Urocortin modulates dopaminergic neuronal survival via inhibition of glycogen synthase kinase-3β and histone deacetylase

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Abstract

Urocortin (UCN) is a member of the corticotropin-releasing hormone (CRH) family of neuropeptides that regulates stress responses. Although UCN is principally expressed in dopaminergic neurons in rat substantia nigra (SN), the function of UCN in modulating dopaminergic neuronal survival remains unclear. Using primary mesencephalic cultures, we demonstrated that dopaminergic neurons underwent spontaneous cell death when their age increased in culture. Treatment of mesencephalic cultures with UCN markedly prolonged the survival of dopaminergic neurons, whereas neutralization of UCN with anti-UCN antibody accelerated dopaminergic neurons degeneration. UCN increased intracellular cAMP levels followed by phosphorylating glycogen synthase kinase-3β (GSK-3β) on Ser9. Moreover, UCN directly inhibited the histone deacetylase (HDAC) activity and induced a robust increase in histone H3 acetylation levels. Using pharmacological approaches, we further demonstrated that inhibition of GSK-3β and HDAC contributes to UCN-mediated neuroprotection. These results suggest that dopaminergic neurons-derived UCN might be involved in an autocrine protective signaling mechanism.

Keywords: Urocortin; Corticotropin-releasing hormone; Neuropeptide; Dopaminergic neurons; Substantia nigra; cAMP; GSK-3β; HDAC

1. Introduction

Parkinson’s disease (PD), a common age-related degenerative disease, is associated with loss of dopaminergic neurons in the substantia nigra (SN) pars compacta that play a critical role in voluntary movement (Dauer and Przedborski, 2003). Although the exact etiology of PD remains elusive, several risk factors have been identified, including genetic predisposition, neuroinflammation, exposing to environmental toxins and aging (Le Couteur et al., 2002). The identification of signals or factors that control the survival and function of dopaminergic neurons is therefore of interest, because it might not only provide a better understanding of the pathophysiological mechanisms of this disease but also foster the development of new therapeutic strategies to halt their progression (Dawson and Dawson, 2002).

Neuropeptides can act as neurotransmitters/neuromodulators in the brain and exhibit intrinsic neuroprotective properties. Disruption of the signaling pathways of various neuropeptides, including corticotropin-releasing hormone (CRH), has been shown to be closely associated with the progression of Alzheimer’s disease (AD) (Bayatti and Behl, 2005; Ishunina and Swaab, 2002). Several lines of evidence have indicated that SN neuropeptides, such as neurokinins, might be important molecules that are associated with func-

tions and survival of dopaminergic neurons (Chen et al., 2004a,b; Fernandez et al., 1996). Urocortin (UCN) is a member of the CRH family of peptides that was first identified in rat midbrain (Vaughan et al., 1995). In the brain, UCN participates in the regulation of anxiety, learning, memory, and body temperature (Pan and Kastin, 2008). UCN has been shown to be distributed in a variety of rat brain regions (Pan and Kastin, 2008). UCN mediates its effects through at least two high-affinity membrane receptors, CRHR1 and CRHR2, which are expressed in neurons and glial cells in the brain (Pedersen et al., 2002; Perrin and Vale, 1999; Stevens et al., 2003; Van Pett et al., 2000). Both CRHRs are coupled to multiple G-proteins which activate intracellular signaling pathways (Grammatopoulos et al., 2001; Perrin and Vale, 1999). UCN has been shown to possess a direct anti-inflammatory effect in rat Kupffer cells and microglia (Agnello et al., 1998; Wang et al., 2007). Accumulating studies showed that UCN appears to protect cultured hippocampal neurons, cortical neurons and cerebellar granule cells against neurotoxins-induced cell death (Bayatti et al., 2003; Facci et al., 2003; Pedersen et al., 2002).

Our previous study demonstrated that dopaminergic neurons are the primary source of UCN in the SN (Wang et al., 2007). Moreover, CRHR1 was previously found to be expressed in dopaminergic neurons (Sauvage and Steckler, 2001). Whether UCN modulates dopaminergic neuronal survival is, as yet, unclear. In this study, we used a model of mesencephalic cultures in which dopaminergic neurons degenerate spontaneously and progressively as a function of time (Chen et al., 2006). The goal of the present study was to investigate the role of UCN in regulating dopaminergic neuronal survival and to delineate the precise molecular pathways underlying this effect.

2. Materials and methods

2.1. Materials

Rat urocortin, mouse urocortin II and human urocortin III were obtained from Sigma (St. Louis, MO). Cell culture ingredients were purchased from Invitrogen (Grand Island, NY). [3H]Dopamine (DA) (60 Ci/mmol) was bought from PerkinElmer Life Sciences (Boston, MA). Polyclonal mouse anti-tyrosine hydroxylase (TH) antibody was from Sigma. Polyclonal goat anti-UCN antibody and polyclonal goat anti-CRHR1 antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal rabbit anti-CRHR2 antibody was from Novus Biologicals (Littleton, CO). Anti-actetyl histone H3 was purchased from Upstate (Lake Placid, NY). All other antibodies were from Cell Signaling (Beverly, MA). The biotinylated secondary antibodies and Vectastain avidin–biotin–peroxidase (ABC) kit were from Vector Laboratories (Burlingame, CA). Carbamylatelet activating factor (cPAF) was obtained from Biorex (Plymouth Landing, PA). L803-mts, TDZD-8, H89, 2′,5′-dideoxyadenosine (dd-Ado) and N-benzoyloxyccarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD-fmk) were obtained from Calbiochem (San Diego, CA). All other reagents were purchased from Sigma.

2.2. Mesencephalic cultures

Primary rat ventral mesencephalic cultures were prepared following our published protocol (Wang et al., 2007). Briefly, ventral mesencephalic tissues were dissected from embryonic day 14/15 Sprague–Dawley rats and dissociated enzymatically (0.1% trypsin) and mechanically. Cells were seeded to 24-well (3 × 10⁵/well) culture plates precoated with poly-d-lysine (20 µg/ml) and maintained in 0.5 ml/well of minimum essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 10% heat-inactivated horse serum (HS), 1 g/l glucose, 2 mM l-glutamine, 1 mM sodium pyruvate, 100 µM nonessential amino acids, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. Cultures were replenished with 0.5 ml/well fresh medium 3 days later and were used for treatment 6 days later. For survival-promoting effects assay, mesencephalic cultures were maintained in 1 ml/well of MEM containing 2% FBS, 2% HS, 2 mM l-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were treated with various concentrations of UCN for the indicated times. Degeneration of dopaminergic neurons was assessed by measuring the ability of cultures to take up [3H]DA, and counting the number of TH-positive cells following immunostaining in mesencephalic cultures.

For mesencephalic neuron-enriched cultures, dissociated rat ventral mesencephalic cells were resuspended in neurobasal medium containing 0.5 mM glutamine, 25 µM glutamate and B27 supplement and seeded to 35-mm culture dishes (1.5 × 10⁵/cm²). Four days later, the medium was changed to fresh neurobasal/B27 medium without glutamate. The cells were incubated for another 2 days prior to the experiments. In the neuron-enriched cultures up to ~95% of the total populations were neurons. Dopaminergic neurons routinely comprised 12–16% of the total population of cells.

2.3. Immunocytochemistry

Dopaminergic neurons were detected with anti-TH antibody. Briefly, cells were fixed with 3.7% paraformaldehyde followed by blocking with PBS containing 0.4% Triton X-100, 2% bovine serum albumin (BSA) and 3% normal goat serum. After blocking, cells were incubated with primary antibody at 4°C for overnight. The bound primary antibody was visualized by incubation with an appropriate biotinylated secondary antibody followed by the Vectastain ABC reagents and color development with 3,3′-diaminobenzidine. The numbers of TH-positive neurons were counted in the entire surface area of a culture well. For fluorescent double-labeling experiments, primary antibodies were incubated...
overnight at 4 °C. Cells were then washed with PBS and incubated for 1 h with the secondary antibodies (anti-rabbit-FITC, anti-goat-FITC and anti-mouse-Rhodamine, Jackson ImmunoResearch, West Grove, PA) at room temperature. After rinsing with PBS buffer, cells were examined under an Axiosvert 200 M fluorescent microscope or a confocal microscope (Zeiss, Zena, Germany). Activated caspase-3-positive neurons among TH-positive neurons were scored by examining 8–9 semi-random fields across the glass coverslips. Approximately 200–350 TH-positive neurons were counted in the controls or treated groups.

2.4. Uptake assays for [3H]-dopamine

Cells were washed twice with warm Krebs–Ringer buffer (16 mM sodium phosphate, 119 mM NaCl, 4.7 mM KCl, 1.8 mM CaCl2, 1.2 mM MgSO4, 1.3 mM EDTA and 5.6 mM glucose, pH 7.4), and then incubated with 1 μM [3H]-dopamine (DA) in Krebs–Ringer buffer at 37 °C for 20 min. After washing with ice-cold Krebs–Ringer buffer, cells were collected in 1N NaOH and radioactivity was counted with a liquid scintillation counter. Nonspecific uptake was determined in parallel wells that received both the tritiated tracer and 10 μM mazindol. The specific [3H] DA was calculated by subtracting the amount of radioactivity obtained in the presence of mazindol from that obtained in the absence of mazindol.

2.5. Measurement of intracellular cAMP

Cells were preincubated for 90 min in serum-free MEM. After this time, the medium was replaced with serum-free MEM containing 1 mM 3-isobutyl-1-methyxanthine (IBMX, Sigma) to inhibit cAMP phosphodiesterase activity. After 60 min, medium was replaced with fresh MEM containing 2% FBS, 1 mM IBMX and various concentrations of UCN, cells were then incubated for 15 min at 37 °C. Intracellular cAMP was measured using the Amersham cAMP EIA system (Piscatway, NJ) according to the manufacturer’s instructions.

2.6. Preparation of cell extracts

Cells cultured in 35-mm dishes were washed twice with ice-cold PBS and lysed in 150 μl of lysis buffer (50 mM Hepes, pH 7.5, 100 mM NaCl, 2 mM EDTA, 10% glycerol, 1% Triton X-100, 3 mM benzamidine, 1 mM sodium pyrophosphate, 10 mM sodium orthovanadate, 100 mM NaF, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 5 μg/ml pepstatin A). After incubation on ice for 30 min, cell lysates were centrifuged and the supernatants were collected. Protein concentration of samples was determined by Bradford assay (Bio-Rad, Hercules, CA), and samples were equilibrated to 2 mg/ml with lysis buffer.

2.7. Histone deacetylase (HDAC) assay

HDAC activity was measured using the HDAC Colorimetric Detection kit (Upstate), according to the protocol of the manufacturer. Briefly, 60 μg or 40 μg of protein from mesencephalic neuron lysates or HeLa nuclear extracts (Upstate), respectively, was incubated with HDAC assay substrate for 60 min at 37 °C, allowing deacetylation of the substrate. The HDAC inhibitor trichostatin A (TSA), sodium butyrate (SB) or UCN were added 15 min prior to addition of substrate. After incubation, the activator solution was added to stop the reaction and release p-nitroanilide from the deacetylated substrate. The absorbance at 405 nm was determined using a microplate reader.

2.8. Western blotting

Protein samples containing 50–100 μg of protein were separated on 10% sodium dodecyl sulphate–polyacrylamide gels and transferred to immobilon polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were incubated in TBST buffer (0.1 M Tris/HCl, pH 7.4, 0.9% NaCl, 0.1% Tween 20) supplemented with 5% dry skim milk for 1 h to block nonspecific binding. After rinsing with TBST buffer, they were incubated with primary antibodies. The membranes were washed twice with TBST buffer followed by incubation with appropriate streptavidin–horseradish peroxidase-conjugated secondary antibodies. The antigen–antibody complexes were detected by using a chemiluminescence detection system (ECL, Amersham, Berkshire, England). The intensity of the band was quantified with a densitometric analysis (GS-800 Calibrated Densitometer, Bio-Rad), and calculated as the optical density × area of band.

2.9. Statistical analysis

All data are expressed as the mean± S.E.M. Data were analyzed by one-way ANOVA followed by Scheffe’s test. A p value less than 0.05 was considered statistically significant.

3. Results

3.1. Expression of UCN and CRHRs in dopaminergic neurons from mesencephalic neuron-enriched cultures

UCN has been shown to be localized principally to dopaminergic neurons in rat SN (Wang et al., 2007). To test whether UCN and CRHRs are also expressed in cultured dopaminergic neurons, we evaluated the expression of these proteins in dopaminergic neurons by double-immunocytochemistry. The results showed that the immunoreactivity for both UCN and CRHRs was localized in dopaminergic neurons from the mesencephalic neuron-enriched cultures (Fig. 1). Simultaneously, both CRHR1 and CRHR2 were also expressed in other neuronal cells.

3.2. Survival-promoting effect of UCN against spontaneous dopaminergic neuronal death

As mentioned above, both CRH receptors were expressed in cultured dopaminergic neurons. To determine the role of UCN in modulating dopaminergic neuronal survival, we used a model of mesencephalic cultures in which dopaminergic neurons degenerate spontaneously and progressively as a function of time. Dopaminergic neurons underwent spontaneous cell death as their age increased in culture. Exposure of mesencephalic cultures to UCN starting from day 6 in vitro markedly suppressed spontaneous cell death assessed by measuring the TH-positive cells counted within a time frame of 16 days after plating (Fig. 2A). The spontaneous, age-induced dopaminergic neuronal death was robustly suppressed by UCN in a dose-dependent manner (Fig. 2B and C). Moreover, UCN appeared to have subtle effects of increasing the length and number of neurites of dopaminergic neurons after 10 days of treatment (Fig. 2D and E). These morphological observations were confirmed by measurement of the number of neurites per TH+ cell from randomly selected TH+ cells (450–500) in each treatment group. The majority of TH+ cells (75%) treated with UCN had 2–5 neurites, whereas 70% of TH+ cells in control cultures had only 1–2 neurites. Similar to the survival-promoting effect of UCN, application of UCN II or UCN III to mesencephalic cultures significantly ameliorated the degeneration of dopaminergic neurons (Fig. 2F).

Additionally, to investigate whether endogenous UCN plays an active role in this process, neutralizing anti-UCN
antibody was added to mesencephalic cultures at 6 DIV. We found that anti-UCN antibody significantly accelerated the loss of TH-positive neurons and reduced DA uptake (Fig. 2G). To further verify that anti-UCN antibody-induced neuronal death is resulted from blocking UCN produced by the neurons but not the peptide presented in the culture medium, the death-promoting effects of anti-UCN antibody on dopaminergic neurons cultured under serum-free conditions was determined. The results showed that the number of TH-positive neurons and the DA uptake was reduced to ∼64.9 ± 2.6% and 58.3 ± 5.9%, respectively, following exposure of cultures to anti-UCN antibody at concentrations as low as 1 μg/ml. These data indicate that endogenous UCN is indeed required to maintain the survival of dopaminergic neurons.

To explore whether astroglia are involved in the UCN-induced survival-promoting effects in this in vitro system, mesencephalic neuron-enriched cultures were treated with 1 μM UCN and TH-positive neurons were counted 10 days after UCN treatment. The results showed that no significant difference in the survival of dopaminergic neurons in mesencephalic neuron-enriched cultures and mesencephalic cultures (data not shown), suggesting that a direct neuronal effect of UCN play a major role in UCN-induced neuroprotective effects. Therefore, the mesencephalic neuron-enriched cultures were used to investigate the underlying mechanisms that mediated the neuroprotective action of UCN.

3.3. UCN promotes survival through a cAMP-dependent pathway

All of the known effects of UCN involve receptor-coupled activation of adenylate cyclase and an increase in cellular levels of cAMP (Dautzenberg et al., 2001). Thus we performed experiments to provide evidence for involvement of the cAMP pathway in the protective effect of UCN. Treatment of mesencephalic neuron-enriched cultures with UCN caused an increase in cellular cAMP levels (Fig. 3A). The effect of UCN on cAMP levels was concentration-dependent. Next, to determine whether cAMP activation mimics UCN action, we incubated mesencephalic cultures with dibutylryl cAMP (dbcAMP), a stable, cell-permeable cAMP analogue, for 8 days. Fig 3B shows dbcAMP protected dopaminergic neurons from spontaneous cell death in a dose-dependent manner.

3.4. GSK-3β inhibition mediates UCN action

It has been recognized that GSK-3β is an important modulator of apoptosis (Grimes and Jope, 2001; Kaytor and Orr, 2002). A previous study indicated that CRH may inactivate GSK-3β by inducing phosphorylation at Ser9 (Bayatti et al., 2003). Thus, we sought to determine whether UCN could regulate GSK-3β phosphorylation in mesencephalic neurons-enriched cultures. The activity of GSK-3β is regulated negatively by the phosphorylation of Ser9. As shown in Fig. 4A, UCN significantly increased levels of phosphorylated GSK-3β within minutes of UCN addition and GSK-3β phosphorylation was sustained and lasted for at least 24 h with a single application of UCN. Similarly, UCN-mediated phosphorylation of GSK-3β was also observed at the cellular level that phospho-GSK-3β-immunoreactivity increased in dopaminergic neurons and other neuronal cells (Fig. 4B). Thus, we sought to determine whether UCN-induced neuronal survival was mediated by inactivation of GSK-3β, we blocked GSK-3β activity by incubating mesencephalic cultures with L803-mts or TDZD-8. Both inhibitors have been shown to specifically inhibit GSK-3β activity with little effect on other kinases. After treatment with GSK-3β inhibitors for 8 days, the viability of dopaminergic neurons was assessed by DA uptake. Fig. 4C shows both GSK-3β inhibitors markedly protected dopaminergic neurons from spontaneous neuronal death, although the protective effect of L803-mts was more potent than TDZD-8. Our data are consistent with a previous report showing that TDZD-8 is not an effective inhibitor of GSK-3β activity in cerebellar granule neurons (Chin et al., 2005).

To further confirm that increased GSK-3β inactivation results in dopaminergic neuronal survival following exposure to UCN, we examined whether platelet activating factor (PAF), an activator of GSK-3β (Maggirwar et al., 1999; Tong et al., 2001), could reverse the effects of UCN. Initially, we confirmed that cPAF, a nonhydrolyzable PAF analogue, increased GSK-3β activity in mesencephalic neuron-enriched cultures by assaying phosphorylation of the GSK-3β target β-catenin. Inhibition of GSK-3β activity results in dephosphorylation of its substrate β-catenin and hence accumulation of this transcription factor. Neurons exposure to UCN resulted in a decrease in β-catenin phosphorylation and an increase in the levels of total β-catenin. These changes were reversed by cPAF (Fig. 5A). Furthermore, restoration of UCN-induced GSK-3β inactivation by cPAF increased the degeneration of dopaminergic neurons (Fig. 5B). To determine whether cPAF itself might be toxic to dopaminergic neurons, mesencephalic cultures were treated with 1 μM cPAF alone or coinubcation with 5 μM L803-mts for 8 days. The DA uptake for cultures treated with cPAF or cPAF plus L803-mts was 60.2 ± 5.3% and 91 ± 5.8% of control, respectively. These results indicate that the ability of cPAF to reduce the protective effect of UCN is through activation of GSK-3β but not reflects its intrinsic toxicity.

3.5. cAMP mediates UCN-induced GSK-3β phosphorylation

In this study, we demonstrated that elevation of intracellular cAMP levels in UCN-treated mesencephalic cultures lead to survival of dopaminergic neurons. In cerebellar granule neurons, cAMP has been shown to inhibit GSK-3β activity by phosphorylation of GSK-3β at Ser9. Transfection of neurons with a GSK-3β mutant that cannot be phosphorylated interferes with the prosurvival effects of cAMP (Li et al., 2000). To investigate the potential role of GSK-3β in mediating the
Fig. 2. UCN induces survival-promoting effect against spontaneous dopaminergic neuronal death. (A) Mesencephalic cultures were treated with 1 μM UCN from day 6 in vitro and viable dopaminergic neurons immunostained with TH were counted at various times thereafter. Data are the mean ± S.E.M. of the present of viable TH-positive cell number on day 6 in vitro in the control culture and are derived from three independent experiments. **p < 0.01 compared with respective control. (B and C) Dose-dependent neuroprotection by UCN. Mesencephalic cultures were exposed to the indicated concentrations of UCN for 8 days. Degeneration of dopaminergic neurons was evaluated by counting the number of TH-positive neurons and by measuring the [3H]DA uptake. Values are represented as the mean ± S.E.M. of the percent of control from three independent experiments. *p < 0.05; **p < 0.01 compared with control. (D and E) Morphological features of mesencephalic cultures were examined after incubation with 1 μM UCN for 10 days starting after day 6 in vitro and then immunostained with TH antibody. The number of neurites per TH+ cell was counted under 40× magnification. Scale bar = 50 μm. Data are the mean ± S.E.M. of two independent experiments. *p < 0.01 compared with respective control. (F) Other members of CRH family possess neuroprotective activities.

Fig. 3. UCN-mediated survival effect is through intracellular cAMP elevation. (A) Mesencephalic neuron-enriched cultures were exposed to MEM with 1 mM 3-isobutyl-1-methyloxanthine (IBMX) for 1 h and then were exposed for 15 min to MEM containing 2% FBS and various concentrations of UCN in the continued presence of IBMX. Intracellular cAMP levels were measured as described in Section 2. (B) Mesencephalic cultures were treated with various concentrations of dibutyryl cAMP (dbcAMP) for 8 days, and then DA uptake was assessed. Values are represented as the mean ± S.E.M. of three independent experiments. *p < 0.05; **p < 0.01 compared with control.

protective effects of cAMP evoked by UCN, we first examined the phosphorylation state of GSK-3β in mesencephalic neuron-enriched cultures stimulated with agents that elevate cAMP levels. After 6 days in culture, mesencephalic neuron-enriched cultures were incubated with forskolin, an activator of adenylate cyclase, to elevate intracellular cAMP levels. After 6 days in culture, mesencephalic neuron-enriched cultures were incubated with forskolin, an activator of adenylate cyclase, to elevate intracellular cAMP levels. Following these treatments, the phosphorylation state of GSK-3β was determined by Western blot analysis. As shown in Fig. 6A, forskolin increased the phosphorylation of GSK-3β on Ser9 determined by Western blot analysis. Moreover, the ability of forskolin to stimulate phosphorylation of GSK-3β was mimicked by incubating the cultures with dbcAMP. Activation of adenylate cyclase leads to the increase in cellular levels of cAMP, which in turn induces the activation of PKA. Therefore, we examined the effects of compounds that inhibit the adenylate cyclase or PKA on the ability of UCN to elicit GSK-3β phosphorylation. Fig. 6B shows the phosphorylation of GSK-3β by UCN or forskolin was markedly blocked when mesencephalic neuron-enriched cultures were preincubated with the adenylate cyclase inhibitor 2′,5′-dideoxycytidine (dd-Ado) or a PKA selective inhibitor H89. At the same time, dd-Ado or H89 had no appreciable effects on neuronal survival, whereas UCN-mediated survival effect was significantly blocked by both inhibitors (Fig. 6C). These data taken together suggest that GSK-3β inactivation-mediated neuroprotection induced by UCN is mediated through production of cAMP.

3.6. Histone deacetylases (HDACs) inhibition by UCN

Recent in vivo studies have shown that treatment with HDAC inhibitors of a wide range of structures provide neuroprotective effects in models of various motor neuron disorders (Ferrante et al., 2003; Minamiyama et al., 2004). Moreover, valproate (VPA), a HDAC inhibitor of short-chain fatty acid structure, has been shown to protect cerebral cortical neurons and dopaminergic neurons from spontaneous cell death (Chen et al., 2006; Jeong et al., 2003). Thus, we tested whether UCN can lead to HDAC inhibition by analyzing the degree of histone acetylation with an antibody specific for hyperacetylated histone H3 (Fig. 7A). Only minute amounts of acetylated histones were detected in untreated mesencephalic neuron-enriched cultures. UCN markedly increased the amount of acetylated histone H3 (acetyl-H3) after treatment for 1–8 days. To further verify that UCN was acting as HDAC inhibitor in dopaminergic neurons, we stained control and treated cultures with an antibody specific for acetylated histone H3. Increased levels of acetylated histone H3 in the nucleus was shown in both non- or dopaminergic neurons (Fig. 7B). UCN-induced the increase in the acetylation of histone H3 may be due to the inhibition of HDAC or the increase in the activity of histone acetyltransferases. To test whether UCN can lead to HDAC inhibition, the HeLa nuclear extracts or mesencephalic neuron lysates were used as a source of HDAC enzymatic activity. UCN inhibited HDAC activity in mesencephalic neurons as determined by immunoblot analyses in which nuclear extracts derived from HeLa cells, as well as, whole-cell extracts of mesencephalic neurons was used (Fig. 7C).

3.7. UCN-mediated neuroprotection involves UCN-dependent inhibition of HDACs

To study whether HDACs inhibition plays a critical role in the neuroprotective effect of UCN, mesencephalic neuron-enriched cultures were treated with sodium butyrate (SB), a chemical inhibitor of HDACs, for 1–8 days. Immunolabeling of acetylated histone H3 confirmed the efficacy of treatment for 1–8 days. Data are the mean ± S.E.M. of three independent experiments. **p < 0.01 compared with control.
Fig. 4. GSK-3β inactivation mediates UCN-induced increase in the survival of dopaminergic neurons. (A) Mesencephalic neuron-enriched cultures were treated with 1 μM UCN for the indicated times. Whole cell lysates were prepared and subjected to Western blotting using Abs specific for phosphorylated (Ser9) or total form of GSK-3β. The immunoblots are representative of three independent experiments. (B) Mesencephalic neuron-enriched cultures were treated with 1 μM UCN for 30 min. Cultures were double immunolabeled with a polyclonal mouse anti-TH and a polyclonal rabbit anti-phospho-GSK-3β as described in Section 2. Scale bar = 50 μm. (C) Mesencephalic cultures were treated with various concentrations of GSK-3β inhibitors L803-mts or TDZD for 8 days. The viability of dopaminergic neurons was assessed by DA uptake assays. Values are represented as the mean ± S.E.M. of three independent experiments. *p < 0.05; **p < 0.01 compared with control.

SB under our experimental conditions (Fig. 8A). Densitometry analysis revealed a significant increase in acetyl-H3/actin ratios induced by SB. Furthermore, SB treatment was showed to reduce the loss of TH-positive neurons and attenuate the decrease of [H3]DA uptake (Fig. 8B).

To further confirm that the effects of UCN were mediated through HDAC inhibition we investigated the effect of the HDAC stimulator theophylline (Cosio et al., 2004; Ito et al., 2002) on repression of UCN-elicited histone H3 acetylation and cell survival. As shown in Fig. 8C, theophylline treatment alone decreased the basal levels of acetylated histone H3. The increase in histone H3 acetylation caused by UCN treatment was blocked by theophylline. Furthermore, UCN-induced increase in DA uptake was significantly reduced after mesencephalic cultures were pretreated with theophylline (Fig. 8D). These data taken together suggest that the increase in dopaminergic neurons survival by UCN is associated with a decrease in HDAC activity.

3.8. UCN reduces caspase-3 activation by inhibiting GSK-3β and HDAC

As apoptotic cell death is almost certainly one of the central components in selective nigrostriatal neuronal death (Schapira, 2001). A double-immunofluorescent localization of activated caspase-3 and TH was performed in
cPAF, a GSK-3β activator, blocks the UCN-induced GSK-3β inactivation and neuroprotection. Mesencephalic neuron-enriched cultures or mesencephalic cultures were treated with 0.5 μM UCN in the presence or absence of cPAF for 60 min (A) or 8 days (B), respectively. Whole cell lysates were prepared and subjected to Western blotting using antibodies specific for phosphorylated (Ser33/37/Thr41) or total form of β catenin. The immunoblots are representative of three independent experiments (A). The viability of dopaminergic neurons was assessed by DA uptake assays (B). Values are represented as the mean ± S.E.M. of three independent experiments. *p < 0.05 or **p < 0.01 compared with control or UCN-treated cultures, respectively.

4. Discussion

In the present study, we have demonstrated the downstream neuroprotective signaling pathways initiated by UCN in an in vitro model in which mesencephalic dopaminergic neurons undergo spontaneous degeneration. UCN could rescue dopaminergic neurons from spontaneous cell death and blockade of UCN actions by treatment with UCN antibody accelerates these neurons degeneration. The investigation of the intracellular mechanisms demonstrated that inhibition of both GSK-3β and HDAC contributes to UCN-induced neuroprotection.

UCN has been shown to be distributed in a variety of rat brain regions including the supraoptic nucleus, the median eminence, Edinger–Westphal nucleus and the sphenoid nucleus (Pan and Kastin, 2008). Our previous study demonstrated that UCN was principally expressed in dopaminergic neurons in the SN (Wang et al., 2007). UCN’s actions in the brain appear to be specifically mediated through the CRH receptors, which are also found to be localized to dopaminergic neurons (Sauvage and Steckler, 2001). In the present study, we further demonstrated that both UCN and its receptors were expressed in dopaminergic neurons in the mesencephalic neuron-enriched cultures. These findings indicate that dopaminergic neurons may be under the physiological regulation of UCN. We used rat primary mesencephalic cultures in which cultured dopaminergic neurons undergoing spontaneous cell death to investigate the survival-promoting effects of UCN. Just as expected, our data demonstrated that UCN markedly prolonged the survival of dopaminergic neurons when added to maturing cultures. These findings are in agreement with recent reports showing that exogeneous administration of UCN is capable of preventing dopaminergic neurons degeneration in the rat 6-OHDA and LPS paradigms of PD (Abuirmeileh et al., 2007a,b), although in which the molecular mechanisms responsible for UCN’s actions is unclear. Similarly, other member of CRH family UCN II or UCN III was also shown to possess neuroprotective activities. Because these two members are not yet reported to express in the SN (Lewis et al., 2001; Reyes et al., 2001), whether they could also be as intrinsic neuroprotective peptides in the SN needs further investigation. Our data presented here also showed that blocking of UCN by anti-UCN antibody increased degeneration of dopaminergic neurons. These results suggest that dopaminergic neurons-derived UCN and its receptors might be involved in an autocrine protective signaling mechanism. Moreover, consistent with the findings that CRHRs were expressed in other types of neuron, UCN-induced GSK-3β phosphorylation and histone acetylation was also increased in these neurons.
Fig. 6. cAMP mediates UCN-induced GSK-3β phosphorylation. Mesencephalic neuron-enriched cultures were exposed to forskolin (25 μM) or dbcAMP (200 μM) for the indicated times (A) or pretreated with 2′,5′-dideoxyadenosine (dd-Ado, 10 μM) or H89 (10 μM) for 30 min before the exposure to 1 μM UCN or 25 μM forskolin for another 60 min (B). Western blots were probed with antibodies specific for phosphorylated and total form of GSK-3β. The immunoblots are representative of three independent experiments. Data are the mean ± S.E.M. **p < 0.01 compared with control in (A) and UCN or forskolin-treated cultures.
Fig. 7. UCN induces accumulation of hyperacetylated histone and inhibits HDAC activity. (A) Western blotting analysis of acetylated histone H3 in cultured DIV 6 mesencephalic neuron-enriched cultures exposed to 1 μM UCN for the indicated times. The immunoblots are representative of three independent experiments. Values are represented as the mean ± S.E.M. **p < 0.01 compared with respective control. (B) Immunodetection of acetylated histone H3 in dopaminergic neurons. Mesencephalic neuron-enriched cultures were exposed to 1 μM UCN for 2 days, then fixed and double immunostained using anti-TH antibody and anti-acetylated histone H3 antibody. Scale bar = 50 μm. (C) UCN inhibits endogenous HDACs presented in HeLa cell nuclear extracts or mesencephalic neuron lysates. HDAC activity was assayed in vitro as release of p-nitroanilide from deacetylated substrate in the absence or presence of UCN (1 μM), TSA (1 μM) or SB (5 mM). Percent HDAC activity is shown with respect to the activity of HDAC alone (100%). Data are the mean ± S.E.M. of three independent experiments. **p < 0.01 compared with control.

The determination of whether UCN also exerts neuroprotective effects in other neuronal cells under our experimental conditions is, however, beyond the scope of the present study.

GSK-3α and -3β, especially GSK-3β, are critical in regulating multiple biological processes of neurons. GSK-3αβ is unusual that it can be inactivated through phosphorylation of serine 21/9. Emerging evidence suggests that GSK-3β is an important modulator of apoptosis. It appears that GSK-3β activity correlates inversely with neuronal viability (Hetman et al., 2000; Lucas et al., 2001; Song et al., 2002). GSK-3β has been implicated as contributing to neuronal cell death induced by ischemia (Bhat et al., 2000), excitotoxicity induced by glutamate receptor activation (Kelly et al., 2004), and models of Alzheimer’s disease (Bhat and Budd, 2002; Kaytor and Orr, 2002). Additionally, neuronal apoptosis induced by trophic withdrawal also appears mediated by GSK-3β activation (Hetman et al., 2000). Therefore, blocking the GSK-3β pathway protects neurons against neurotoxic insults (Xu et al., 2003; Chen et al., 2004a,b; Kelly et al., 2004; Koh et
In agreement with those studies, UCN indirectly inhibited GSK-3β activity together with the observations that UCN-mediated neuroprotection could be mimicked by L803-mts and was blocked by cPAF, suggesting that inhibition of GSK-3β is an essential mechanism responsible for UCN’s actions.

All of the known effects of UCN involve receptor-coupled activation of adenylate cyclase and an increase in cellular levels of cAMP (Dautzenberg et al., 2001). Activation of cAMP-dependent pathways, through elevation of intracellular cAMP levels is known to promote survival of a large variety of central and peripheral neuronal populations, including dopaminergic neurons (Michel and Agid, 1996). In mesencephalic neurons, UCN treatment significantly stimulated the accumulation of cAMP, and increased it in a dose-dependent manner. Exogeneous addition of cAMP analogue could attenuate dopaminergic neurons degeneration. Inactivation of GSK-3β has been shown to mediate cAMP-evoked neuroprotection from serum deprivation and lower of the KCl concentration in rat cerebellar granule neurons (Li et al., 2000). Consistent with this, blocking the cAMP/PKA pathway resulted in reduced GSK-3β phosphorylation and increased cell death, suggesting that UCN-induced GSK-3β phosphorylation-dependent neuroprotection is mediated by a raise in intracellular level of cAMP.

HDAC is an enzyme that catalyzes the removal of acetyl groups from lysine residues of histones (Strahl and Allis, 2000). A growing body of evidence supports the notion that HDAC inhibitors, including VPA, SB, and TSA, protect neurons against glutamate-induced excitotoxicity.
Fig. 9. UCN attenuates caspase-3 activation via inhibition of GSK-3β and HDAC. (A) Mesencephalic cultures were treated with 1 μM UCN for the indicated times. Cultures were double immunolabeled with a polyclonal mouse anti-TH and a polyclonal rabbit anti-activated caspase-3. Cultures then were examined with fluorescent microscopy. Neurons that were positive for activated caspase-3 among TH-positive neurons were counted as described in Section 2. Values are represented as the mean ± S.E.M. of three independent experiments. **p < 0.01 compared with respective control. (B and C) Mesencephalic cultures were treated with UCN (1 μM), L803-mts (10 μM) or SB (2.5 mM) for 8 days. Double-immunostaining of TH and activated caspase-3 were performed and the ratio of activated caspase-3+/TH+ were counted as described above. Arrows indicate neurons that are positive for both activated caspase-3 and TH in the merge of images. Scale bar = 50 μm. Data are the mean ± S.E.M. of three independent experiments. **p < 0.01 compared with control.
Apoptosis has been shown to occur in dopaminergic neurons of the human SN during normal aging or in PD (Anglade et al., 1997a,b), suggesting that cell death of dopaminergic neurons in PD may occur, at least in part, by apoptosis (Schaapira, 2001). Ishitani et al. (1996a,b) found that mature cerebellar granule cells and cerebrocortical neurons undergo an age-induced apoptotic death in long-term cultures. In this report, we present data to show that caspase-3 activation in primary cultures of dopaminergic neurons was gradually increased as mesencephalic cell cultures age. Thus, the capacity of UCN as an anti-apoptotic molecule was examined. In mesencephalic dopaminergic neurons, UCN treatment was effective in diminishing caspase-3 activation. GSK-3β has been shown to be a key intermediate in several apoptotic signaling pathways that lead to activation of caspase-3. This was shown by the findings that GSK-3β inhibitors blocked caspase-3 activation induced by ischemia, endoplasmic reticulum stress, 6-OHDA and amyloid-β-peptide (Chen et al., 2004a,b; Kim et al., 2005; Koh et al., 2008; Xu et al., 2003). Furthermore, several lines of investigation support the notion that HDAC inhibition suppresses ischemia- or excitotoxicity-induced neuronal caspase-3 activation (Kami et al., 2004; Ren et al., 2004). Our studies showed that inhibition of GSK-3β and HDAC by UCN resulted in decreased caspase-3 activation and treatment with a caspase inhibitor deterred the degeneration of dopaminergic neurons, indicating that UCN acts as a true neuroprotective factor by preventing the spontaneous demise of dopaminergic neurons.

In conclusion, on the basis of the data shown here, we propose a model for the neuroprotective action of UCN (Fig. 10). UCN-elevated cAMP activates PKA and leads to the phosphorylation and inactivation of GSK-3β. On the other hand, exogeneous UCN could enter the cells by CRHRS-mediated uptake (Tu et al., 2007a,b) followed by inhibiting HDAC activity directly. Both events subsequently reduce the activation of caspase-3, thereby promoting cell survival. Increasing incidence of PD with advancing age suggests the age-related processes predispose the nigrostriatal dopaminergic system to neurodegeneration but the cause of neuronal death is largely unknown. The identification of signals or factors that control the survival and function of dopaminergic neurons is therefore of interest. Given that our results suggest that UCN could be as a potential candidate factor which is secreted principally by dopaminergic neurons and exerts autocrine neuroprotective actions. In vivo investigations focusing on the regulation of UCN and its receptor may further elucidate the role of UCN in neuropathology of PD.

**Disclosure statement**

The authors declare no conflict of interest. All animals were in compliance with protocols approved by the Buddhist Tzu Chi General hospital Institutional Animal Care and Use Committee.

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