*, 1319-1335.

Kanwar, Y.S., and Sun, L. (2008). Shuttling of calcium between endoplasmic reticulum and mitochondria in the renal vasculature. Am J Physiol Renal Physiol *295*, F1301-1302.

Kettenmann, H., Hanisch, U.K., Noda, M., and Verkhratsky, A. (2011). Physiology of microglia. Physiol Rev *91*, 461-553.

Khan, M.T., Wagner, L., 2nd, Yule, D.I., Bhanumathy, C., and Joseph, S.K. (2006). Akt kinase phosphorylation of inositol 1,4,5-trisphosphate receptors. J Biol Chem *281*, 3731-3737.

Kim, J.J., Choi, Y.M., Hong, M.A., Hwang, S.S., Yoon, S.H., Chae, S.J., Jee, B.C., Ku, S.Y., Kim, J.G., and Moon, S.Y. (2009). Phosphatidylinositol 3-kinase p85alpha regulatory subunit gene Met326Ile polymorphism in women with polycystic ovary syndrome. Hum Reprod *24*, 1184-1190.

Kruzel, M.L., Bacsi, A., Choudhury, B., Sur, S., and Boldogh, I. (2006). Lactoferrin decreases pollen antigen-induced allergic airway inflammation in a murine model of asthma. Immunology *119*, 159-166.

Lang, J., Yang, N., Deng, J., Liu, K., Yang, P., Zhang, G., and Jiang, C. (2011). Inhibition of SARS pseudovirus cell entry by lactoferrin binding to heparan sulfate proteoglycans. PloS One *6*, e23710.

Levay, P.F., and Viljoen, M. (1995). Lactoferrin: a general review. Haematologica *80*, 252-267.

Leveugle, B., Faucheux, B.A., Bouras, C., Nillesse, N., Spik, G., Hirsch, E.C., Agid, Y., and Hof, P.R. (1996). Cellular distribution of the iron-binding protein lactotransferrin in the mesencephalon of Parkinson's disease cases. Acta Neuropathol *91*, 566-572.

Lotharius J, Dugan LL, O'Malley KL. (1999). Distinct mechanisms underlie neurotoxin-mediated cell death in cultured dopaminergic neurons. J Neurosci *19*, 1284-93.

Metz-Boutigue, M.H., Jolles, J., Mazurier, J., Schoentgen, F., Legrand, D., Spik, G., Montreuil, J., and Jolles, P. (1984). Human lactotransferrin: amino acid sequence and structural comparisons with other transferrins. Eur J Biochem *145*, 659-676.

Michel, P.P., Toulorge, D., Guerreiro, S., and Hirsch, E.C. (2013). Specific needs of dopamine neurons for stimulation in order to survive: implication for Parkinson disease. FASEB J *27*, 3414-23.

Moon, C., Han, J.R., Park, H.J., Hah, J.S., and Kang, J.L. (2009). Synthetic RGDS peptide attenuates lipopolysaccharide-induced pulmonary inflammation by inhibiting integrin signaled MAP kinase pathways. Respir Res *10*, 18.

Mourlevat, S., Troadec, J.D., Ruberg, M., and Michel, P.P. (2003). Prevention of dopaminergic neuronal death by cyclic AMP in mixed neuronal/glial mesencephalic cultures requires the repression of presumptive astrocytes. Mol Pharmacol *64*, 578-586.

Muriel, M.P., Lambeng, N., Darios, F., Michel, P.P., Hirsch, E.C., Agid, Y., and Ruberg, M. (2000). Mitochondrial free calcium levels (Rhod-2 fluorescence) and ultrastructural alterations in neuronally differentiated PC12 cells during ceramide-dependent cell death. J Comp Neurol *426*, 297-315.

Okazaki, Y., Kono, I., Kuriki, T., Funahashi, S., Fushimi, S., Iqbal, M., Okada, S., and Toyokuni, S. (2012). Bovine lactoferrin ameliorates ferric nitrilotriacetate-induced renal oxidative damage in rats. J Clin Biochem Nutr *51*, 84-90.

Orth, M., and Schapira, A.H. (2002). Mitochondrial involvement in Parkinson's disease. Neurochem Int *40*, 533-541.

Pierce, A., Legrand, D., and Mazurier, J. (2009). [Lactoferrin: a multifunctional protein]. Med Sci (Paris) *25*, 361-369.

Ries, V., Cheng, H.C., Baohan, A., Kareva, T., Oo, T.F., Rzhetskaya, M., Bland, R.J., During, M.J., Kholodilov, N., and Burke, R.E. (2009). Regulation of the postnatal development of dopamine neurons of the substantia nigra in vivo by Akt/protein kinase B. J Neurochem *110*, 23-33.

Sakamoto, K., Ito, Y., Mori, T., and Sugimura, K. (2006). Interaction of human lactoferrin with cell adhesion molecules through RGD motif elucidated by lactoferrin-binding epitopes. J Biol Chem *281*, 24472-24478.

Salthun-Lassalle, B., Hirsch, E.C., Wolfart, J., Ruberg, M., and Michel, P.P. (2004). Rescue of mesencephalic dopaminergic neurons in culture by low-level stimulation of voltage-gated sodium channels. J Neurosci *24*, 5922-5930.

Sarrazin, S., Lamanna, W.C., and Esko, J.D. (2011). Heparan sulfate proteoglycans. Cold Spring Harb Perspect Biol *3:a004952*.

Sun, X., Jiang, R., Przepiorski, A., Reddy, S., Palmano, K.P., and Krissansen, G.W. (2012). "Iron-saturated" bovine lactoferrin improves the chemotherapeutic effects of tamoxifen in the treatment of basal-like breast cancer in mice. BMC Cancer *12*, 591.

Suzuki, Y.A., Lopez, V., and Lonnerdal, B. (2005). Mammalian lactoferrin receptors: structure and function. Cell Mol Life Sci *62*, 2560-2575.

Thorne, R.G., Lakkaraju, A., Rodriguez-Boulan, E., and Nicholson, C. (2008). In vivo diffusion of lactoferrin in brain extracellular space is regulated by interactions with heparan sulfate. Proc Natl Acad Sci U S A *105*, 8416-8421.

Toulorge, D., Guerreiro, S., Hild, A., Maskos, U., Hirsch, E.C., and Michel, P.P. (2011). Neuroprotection of midbrain dopamine neurons by nicotine is gated by cytoplasmic Ca2+. FASEB J *25*, 2563-2573.

Toulorge, D., Guerreiro, S., Hirsch, E.C., and Michel, P.P. (2010). KATP channel blockade protects midbrain dopamine neurons by repressing a glia-to-neuron signaling cascade that ultimately disrupts mitochondrial calcium homeostasis. J Neurochem *114*, 553-564.

Traver, S., Marien, M., Martin, E., Hirsch, E.C., and Michel, P.P. (2006). The phenotypic differentiation of locus ceruleus noradrenergic neurons mediated by brain-derived neurotrophic factor is enhanced by corticotropin releasing factor through the activation of a cAMP-dependent signaling pathway. Mol Pharmacol *70*, 30-40.

Tung, Y.T., Chen, H.L., Yen, C.C., Lee, P.Y., Tsai, H.C., Lin, M.F., and Chen, C.M. (2013). Bovine lactoferrin inhibits lung cancer growth through suppression of both inflammation and expression of vascular endothelial growth factor. J Dairy Sci *96*, 2095-2106.

Wally, J., and Buchanan, S.K. (2007). A structural comparison of human serum transferrin and human lactoferrin. Biometals *20*, 249-262.

Ward, P.P., and Conneely, O.M. (2004). Lactoferrin: role in iron homeostasis and host defense against microbial infection. Biometals *17*, 203-208.

Zemann, N., Klein, P., Wetzel, E., Huettinger, F., and Huettinger, M. (2010). Lactoferrin induces growth arrest and nuclear accumulation of Smad-2 in HeLa cells. Biochimie *92*, 880-884.

FOOTNOTES:

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Fig. 1 Protective effects of lactoferrin for spontaneously dying DA neurons in midbrain culture. A, Number of DA (TH^+) neurons in 10 DIV control cultures or in sister cultures treated with Lf or mLf (0.1–30 μM). B, Number of TH^+ neurons as a function of the culture age in control conditions or after a chronic treatment with Lf (10 μM) or GDNF (20 ng/ml). C, Immunodetection of TH^+ neurons in control, Lf (10 μM) or GDNF (20 ng/ml)-treated cultures. Scale bar: 30 μm. D, Quantification of MAP-2^+ neurons in control, and Lf (10 μM)-treated cultures. Insert depicts MAP-2^+ cells in control and Lf-treated cultures. E, Uptake of [^{3}H]-DA at 10 DIV in control cultures or in cultures chronically treated with Lf (0.1-30 μM). F, Number of TH^+ neurons in 10 DIV cultures in which the addition of Lf (10 μM) was postponed until 5 or 7 DIV. *$P < 0.05$ *vs*. corresponding control.

Fig. 2 Lactoferrin also protects DA neurons from GDNF deprivation. A, Impact of a treatment with Lf (10 μM) in cultures initially exposed to 20 ng/ml GDNF for 10 DIV and then deprived of the peptide between 11 and 15 DIV. B, Microphotographs illustrating the effects of Lf after GDNF withdrawal. Scale bar: 20 μm. *$P < 0.05$ *vs*. GDNF-treated cultures; $^{\#}P < 0.05$ *vs* cultures deprived of GDNF.

Fig. 3 Iron-binding capability of lactoferrin and DA cell rescue. A, Midbrain cultures maintained with standard N5 medium and exposed chronically to Lf, hLf, aTf, hTf or DEF (all at 10 μM) before quantification of TH^+ cell survival at 10 DIV. B, Counts of TH^+ neurons in 7 DIV midbrain cultures that were maintained with a chemically defined medium supplemented with Fe^{3+} (1.5 μM) and exposed chronically to the same treatments as before. C, ROS production detected with DHR-123 in 5 DIV midbrain cultures maintained in the same experimental conditions as in B. D, Microphotographs illustrating the impact of Lf, aTf and DEF treatments on ROS production in all midbrain neurons. E, Culture imaging by fluorescence and phase contrast optics showing the impact of Lf on ROS produced by DA neurons. The white dotted line indicates the contour of DA neurons

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and the small white arrow points to a non-dopaminergic neuron producing high levels of ROS. Scale bars: 10 µm. *$P < 0.05$ *vs*. corresponding controls.

Fig. 4 Role of glial cells in the protective action of lactoferrin for DA neurons. A, shows the number of TH^+ neurons at 10 DIV, and B the incorporation of [^{3}H]-thymidine at 6 DIV in midbrain cultures maintained in the presence of Lf (10 µM) or ARA-C (1 µM). C, Impact of a treatment with Lf (10 µM) on microglial ($CD11b^+$) cell numbers in 6 DIV cultures treated or not transiently with 1 mM dbcAMP between 0 and 1 DIV. D, Illustrations of the effects of the previous treatments on microglial cell numbers. E, Impact of a treatment with Lf (10 µM) on TH^+ cells numbers in 10 DIV cultures treated or not transiently with 1 mM dbcAMP between 0 and 1 DIV. *$P < 0.05$ *vs*. controls; $^{\#}P < 0.05$ *vs*. corresponding cultures not exposed to dbcAMP.

Fig. 5 Lactoferrin-mediated protection of DA neurons: role of Ca^{2+}_{mit}. A, Relative changes in Ca^{2+}_{cyt} levels measured with fluo-8 in 7 DIV cultures exposed chronically to Lf (10 µM) or to VER (0.8 µM) used as a positive control. B, Relative changes in Ca^{2+}_{mit} levels evaluated with rhod-4 in 7 DIV cultures exposed chronically (0–7 DIV) or transiently (0–5 DIV) to Lf (10 µM). In chronically treated cultures, the effect of Lf was challenged with the IP3R blocker XEC (2.5 µM) or the MCU inhibitor Ru360 (0.6 µM). C, Survival of TH^+ neurons in 10 DIV cultures exposed chronically (0–10 DIV) or transiently (0–5 DIV) to Lf (10 µM). In chronically treated cultures, the effect of Lf was challenged with XEC (2.5 µM), Ru360 (0.6 µM) or the ryanodine receptor blocker dantrolene (DANT; 30 µM). *$P < 0.05$ *vs*. corresponding controls; $^{\#}P < 0.05$ reduced *vs*. chronic Lf treatment, alone. § Signifies that the treatment with Lf was interrupted prematurely at 5 DIV for subsequent assessment.

Fig. 6 Role of P-AKT in lactoferrin-mediated rescue of DA neurons. A, Immunofluorescence detection of P-AKT (red labeling) in TH^+ neurons (green labeling) from 7 DIV cultures exposed

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chronically to Lf (10 µM) in the presence or not of LY (5 µM), or maintained with PF (3 µM) only. Scale bar: 15 µm. B, Variations of P-AKT fluorescence intensities analyzed in TH^+ neurons from cultures exposed to the same treatments as in A. Fluorescence signals are expressed in arbitrary units (A.U.). C, Survival of DA neurons in 10 DIV midbrain cultures exposed to Lf in the presence or not of LY. D, Ca^{2+}_{mit} levels in 7 DIV midbrain cultures exposed to the same treatments as in C. **P* < 0.05 *vs*. corresponding controls. $^{\#}P$ < 0.05 reduced *vs*. Lf alone.

Fig. 7 Binding sites of lactoferrin in midbrain cultures. A, Immunofluorescence detection of Lf in midbrain cultures treated chronically with Lf (10 µM) and exposed or not for one hour, just before termination of the cultures, to 10 UI/ml Hep. B, Immunofluorescence detection of Lf (red signal) in TH^+ (green signal) neurons from Lf-treated cultures exposed or not acutely to Hep. Scale bar: 10 µm. C, Western immunoblotting analysis of total protein extracts obtained from 10 DIV control and Lf-treated cultures exposed or not acutely to Hep. D, Variations of Lf fluorescence intensities in TH^+ neurons from Lf-treated cultures exposed or not acutely to Hep. Data are expressed in arbitrary units (A.U.). $^{\#}P$ < 0.05 reduced *vs*. Lf, alone.

Fig. 8 Lactoferrin-mediated rescue of DA neurons requires P-FAK inhibition. A, Immunofluorescence detection of P-FAK (red labeling) and TH (green labeling) in 7 DIV cultures exposed or not chronically to Lf (10 µM) or PF (3 µM). Scale bar: 15 µm. B, Variations of P-FAK fluorescence intensities analyzed in TH^+ neurons from cultures exposed or not chronically to Lf or PF. Fluorescence signals are expressed in arbitrary units (A.U.). C, Survival of DA neurons in 10 DIV midbrain cultures exposed chronically to Lf, PF or PF + LY (5 µM). D, Ca^{2+}_{mit} levels in 7 DIV midbrain cultures exposed to the same treatments as in C. **P* < 0.05 *vs*. corresponding controls. $^{\#}P$ < 0.05 reduced *vs*. PF, alone.

Fig. 9 Protective effects of lactoferrin against MPP^+-induced DA cell death. A, DA cell survival

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in midbrain cultures treated with MPP^+ (3 µM) between 5 and 7 DIV and exposed continuously to Lf (10 µM), Lf + LY (5 µM) or PF (3 µM), alone from 2-10 DIV. B, Assessment of Ca^{2+}_{mit} in 5 DIV midbrain cultures treated or not chronically with Lf, Lf + LY or PF, alone and exposed for only 6 h to MPP^+ (50 µM). Note that in all conditions, the culture medium was supplemented with K^+ (30 mM) and MK801 (2 µM) to prevent the spontaneous demise of DA neurons. $*P < 0.05$ *vs*. corresponding controls; $^{\#}P < 0.05$ *vs*. corresponding treatment with MPP^+ only. $^{\#\#}P < 0.05$ *vs*. corresponding treatment with MPP^+ and Lf.

Fig. 10 Schematic representation of the mechanisms by which lactoferrin may prevent spontaneous DA cell demise in midbrain cultures. In control conditions, deleterious contacts between integrins located on the plasma membrane of DA neurons and ECM components secreted presumably by immature astroglial cells, trigger the progressive demise of DA neurons through a mechanism that requires activation of FAK, the main effector kinase of integrins. In Lf-treated cultures, Lf interacts with HSPGs located on DA cell membranes and presumably with ECM components to prevent this deleterious contact. This reduces FAK activation and promotes AKT phosphorylation via PI3K activation. An increase in P-AKT permits the mobilization of intracellular Ca^{2+} stores through IP3Rs. Calcium is then taken up by mitochondria through the MCU, an effect that may stimulate mitochondrial bioenergetics in DA neurons. Protection of Lf against MPP^+-induced DA cell death may also require a rise in Ca^{2+}_{mit} produced via a similar mechanism (not shown here for simplification).

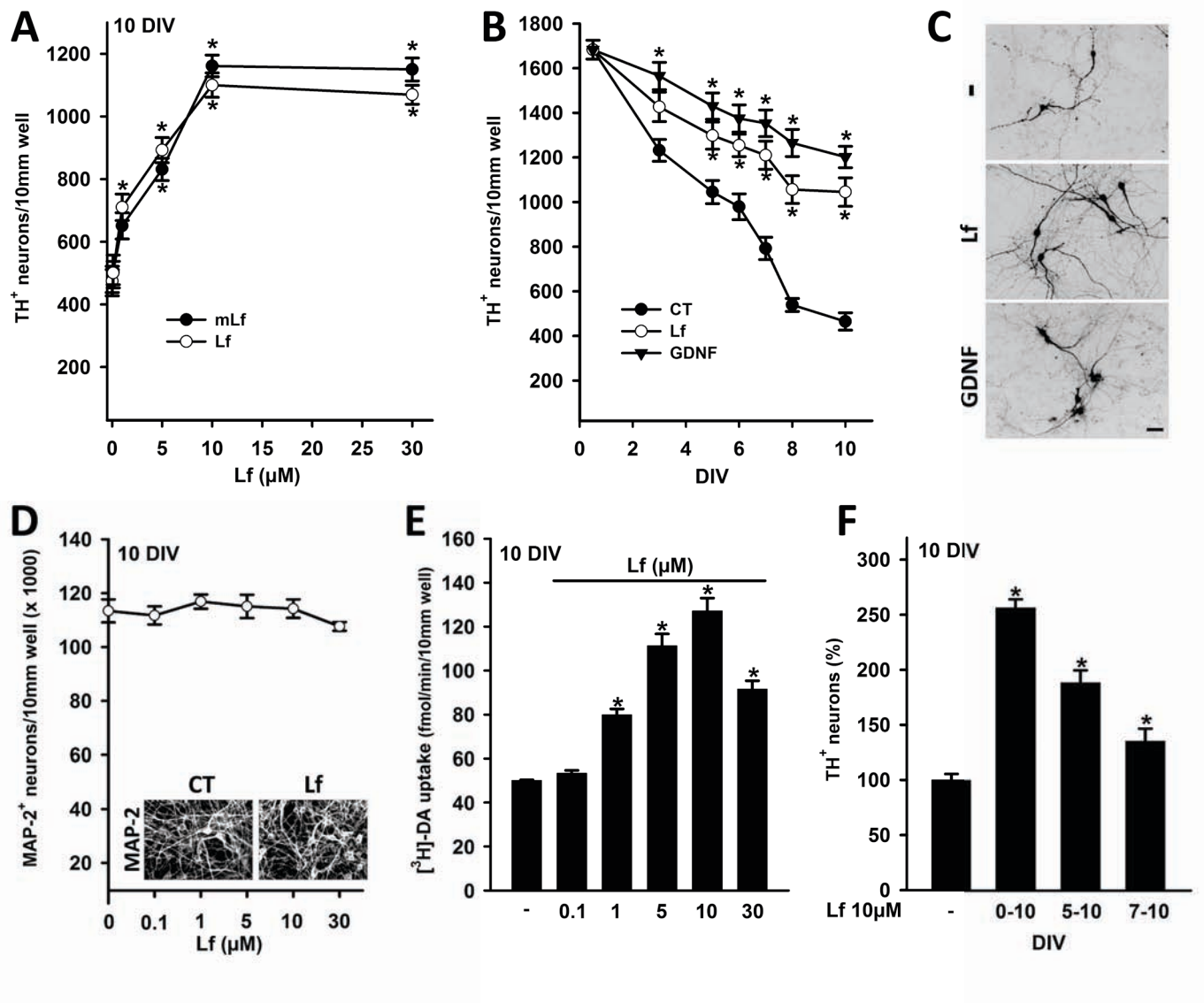
A
10 DIV
TH+ neurons/10mm well
mLf
Lf
Lf (μM)
B
TH+ neurons/10mm well
CT
Lf
GDNF
DIV
C
-
Lf
GDNF
D
10 DIV
MAP-2+ neurons/10mm well (x 1000)
CT
Lf
MAP-2
Lf (μM)
E
10 DIV
Lf (μM)
[3H]-DA uptake (fmol/min/10mm well)
F
10 DIV
TH+ neurons (%)
Lf 10μM
DIV

Figure 1

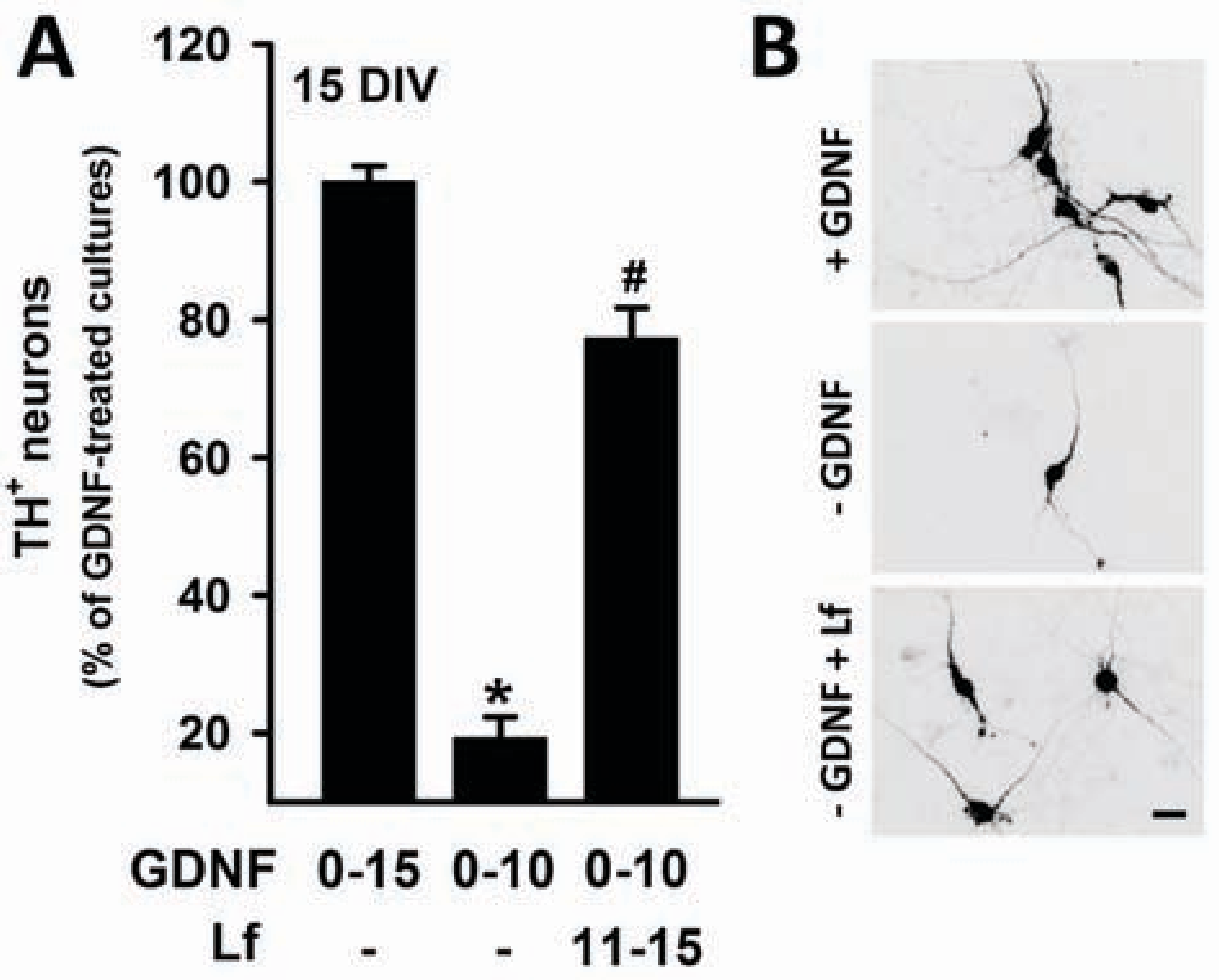
A
120
15 DIV
100
80
60
40
20
TH+ neurons
(% of GDNF-treated cultures)
*
#
GDNF 0-15 0-10 0-10
Lf - - 11-15
B
+ GDNF
- GDNF
- GDNF + Lf

Figure 2

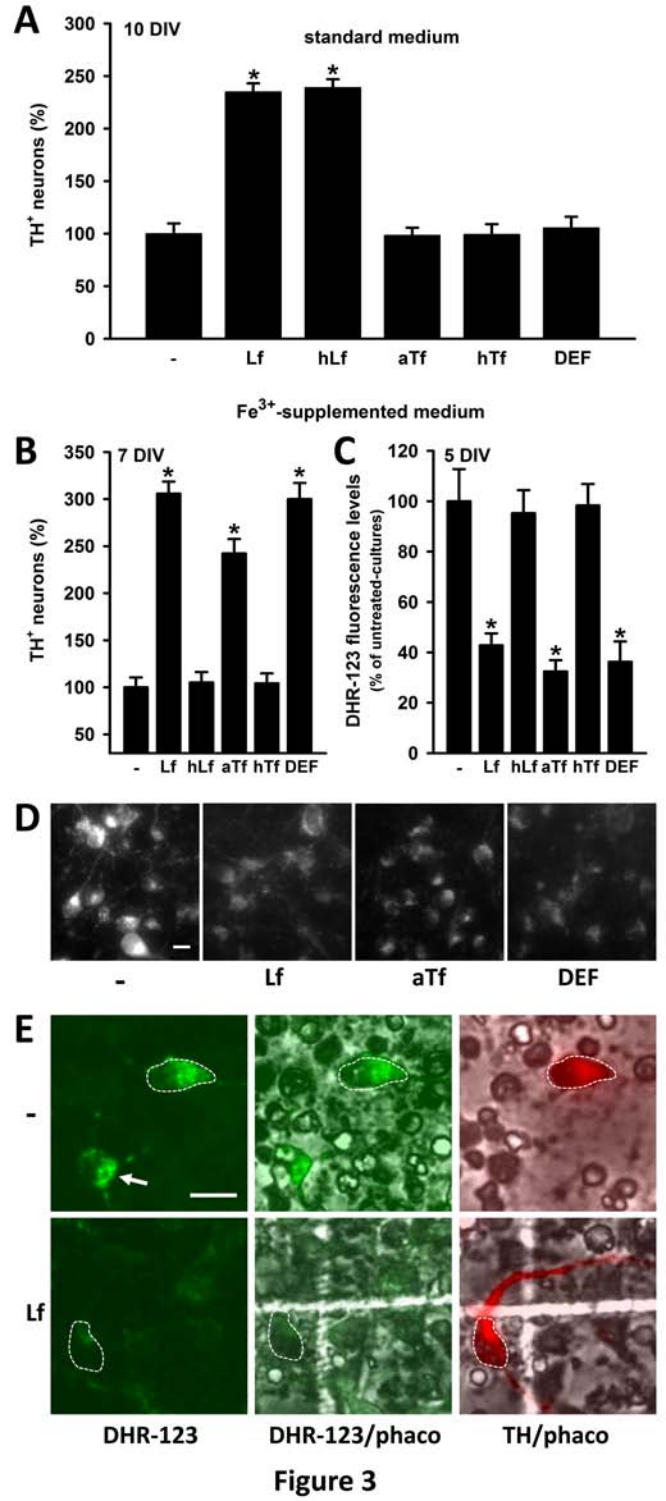
A
10 DIV
standard medium
TH+ neurons (%)
300
250
200
150
100
50
0
*
*
-
Lf
hLf
aTf
hTf
DEF
Fe3+-supplemented medium
B
7 DIV
TH+ neurons (%)
350
300
250
200
150
100
50
*
*
*
-
Lf
hLf
aTf
hTf
DEF
C
5 DIV
DHR-123 fluorescence levels
(% of untreated-cultures)
120
100
80
60
40
20
0
*
*
*
-
Lf
hLf
aTf
hTf
DEF
D
-
Lf
aTf
DEF
E
-
Lf
DHR-123
DHR-123/phaco
TH/phaco

Figure 3

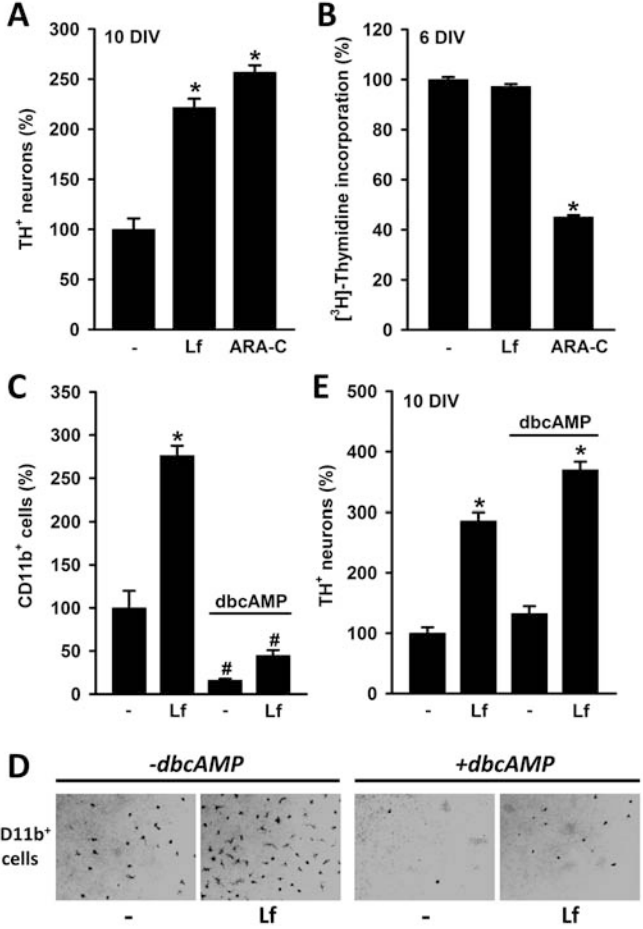
A
10 DIV
TH+ neurons (%)
300
250
200
150
100
50
0
*
*
-
Lf
ARA-C
B
6 DIV
[3H]-Thymidine incorporation (%)
120
100
80
60
40
20
0
*
-
Lf
ARA-C
C
CD11b+ cells (%)
350
300
250
200
150
100
50
0
*
dbcAMP
#
#
-
Lf
-
Lf
E
10 DIV
TH+ neurons (%)
500
400
300
200
100
0
dbcAMP
*
*
-
Lf
-
Lf
D
-dbcAMP
+dbcAMP
CD11b+ cells
-
Lf
-
Lf

Figure 4

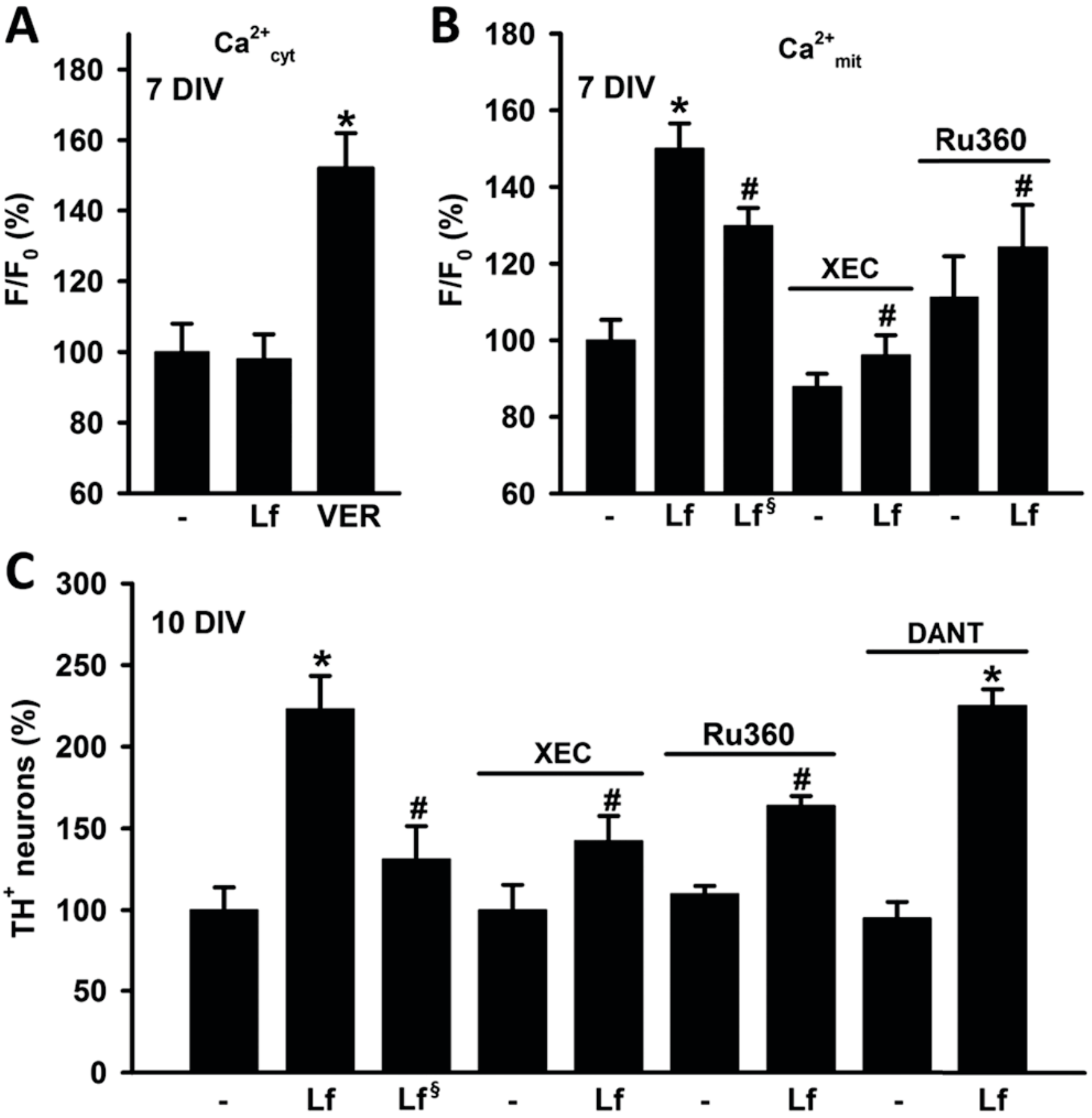
A
Ca^{2+}_{cyt}
7 DIV
F/F0 (%)
180
160
140
120
100
80
60
*
-
Lf
VER
B
Ca^{2+}_{mit}
7 DIV
F/F0 (%)
180
160
140
120
100
80
60
*
#
Ru360
XEC
-
Lf
Lf§
C
10 DIV
TH+ neurons (%)
300
250
200
150
100
50
0
DANT
XEC
Ru360

Figure 5

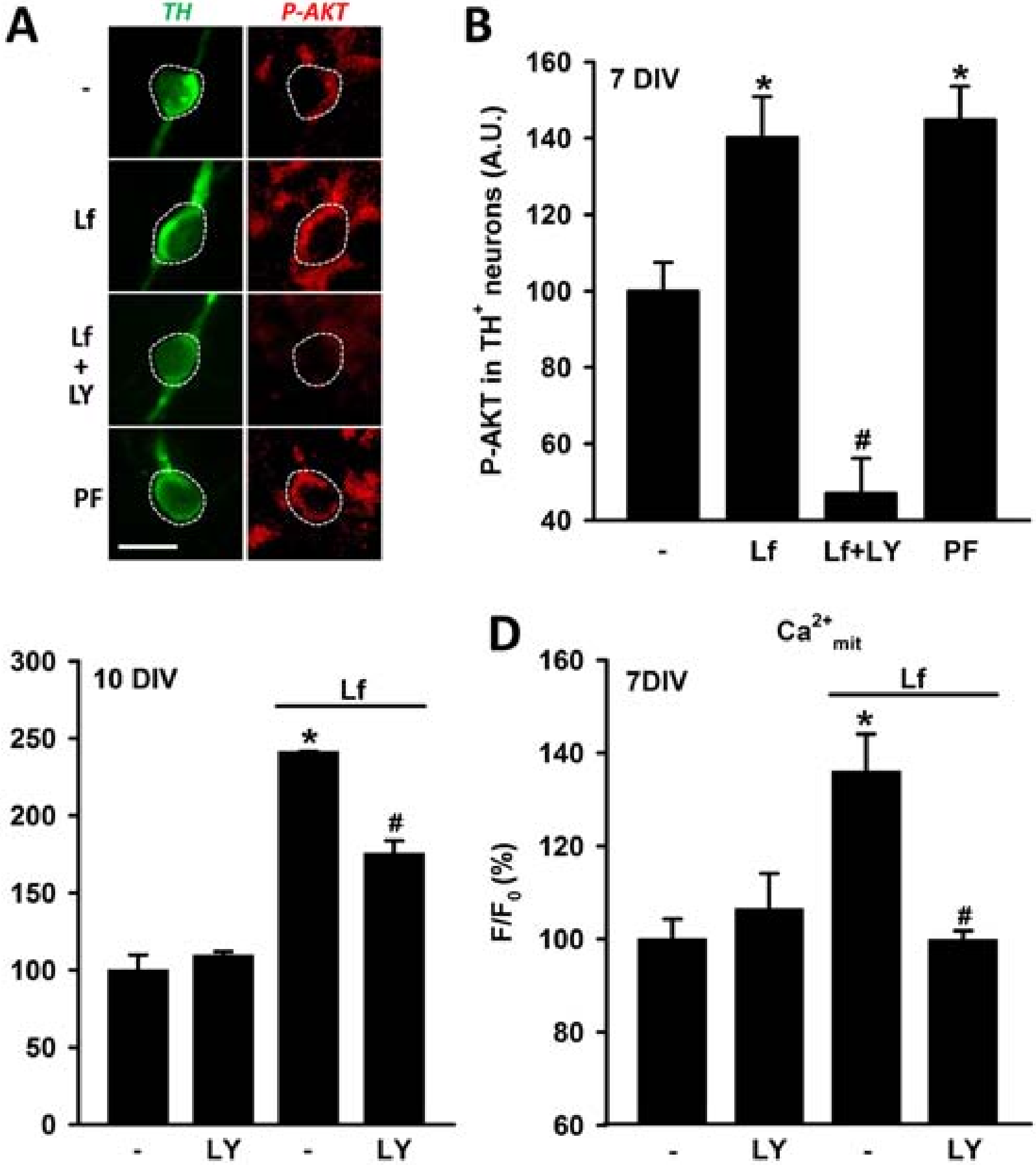
A
TH
P-AKT
-
Lf
Lf + LY
PF
B
7 DIV
P-AKT in TH+ neurons (A.U.)
160
140
120
100
80
60
40
*
*
#
-
Lf
Lf+LY
PF
C
10 DIV
Lf
TH+ neurons (%)
300
250
200
150
100
50
0
*
#
-
LY
-
LY
D
Ca2+mit
7DIV
Lf
F/F0 (%)
160
140
120
100
80
60
*
#
-
LY
-
LY

Figure 6

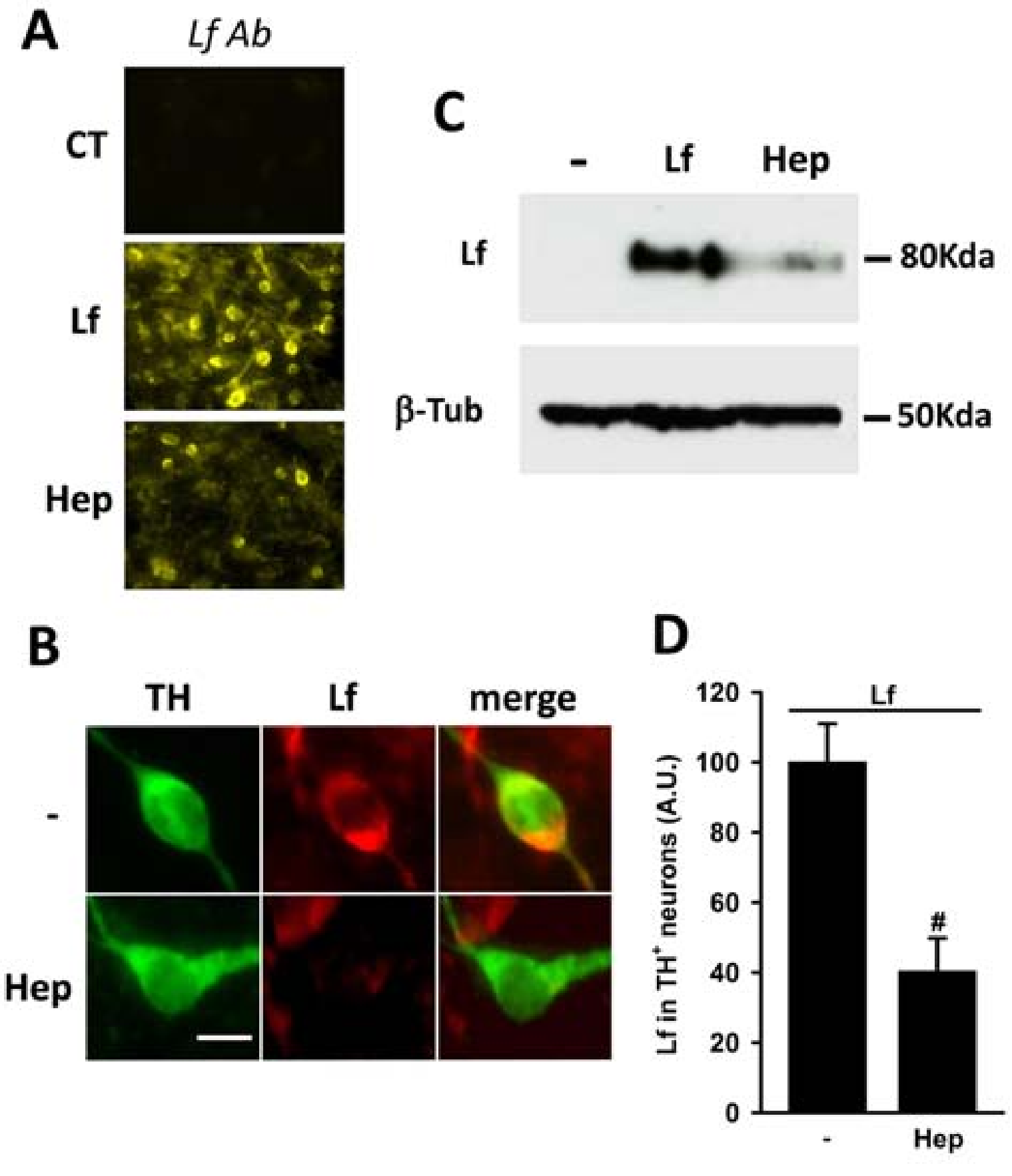
A
Lf Ab
CT
Lf
Hep
C
-
Lf
Hep
Lf
80Kda
β-Tub
50Kda
B
TH
Lf
merge
-
Hep
D
Lf
120
100
80
60
40
20
0
Lf in TH+ neurons (A.U.)
#
-
Hep

Figure 7

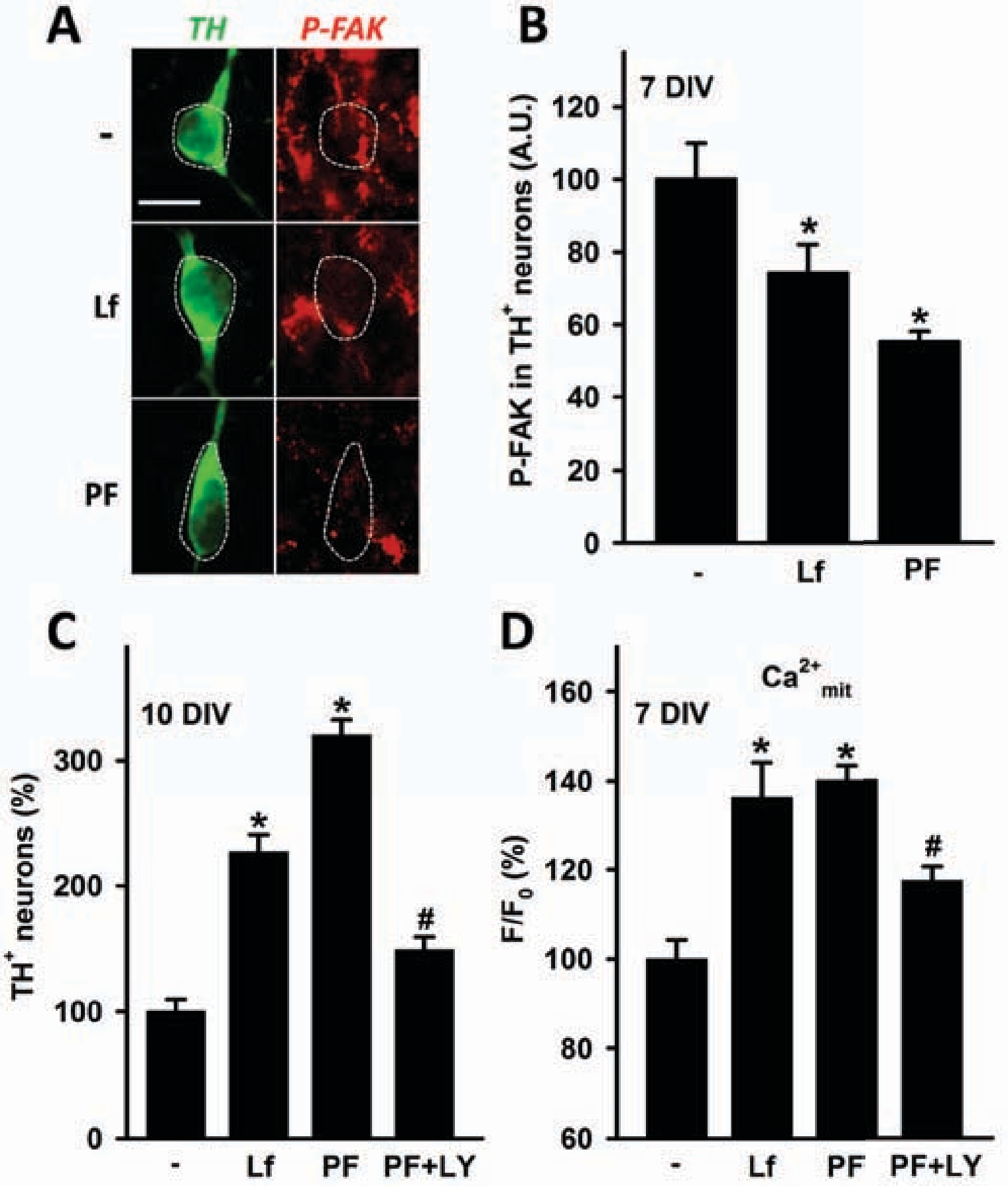
A
TH
P-FAK
-
Lf
PF
B
7 DIV
P-FAK in TH+ neurons (A.U.)
120
100
80
60
40
20
0
*
*
-
Lf
PF
C
10 DIV
TH+ neurons (%)
300
200
100
0
*
*
#
-
Lf
PF
PF+LY
D
7 DIV
Ca2+ mit
F/F0 (%)
160
140
120
100
80
60
*
*
#
-
Lf
PF
PF+LY

Figure 8

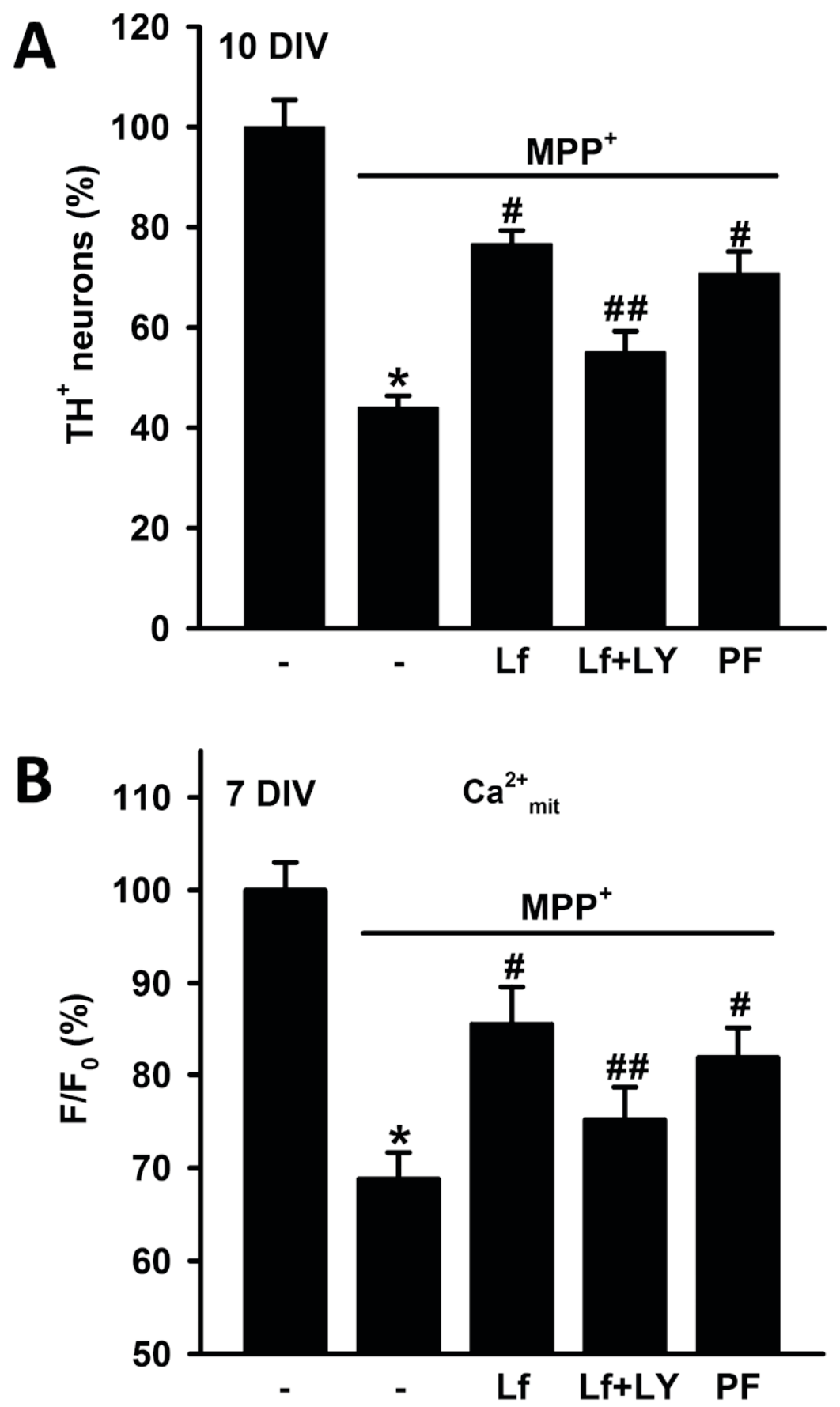
A
10 DIV
MPP+
TH+ neurons (%)
120
100
80
60
40
20
0
*
#
##
#
-
-
Lf
Lf+LY
PF
B
7 DIV
Ca2+ mit
MPP+
F/F0 (%)
110
100
90
80
70
60
50
*
#
##
#
-
-
Lf
Lf+LY
PF

Figure 9

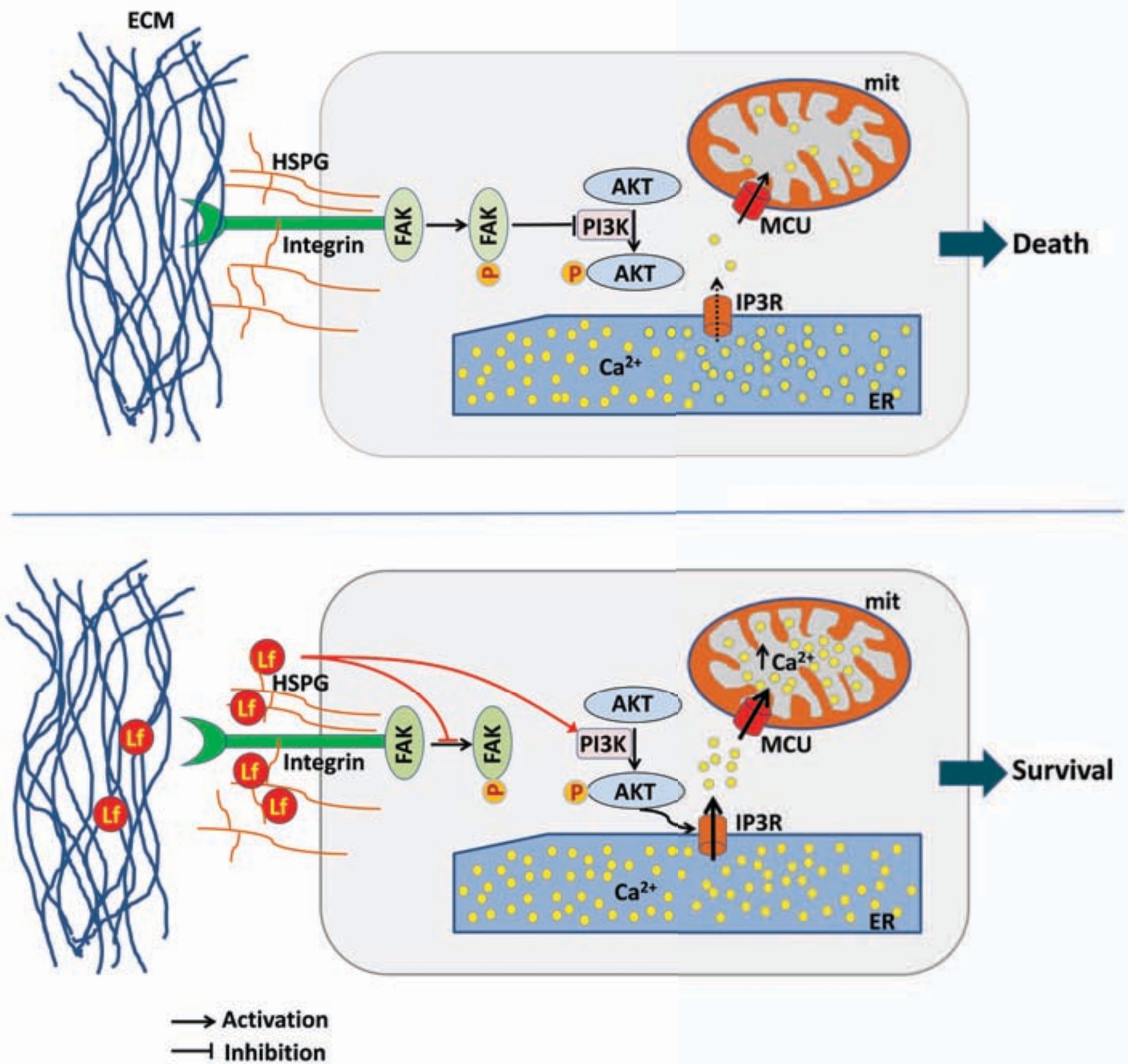
ECM
HSPG
Integrin
FAK
FAK
P
AKT
PI3K
P
AKT
mit
MCU
IP3R
Ca2+
ER
Death
Lf
HSPG
Lf
Lf
Lf
Lf
Integrin
FAK
FAK
P
AKT
PI3K
P
AKT
mit
↑Ca2+
MCU
IP3R
Ca2+
ER
Survival
Activation
Inhibition

Figure 10