UNCOUPLING PROTEIN 2/3 IMMUNOREACTIVITY AND THE ASCENDING DOPAMINERGIC AND NORADRENERGIC NEURONAL SYSTEMS: RELEVANCE FOR VOLUME TRANSMISSION

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Abstract—Uncoupling proteins in the inner mitochondrial membrane uncouples oxidative phosphorylation from ATP synthesis. It has been suggested that these proteins are involved in thermogenesis as well as in the regulation of reactive oxygen species production in the mitochondria. The present work was conducted to investigate the localization of the uncoupling protein 2/3-immunoreactivity (uncoupling protein 2/3 immunoreactivity) in the main catecholaminergic projection fields in the rat brain as well as in the areas of the dopaminergic and noradrenergic nerve cell groups. In particular, the relationships of tyrosine hydroxylase, dopamine β-hydroxylase and uncoupling protein 2/3 immunoreactivity were assessed by double immunolabeling and confocal laser microscopy analysis associated with computer-assisted image analysis. Uncoupling protein 2/3 immunoreactivity was observed in discrete dopaminergic terminals in the nucleus accumbens and in the cerebral cortex whereas it was found in scattered noradrenergic terminals in the caudate putamen and Islands of Calleja Magna. One interesting finding was that uncoupling protein 2/3 immunoreactivity together with tyrosine hydroxylase immunoreactivity in the shell of the nucleus accumbens was observed surrounding the previously characterized D2 receptor rich nerve cell column system characterized by a relative lack of tyrosine hydroxylase immunoreactivity. Moreover, in animal models of dopaminergic pathway degeneration, plastic changes in uncoupling protein 2/3 terminals have been shown in the cerebral cortex and striatum as seen from the increased size and intensity of uncoupling protein 2/3 immunoreactivity of their varicosities. Taken together, these findings open up the possibility that uncoupling protein 2/3 could play an important role modulating the dopaminergic and noradrenergic neurotransmission within discrete brain regions. © 2005 Published by Elsevier Ltd on behalf of IBRO.

Key words: uncoupling protein, intercellular communication, catecholamines, 6-OHDA, rat brain, UCP2/3 overexpressing mice.

In the inner membrane of the mitochondria, the uncoupling proteins (UCPs) mediate the passive transport of hydrogen protons from the intermembrane space to the matrix compartment. Through this process, UCPs dissipate the proton gradient across the inner membrane diminishing the ATP synthesis and generating an important amount of heat (Bouillaud et al., 1985; Horvath et al., 2003a; Nicholls and Locke, 1984; Palou et al., 1998; Richard et al., 2001). UCPs have also been demonstrated to reduce the production of superoxides and increase the calcium efflux from the mitochondria with the reduction of the mitochondrial membrane potential (Arnesenjovic et al., 2000; Horvath et al., 2003a; Negre-Salvayre et al., 1997).

UCP proteins are expressed in a variety of tissues, including the brown adipose tissue where an important role in the processes of thermogenesis has been assigned to them (Bouillaud et al., 1985; Nicholls and Locke, 1984). The proteins UCP2 and UCP3 are members of this protein family and at least UCP2 is expressed in the CNS (Diano et al., 2000; Fleury et al., 1997; Horvath et al., 1999; Lengacher et al., 2004; Richard et al., 1998). The role played by UCP2 in the brain has yet to be fully described, but it has been suggested that UCP2 could contribute to the control of neurotransmission (Horvath et al., 1999, 2003a) and exert neuroprotective actions (Bechmann et al., 2002; Diano et al., 2003; Horvath et al., 2003a,b; Paradis et al., 2003).

It is now generally accepted that brain communication operates not only by synaptic transmission but also by volume transmission involving diffusion and convection of the transmitters in the extracellular fluid (ECF) and in the cerebrospinal fluid (CSF) (Agnati et al., 1994, 2000, 2005; Fuxe and Agnati, 1991). The present study was initiated to test our hypothesis that UCP2/3 may be involved in volume transmission in the brain. In view of the previous work indicating that the ascending dopaminergic (DAergic) and noradrenergic (NAergic) systems operate mainly by volume transmission (Jansson et al., 2002), an immunohistochemical analysis was performed to characterize the relationship of the UCP2/3 and tyrosine hydroxylase (TH) and dopamine β-hydroxylase (DBH) immunoreactivity (IR) in cell bodies, dendrites and nerve terminals using double immunolabeling procedures in combination with confocal laser microscopy. Of special interest was the nucleus ac-
cumbens where clear dopamine (DA) terminal/DA receptor mismatches have been observed (Jansson et al., 1999). We also explored the functional role of UCP2/3 by using a rat model of Parkinson’s disease with 6-hydroxydopamine (6-OHDA) induced degeneration of the ascending DA neurons on one side, where compensatory mechanisms have previously been demonstrated increasing DA release and promoting volume transmission (Zoli et al., 1998, 1999). Finally, we used UCP2/3 overexpressing mice and their littermates to test the specificity of the UCP2/3 antibody used in the present work.

**EXPERIMENTAL PROCEDURES**

**Animals**

Male Sprague–Dawley rats weighing 250 g (n=10) and male UCP2/3 overexpressing mice (n=4) and their wild-type littermates (n=4) (Fuller et al., 2000) were used. The animals were kept under a standard 12-h light/dark cycle and constant room temperature (23 °C), and had free access to tap water and food pellets. Animal care and use followed the directives of the Council of European Communities (86/609/EEC). All the experiments were approved by the appropriate animal care committee at the University of Málaga, and all efforts were made to minimize the number of animals used and their suffering.

**Lesion of the ascending DA pathways**

Ascending DA axon bundles to the forebrain were lesioned with local administration of the catecholamine (CA) neurotoxin 6-OHDA into the medial forebrain bundle (MFB). The rats (n=4) were pretreated 30 min prior to surgery with desipramine (25 mg/kg i.p. in saline solution) to protect NA projections (Breese and Traylor, 1970; Jacks et al., 1972) and then anesthetized with a mixture of ketamine (75 mg/kg i.p.) and medetomidine (0.02% of ascorbic acid) was infused for 2 min in the MFB using a 26-gauge cannula at the coordinates AP −3.7 mm, L −1.4 mm and V −8.6 mm from Bregma (Paxinos and Watson, 1986). Two weeks after the surgery, the animals were perfused and the brains processed for immunohistochemistry (as described below).

**Tissue processing**

The animals were deeply anesthetized with sodium pentobarbital (60 mg/kg i.p.) and perfused transcardially with 0.1 M phosphate-buffered saline, pH 7.4 (PBS) followed by 4% paraformaldehyde (w/v) in 0.1 M phosphate buffer, pH 7.4 (PB). The brains were dissected out, post-fixed in the same fixative for 2 h, immersed in 30% sucrose in PBS for at least 48 h and frozen in dry ice. Coronal free-floating sections (20 μm thick) were obtained and stored in PBS with 0.02% sodium azide at 4 °C until use for immunohistochemistry.

**Antibodies**

Affinity-purified rabbit polyclonal UCP2 antisem developed by Horvath et al. (1999) was used at a 1:500 (in the double immunofluorescence stainings) or 1:1000 dilution (in the avidin–biotin immunohistochemistry experiments). The UCP2 antibody has been previously characterized and used to visualize the UCP2 protein distribution in the brain especially the hypothalamus and entorhinal cortex (Bechmann et al., 2002; Diano et al., 2000; Horvath et al., 1999). This antisem also recognizes UCP3, an UCP that we recently observed to be present in certain brain areas (Horvath et al., unpublished observations). Thus, we refer to UCP2/3 IR throughout the manuscript. TH was detected using a mouse monoclonal antibody (DiaSorin, SpA, Saluggia, Italy) diluted at 1:1000 or 1:10,000. DBH was detected with a mouse monoclonal antibody (Chemicon International Inc., Temecula, CA, USA) diluted at 1:1000 or 1:10,000. High dilutions (1:10,000) were used for TH and DBH antibodies at the substantia nigra (SN) and the locus coeruleus (LC) level due to the high expression of TH and DBH in these areas. Low dilutions (1:1000) of TH and DBH antibodies were used to analyze the other brain regions, where a higher sensitivity is required to label varicose terminal-like fibers. All antibodies were diluted in phosphate-buffered saline containing 0.2% Triton X-100 (PBS-TX) and 0.1% sodium azide.

**Immunohistochemistry**

Coronal sections from different bregma levels according to the atlas of Paxinos and Watson (1986) were processed for either single or double immunolabeling procedures. The bregma levels used were: +1.70 mm to +0.70 mm (striatum, nucleus accumbens, Islands of Calleja, olfactory tubercle and cingulate cortex); −4.8 mm to −5.8 mm (SN and ventral tegmental area, VTA); and −9.6 mm to −10.0 mm (LC).

Single antigen immunohistochemistry was performed to compare UCP2/3 IR on the unoperated vs the DA denervated side in rat and UCP2/3 IR in the wild-type vs UCP2/3 overexpressing mice. Endogenous peroxidase activity was quenched by incubation for 10 min with 3% hydrogen peroxide in PBS. After washing with PBS, sections were incubated for 48 h at 4 °C with UCP2/3 antibody. After washing with PBS sections were incubated for 1 h in biotin-conjugated goat anti-rabbit IgG diluted 1:500 in PBS-TX (Vector Laboratories, Burlingame, CA, USA). The sections were washed again and incubated for 1 h in a streptavidin–peroxidase complex (Sigma, St. Louis, MO, USA) diluted 1:2000 in PBS-TX. Peroxidase activity was visualized with 0.05% 3,3’-diaminobenzidine (DAB, Sigma) and 0.002% H2O2, and staining was intensified with 0.8% nickel ammonium sulfate. The sections were mounted on gelatin-coated slides, air dried, dehydrated in xylene and coverslipped with DPX mounting medium.

Double immunohistochemistry for UCP2/3 and TH or DBH IR was performed in rat sections using green and red fluorescent staining, respectively. Non-specific binding sites were blocked for 30 min with 5% bovine serum albumin in PBS-TX. Sections were washed in PBS and incubated for 48 h at 4 °C with the UCP2/3 antibody followed by incubation for 24 h at 4 °C with TH or DBH antibodies. After washing in PBS, the sections were incubated for 1 h at room temperature with goat biotinylated anti-rabbit IgG (1:500, Vector Laboratories), washed again and incubated in a mixture of streptavidin conjugated with Alexa 488 (1:2000, Molecular Probes, Carlsbad, CA, USA) and goat anti-mouse IgG conjugated with Cy3 (1:200, Jackson Immunoresearch, West Grove, PA, USA). Finally, the sections were rinsed in PBS and mounted on gelatin-coated slides with an antifading mounting medium (Dako Corporation, Carpinteria, USA).

**Image analysis**

Quantitative analysis of both size and intensity of UCP2/3 IR varicose terminal-like fibers was performed using the image analyzing system NIH Image (http://rsb.info.nih.gov/nih-image/). Photographs of UCP2/3 IR varicose terminal-like fibers were taken with a digital camera (Coolpix 4500, Nikon, Tokyo, Japan) using a ×100 objective. Measurements of size and UCP2/3 IR intensity of varicosities were only performed from those that were in focus. The size of each varicosity was determined of the average value of four measures of diameter according to different sampling of the same varicosity. The intensity of UCP2/3 IR was expressed as the mean optical density (OD) and the value was corrected with the OD from an immunonegative area. Ten fibers (with three to eight varicosities) were measured from each region on the unoperated
and 6-OHDA-lesioned side as well as in the wild-type and UCP2/3 overexpressing mice. The statistical analysis was performed by Mann Whitney U test.

Specific immunofluorescence signals were visualized by excitation at blue (488 nm) for Alexa 488 (absorption: 490; emission: 519) and green (545 nm) for Cy3 (absorption: 550; emission: 580) using a confocal laser microscope (Leica TCS-NT, Wetzlar, Germany; laser ArKr) or a Nikon Microphot-Fx (Japan; Hg-lamp). In the confocal microscope, the emission signal was separated through a short-pass filter (RSP580), and the short-wavelength signal corresponding to Alexa 488 was band-pass filtered (BP530/30) and collected in the green channel. The long-wavelength signal was long-pass filtered.

Fig. 1. Fluorescence photomicrographs from the rat shell part of the nucleus accumbens showing UCP2/3 IR (A, C) and TH IR (B, D) in the same section. Arrowheads indicate patches displaying low UCP2/3 IR (A, C) where also weak TH IR is shown (B, D). The major part of shell of nucleus accumbens shows strong UCP2/3 IR matched by strong TH IR. Asterisks in C–D show an example of a strongly UCP2/3-immunolabeled patch with weak TH IR. Crossed arrows indicate dorsal and lateral direction in the four panels. AcbSh, shell part of the nucleus accumbens; ICjM, Islands of Calleja Magna. Scale bar= 100 μm in A and B and in C and D 200 μm.
and collected in the red channel. The scanning was made sequentially to avoid crosstalk. In the figures, UCP2/3 IR is shown in green and TH and DBH IR in red.

The analysis of the overlap between UCP2/3 and TH IR was made using the image analyzer KS 400 (Zeiss Kontron, Zeiss, Arese, Italy). Area measurements were obtained from the shell nucleus accumbens from four different rats and expressed as means ± SEM. Field areas (FA) in pixels were determined for TH- and UCP2/3 immunolabelings and were evaluated at a low and high threshold of discrimination. The two thresholds were selected by measuring with an automatic interactive procedure the mean gray values and the standard deviations within the area of interest (X, S.D.) and then using as a “low threshold of discrimination” the value equal to X and as “high threshold of discrimination” the value equal to X + 2SD.

The Boolean operator “AND” was applied to evaluate the overlap between TH IR and UCP2/3 IR at the two different thresholds of discrimination. The FA of these overlaps was determined and expressed in percent of the corresponding TH IR FA.

### RESULTS

#### UCP2/3 IR in catecholaminergic (CAergic) projection fields in the brain

In line with previous results (Diano et al., 2000; Horvath et al., 1999) UCP2/3 IR terminal-like fibers were observed in many brain regions, including CAergic projection fields. Intensely stained UCP2/3 IR terminal-like fibers were found in the ventral striatum (nucleus accumbens and olfactory tubercle) as well as in the lateral septum and the diagonal band of Broca. Less UCP2/3 IR was observed in the dorsal striatum and in the cerebral cortex. A detailed description on the UCP2/3 IR in the main CAergic projection fields and its relationship to the TH and DBH IR nerve terminal plexa will be given.

#### Nucleus accumbens

In the nucleus accumbens prominent UCP2/3 IR terminals were found in the shell and core part but with different distribution patterns. The shell nucleus accumbens showed a heterogeneous pattern with both strongly and weakly UCP2/3 IR patches (Fig. 1A). This pattern was found to be in good register with the TH IR patches identified in the same section (Fig. 1A, B). Nevertheless, certain strong UCP2/3 IR patches were not associated with high levels of TH IR but with low levels (Fig. 1C, D). Based on image analysis, the overlap between UCP2/3 IR and TH IR was about 78% and 96% at the lower and higher threshold of discrimination, respectively when evaluating the UCP2/3 IR and TH IR patches with strong IR (Table 1).

Scattered UCP2/3 IR varicose terminal-like fibers displaying strong intensity of immunostaining were observed in both strongly and weakly UCP2/3 IR patches in the shell nucleus accumbens. A detailed analysis using laser confocal microscopy showed that these UCP2/3 IR varicose terminals like fibers were also TH IR (data not shown) and DBH IR (Fig. 2). However, the vast majority of TH- and DBH IR terminal-like fibers did not express UCP2/3 IR (Fig. 2).

As seen in Fig. 3A, the core part of the nucleus accumbens showed a prominent UCP2/3 IR patch, presumably built up of a nerve terminal plexus surrounding the anterior commissure, where a large number of TH IR terminal-like fibers were also found (Fig. 3B). Analysis with confocal laser microscopy demonstrated a few intensely UCP2/3 IR varicose terminal-like fibers in close proximity

### Table 1. Overlap between TH- and UCP2/3 IR terminal-like fibers in the shell part of the nucleus accumbens

<table>
<thead>
<tr>
<th>FA of IR (pixels)</th>
<th>Low threshold</th>
<th>High threshold</th>
</tr>
</thead>
<tbody>
<tr>
<td>TH</td>
<td>100 ± 4</td>
<td>24 ± 6</td>
</tr>
<tr>
<td>UCP2/3</td>
<td>102 ± 7</td>
<td>28 ± 2</td>
</tr>
<tr>
<td>TH + UCP2/3</td>
<td>78 ± 3</td>
<td>23 ± 8</td>
</tr>
<tr>
<td>TH + UCP2/3 overlap (%)</td>
<td>78 ± 11</td>
<td>96 ± 33</td>
</tr>
</tbody>
</table>

The FA (pixels) values represent the measurement of area of TH- and UCP2/3 IR at two threshold levels (low and high). The FA is expressed as means ± SEM (n = 4). The TH + UCP2/3 field areas overlaps were also evaluated at the two different thresholds of discrimination and expressed as percentages of the respective FA of the TH IR staining.

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to the anterior commissure (Fig. 3C, E). These UCP2/3 IR profiles were found to be immunoreactive also for TH (Fig. 3C, D) but seemed to lack DBH IR (Fig. 3E, F).

**Olfactory tubercle and Islands of Calleja**

A low to high density of UCP2/3 IR terminal-like fibers was found in the olfactory tubercle (Fig. 4A). High density regions in the inner layer were characterized by a densely punctate of UCP2/3 immunofluorescence overlapping with TH IR terminal-like fibers also of a high density. This overlap did not exist in the outer layer. It was not possible to determine if UCP2/3 IR and TH IR were co-located in the same terminals. On the other hand, few scattered UCP2/3 IR varicose terminal-like fibers in the outer layer were

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Fig. 3. Confocal photomicrographs from the rat core part of the nucleus accumbens showing double immunolabeling using UCP2/3 and TH or DBH antibodies. A strongly UCP2/3 IR area surrounds the anterior commissure displaying intense TH IR (arrows in A–B). In high magnification the confocal laser microscopy analysis demonstrated intense large UCP2/3 IR varicose terminal-like fibers displaying also TH IR (arrowheads in C–D), but not DBH IR (E, F). Weakly stained TH IR fibers did not display UCP2/3 IR (arrows in C–D). Crossed arrows indicate dorsal and lateral direction in all the panels. aca, anterior commissure; AcbC, core part of the nucleus accumbens. Scale bar = 200 μm in A and B and in C, D, E and F 25 μm.
found to contain TH IR (Fig. 4C, D) as shown with confocal laser microscopy.

The core of the Islands of Calleja displayed a moderate degree of UCP2/3 IR punctate while the outer part lacked UCP2/3 IR (Fig. 4A). Large number of TH IR terminal-like fibers surrounded these islands but only few were found inside (Fig. 4B). In the Islands of Calleja Magna, as indicated in Fig. 5A and B, low to moderate numbers of TH and DBH IR varicose terminal-like fibers were observed. In contrast only few UCP2/3 IR varicose terminal-like fibers were found in the Islands of Calleja Magna. Double immunolabeling experiments and detailed analysis of optical sections using confocal laser microscopy, revealed that TH- but not DBH IR co-localize with UCP2/3 IR in their varicosities (insets in Fig. 5A and B).

**Caudate putamen**

Scattered strongly UCP2/3 IR cell bodies and varicose terminal-like fibers appeared in the caudate putamen (Fig. 6A, B). They did not seem to have a selective localization within the different compartments (striosomes vs matrix) and topographic regions in the caudate putamen. As seen in Fig. 6C and D, the UCP2/3 IR varicose terminal-like fibers were also immunoreactive for TH and for DBH.
Cerebral cortex

In cortical areas, such as cingulate, frontal, parietal, entorhinal and piriform cortex, scattered UCP2/3 IR cell bodies and varicose terminal-like fibers were observed (Fig. 7A). Most of them were located in the supragranular layers (I, II and III), although they could also be found in the infragranular layers. The UCP2/3 IR nerve cells displayed morphological features of cortical interneurons. TH IR could be demonstrated in all of the UCP2/3 IR varicose terminal-like fibers analyzed characterized by large varicosities and intense IR (Fig. 7B, C). Frequently fine TH IR terminal-like fibers with small varicosities and with less intensity of staining were observed to lack UCP2/3 IR. In optical sections using laser confocal microscopy, the relationship between DBH IR and UCP2/3 IR was further studied. As seen in Fig. 7D and E, UCP2/3 varicose terminal-like fibers were found to lack DBH IR but with DBH IR terminal-like fibers present in the surround.

UCP2/3 expression in DAergic and NAergic nerve cell groups

The existence of UCP2/3 IR was analyzed in the DAergic nerve cell bodies located in the SN and the VTA and in the NAergic nerve cell bodies in the LC.

SN

In the substantia nigra pars compacta (SNc) and VTA prominent UCP2/3 IR was demonstrated in both cell bodies and dendritic branches of DAergic neurons immunostained in the same section with anti-TH antibodies (Fig. 8). Within the substantia nigra pars reticulata (SNr) abundant UCP2/3 IR processes and cell bodies were also present (Fig. 8A, B, D). Double immunolabeling for TH and UCP2/3 IR revealed that TH IR cell bodies and dendrites also contain UCP2/3 IR. However, a large number of...
perikarya and dendritic profiles in SNr were found to express exclusively UCP2/3 IR (Fig. 8D).

LC

In line with the previous results of Horvath et al. (1999), a moderate degree of UCP2/3 IR was found in the LC. The UCP2/3 IR was either found in the cell bodies and their dendritic processes or in terminal-like fibers (Fig. 9A). UCP2/3 IR appeared as punctate in the cytoplasm of NAergic cell bodies, identified in the same section with anti-DBH antibodies (Fig. 9). This analysis was performed by confocal laser microscopy that allows through optical sectioning the localization of the punctate UCP2/3 IR to the cytoplasm of the cell bodies. Most of the DBH IR nerve cells displayed high degrees of UCP2/3 IR, although moderate–low degrees were also observed in some cells. UCP2/3 IR was also demonstrated in varicose terminal-like fibers in close proximity to the NAergic nerve cells (Fig. 9).

Effects of 6-OHDA induced lesions of the ascending DA pathways to the forebrain on UCP2/3 expression

Remaining striatal DA nerve terminals are known to become hypertrophic after 6-OHDA lesions, probably as a result of a compensatory activation of surviving DAergic neurons (Zoli et al., 1998, 1999). To test whether UCP2/3 expression is affected by the 6-OHDA-induced lesion of the ascending DA pathways we analyzed the size of UCP2/3 IR varicosities as well as the intensity of UCP2/3
IR in both cerebral cortex and caudate putamen from the unoperated and 6-OHDA-lesioned side.

The analysis showed a marked increase in the size of UCP2/3 IR varicose terminal-like fibers as well as in their intensity of UCP2/3 IR on the lesioned side as compared with the unoperated side (Fig. 10A, B). The diameter of the UCP2/3 IR varicosities increased by 95% in the cerebral cortex and by 97% in the striatum (Fig. 10C). The OD as a measure of intensity of UCP2/3 IR increased by 37% and 16% in the cerebral cortex and striatum, respectively (Fig. 10D). A detailed analysis of the percentage of varicosities belonging to different categories of size (Fig. 10E, F) and OD (Fig. 10G, H) in both the cerebral cortex and the caudate putamen showed a displacement toward larger categories in those from the DA-lesioned side. It should be noted that the scattered UCP2/3 IR terminal-like fibers remain with a similar distribution pattern after the 6-OHDA-induced lesion. No differences in the OD were observed in the hypothalamus (Fig. 10D).

UCP2/3 expression in UCP2/3 overexpressing mice

UCP2/3 IR was analyzed in UCP2/3 overexpressing mice. No difference was observed in the distribution pattern of UCP2/3 IR in mice compared with that of rat. The areas selected were the cerebral cortex, the caudate putamen and the hypothalamus, where UCP2/3 IR terminal-like fibers were observed.

In the UCP2/3 overexpressing mice significant increases of the intensity of UCP2/3 IR were observed in terminals of the cerebral cortex (by 44%), the caudate putamen (by 23%) and the hypothalamus (by 28%) (Fig. 11A). UCP2/3 IR varicose terminal-like fibers of cerebral cortex and caudate putamen from the unoperated and 6-OHDA-lesioned side.

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Fig. 8. Confocal photomicrographs from the rat SN showing double immunolabeling using the UCP2/3 and TH antibodies (A–D). Strong UCP2/3 IR was found in many TH IR nerve cell bodies especially in the pars compacta but also in the pars reticulata (arrow in A). It was possible to demonstrate UCP2/3 IR in the TH IR dendritic like processes (open arrow in D). Many UCP2/3 IR cell bodies in the pars reticulata did not show TH IR (arrowheads in A and D). Confocal photomicrographs from the VTA showing double immunolabeling using UCP2/3 and TH antibodies (yellow) (E). Strong UCP2/3 IR was found in the majority of the TH IR cell bodies. Scale bar: 100 μm in A, in B–C is 200 μm, D 200 μm and in E 150 μm.
cortex and caudate putamen also showed an increase (by 21% and 23%, respectively) in their size as compared with wild-type (Fig. 11B).

**DISCUSSION**

Strikingly almost 100% overlap exists between UCP2 mRNA expression and UCP2 IR in the brain of both rats (Horvath et al., 1999) and non-human primates (Diano et al., 2000). Additionally, the use of commercially available antisera, which in Western blot analysis of tissues from UCP2 knock mice demonstrated the lack of the UCP2 protein (Horvath et al., 2002), resulted in immunostaining that overlaps UCP2 mRNA expression in mice (Diano et al., 2003). The same was true for immunolabeling obtained by the affinity purified polyclonal antiserum against human UCP2/3 in both mice and rats (Diano et al., 2000, 2003; Horvath et al., 1999). The antiserum of choice in the present study. In further support of our view that the immunolabeling in CAergic cells depicts UCP expression, we recently observed that mitochondrial uncoupling is elevated in SN samples of UCP2/3 overexpressing animals and diminished in UCP2 knockout animals (Andrews et al., 2005). The increase of UCP2/3 IR observed in the UCP2/3 overexpressing mice support the specificity of the antiserum used in the present work. Nevertheless, the possibility exists that the antisera used in the present study may recognize epitopes of other peptides, particularly other UCPs such as UCP3. Thus, throughout the text of this manuscript, we refer to the immunolabeling as UCP2/3.

Previous work by Horvath’s group (Bechmann et al., 2002; Diano et al., 2000; Horvath et al., 1999, 2003b) and others (Richard et al., 1998) has demonstrated the existence of UCP2 producing neurons in the mammalian brain especially in the hypothalamus, where it is found in various types of neuropeptidergic neurons. Previous work also indicates that UCP2 can be both a neuromodulator and a neuroprotector by producing mitochondrial uncoupling with generation of heat leading to modulation of synaptic transmission and reduction of the production of superoxides (Bechmann et al., 2002; Diano et al., 2003; Horvath et al., 1999, 2003a). Immunohistochemistry and in situ hybridization studies showing high levels of UCP2 mRNA and UCP2 IR especially in the hypothalamus indicated an increased role of UCP2 in homeostatic centers involved in the regulation of autonomic, endocrine and metabolic processes (Diano et al., 2000; Horvath et al., 1999; Richard et al., 1998).

The present results show coexistence of UCP2/3 and TH or DBH IR in many cell bodies and discrete terminal-like fibers of the ascending DA and NA neuron systems.

![Confocal photomicrograph from the rat LC showing double immunolabeling using UCP2/3 (A) and DBH (B) antibodies. Co-localization is shown in C. Strong punctate UCP2/3 IR (arrowheads) was demonstrated in the DBH IR cell bodies (C, D). Cells with weak UCP2/3 IR are indicated with open arrows (C, E). UCP2/3 IR was also present in DBH IR dendrites and terminal-like fibers (arrows) (C, E) in close proximity to the DBH IR nerve cell bodies. Scale bar=100 μm in A–C and in D–E is 25 μm.](image-url)
These observations open up the possibility that UCP2/3 also can play a major role in modulation of CA volume transmission in the brain by producing temperature gradients (see Horvath et al., 1999, 2003a) causing convective fluid movements. In this way UCP2/3 may enhance migration of neurotransmitters released from discrete forebrain CAergic nerve terminals and from large numbers of CA cell bodies and their dendrites.

In the shell of nucleus accumbens there exist special nerve cell systems that contain a high density of D1 receptors but very few DA terminals that are surrounded by a high density of DA but by only sparse NA nerve terminals (Jansson et al., 1999). The existence of neuronal cell clusters and neurochemical compartments in the rat nucleus accumbens was demonstrated by Herkenham et al. (1984). In the Jansson et al. (1999) studies it was proposed that DA can diffuse from the surrounding DA terminals to the high numbers of high affinity D1 DA receptors found in these special accumbens cell compartments. In the present studies strongly UCP2/3 IR terminals were
observed surrounding these accumbens nerve cell compartments characterized by a D1/TH IR mismatches as observed in the present images (Fig. 1). These two terminal systems overlap in the distribution but the co-existence of these two IR in the DA terminals remains to be investigated using ultrastructural analysis. Nevertheless, the present findings again open up the possibility that if UCP2/3-generated temperature gradients (Horvath et al., 1999, 2003a) exist in the UCP2/3 IR-enriched patches, they may enhance release of DA from DA terminals and DA migration with the convective fluid movements. In this way DA may reach the D1 receptor rich cell system more rapidly and in biologically relevant concentrations, enhancing the DA communication via volume transmission.

In the core of the nucleus accumbens it was however possible to demonstrate co-localization of UCP2/3 and TH IR in the same varicose nerve terminals, but not of UCP2/3 and DBH IR. These results indicate that UCP2/3 can exist in DAergic terminals where by its postulated generation of heat (Horvath et al., 1999, 2003a) it may facilitate release and migration of DA in the surrounding ECF by the resulting temperature gradient. It should be noted that UCP2/3 receptor rich cell system more rapidly and in biologically relevant concentrations, enhancing the DA communication via volume transmission.

A partial lesion of the DAergic mesostriatal pathway induces an increase in the size and in the content in TH IR of the DA-varicosities, which is interpreted as a compensatory mechanism after the lesion of the DA pathway to increase extrasynaptic DA release and to promote volume transmission (Zoli et al., 1998, 1999). Similar results were observed in the present study on UCP2/3 IR terminals in all cortical regions where only the few strongly TH IR terminals with large varicosities, probably representing DA terminals, were found to contain UCP2/3 IR. The DA character of the UCP2/3-TH co-storing terminals was indicated by the absence of the UCP2/3-DBH co-storage in the cerebral cortex. Thus, the increase in the size and intensity of the UCP2/3 IR terminals on the 6-OHDA-lesioned side may indicate a compensatory increase in their activity in response to the partial forebrain DA denervation with a switch toward the VT mode of communication (see above). In contrast the vast majorities of the cortical TH IR terminals characterized by small varicosities with weak IR lack UCP2/3 IR.
Hypothalamic varicosities with high amount of TH and DBH IR i.e. large NA terminals with high NA synthesis containing UCP2/3 IR are specialized for volume transmission, capable of generating temperature gradients by means of UCP2 resulting in increased migration of NA in the ECF from the release sites (see above).

The scattered UCP2/3 IR NAergic terminals in the striatum are protected against 6-OHDA-induced lesions by pretreatment with desipramine (Breese and Traylor, 1970; Jacks et al., 1972). However, on the 6-OHDA-lesioned side we observed an increase of the size and intensity of the scattered striatal UCP2/3 IR varicose-like terminals, similar to those observed in the cerebral cortex. The observation that in the hypothalamus there were no changes in the UCP2/3 IR intensity supports the view of a specific CA terminal response developed after the lesion of the ascending DAergic pathway. In the caudate putamen the morphological analysis indicates that the compensatory responses occurred in the scattered NA-UCP2/3 terminal system. It has been shown that NA exhibits a high affinity for the D₄ DA receptors (Lanau et al., 1997; Newman-Tancredi et al., 1997). An increased release of NA from the scattered NA-UCP2/3 terminal system could more effectively activate the DA D₄ receptors and partially compensate for the loss of DA. An increase of NA migration may occur by increasing the expression of UCP2/3 with an increase of postulated heat generation.

The confocal analysis elegantly demonstrated the existence of punctate UCP2/3 IR in the cytoplasm of the LC NA cells probably representing aggregates of UCP2/3 IR mitochondria. Like in the case of the DA cell bodies these UCP2/3 IR mitochondria may play a role inter alia in facilitating NA release and migration in the surrounding ECF to reach e.g. α₂ autoreceptors (Aoki et al., 1994). This mechanism may be in operation also in the NA dendrites which also possessed UCP2/3-DBH IR. These results are consistent with the finding of Callado and Stanford (2000) suggesting interactions between NA and α₂ autoreceptors or NA transporters as far as 10 μm from the NA release sites.

It is clear that despite the fact that large numbers of DA and NA cell bodies contain cytoplasmic UCP2/3 IR only a distinct minority of DA and NA nerve terminals contains UCP2/3 IR and is characterized by large varicosities and high amounts of TH and/or DBH IR. It may be that only certain types of DA and NA nerve cells have the capability to transport the UCP2/3 IR mitochondria into the nerve terminal networks by axonal flow, at least in amounts detectable by immunohistochemistry techniques. Under all circumstances there must exist substantial differences in the ability of DAergic and NAergic cells to transport UCP2/3 IR mitochondria to the DAergic and NAergic net-

Fig. 11. Mean OD values (A) (μm) and diameter (B) of UCP2/3 IR varicosities in UCP2/3 overexpressing mice. White bars represent wild-type mice and black bars transgenic mice. Scatter plots show the mean values from each individual animal together with the overall mean value. Statistical analysis was made with Mann-Whitney U test (** P<0.01).
works. It is of particular interest that UCP2/3 IR DA and NA nerve terminals have large varicosities and large amounts of the CA synthesizing enzymes. Such terminals may be highly specialized for volume transmission with the ability to synthesize and release large amounts of CA to facilitate CA migration in the ECF by the UCP2/3-induced temperature gradients.

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REFERENCES


Bouillaud F, Richard D, Collins S, Ricquier D, War-


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