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Vesicular Monoamine Transporters (VMATs) in Adrenal Chromaffin Cells: Stress-Triggered Induction of VMAT2 and Expression in Epinephrine Synthesizing Cells

Andrej Tillinger · Anne Sollas · Lidia I. Serova · Richard Kvetnansky · Esther L. Sabban

Abstract Vesicular monoamine transporters (VMATs) mediate transmitter uptake into neurosecretory vesicles. There are two VMAT isoforms, VMAT1 and VMAT2, encoded by separate genes and displaying different cellular distributions and pharmacological properties. We examined the effect of immobilization stress (IMO) on expression of VMATs in the rat adrenal medulla. Under basal conditions, VMAT1 is widely expressed in all adrenal chromaffin cells, while VMAT2 is co-localized with tyrosine hydroxylase (TH) but not phenylethanolamine N-methyltransferase (PNMT), indicating its expression in norepinephrine (NE)-, but not epinephrine (Epi)-synthesizing chromaffin cells. After exposure to IMO, there was no change in levels of VMAT1 mRNA. However, VMAT2 mRNA was elevated after exposure of rats to 2 h IMO once ($1\times$ IMO) or daily for 6 days ($6\times$ IMO). The changes in VMAT2 mRNA were reflected by increased VMAT2 protein after the repeated IMO. Immunofluorescence revealed an increased number of cells expressing VMAT2 following repeated IMO and its colocalization with PNMT in many chromaffin cells. The findings suggest an adaptive mechanism in chromaffin cells whereby enhanced catecholamine storage capacity facilitates more efficient utilization of the well-characterized heightened catecholamine biosynthesis with repeated IMO stress.

Keywords Adrenal medulla · Chromaffin · Epinephrine · Stress · Vesicular monoamine transporter

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Introduction

Release of the catecholamines, norepinephrine (NE), and epinephrine (Epi) is among the earliest responses of an organism to stress. This is crucial for activation of the "fight or flight" response to deal with a threat to homeostasis (Cannon 1929). In the periphery, the adrenal medulla and sympathetic nervous systems are responsible for the stress-triggered increase of plasma Epi and NE levels. Epi is the main catecholamine produced by the adrenal medulla, while NE in the periphery is mostly produced and released by sympathetic nerve endings, and also by noradrenergic chromaffin cells of the adrenal medulla (Kvetnansky et al. 1992, 2009).

Prior to secretion, the catecholamines, whether newly synthesized or after re-uptake, are stored in neurosecretory vesicles. The vesicular monoamine transporters (VMATs), using energy from proton co-transport gradient, pump monoamines from the cytoplasm into these vesicles, where they are sequestered prior to their secretion (Kanner and Schuldiner 1987; Johnson 1988; Henry et al. 1994). There are two VMAT isoforms, VMAT1 and VMAT2, encoded by two separate genes and displaying different cellular distributions and pharmacological properties (Erickson et al. 1992; Liu et al. 1992). In rodents, VMAT1 is expressed predominantly in neuroendocrine cells (e.g., adrenal medulla, small intensely fluorescent cells), while VMAT2 is expressed in peripheral, central, and enteric

neurons (Weihe et al. 1994; Peter et al. 1995). The two VMATs also differ in substrate affinity. VMAT2 has three times higher affinity for dopamine, NE, and Epi than VMAT1 and 100 times greater affinity for histamine. In addition, VMAT2 has a higher turnover number than VMAT1, which is especially appropriate for rapidly recycling vesicles (Peter et al. 1994; Erickson et al. 1996). Heterotrimeric G proteins have been shown to regulate the monoamine uptake and thus content of both large dense core and small synaptic vesicles. VMAT2 is more sensitive to G-protein regulation than VMAT1 (Holtje et al. 2000).

With stress, secretion of catecholamines is accompanied with the increased biosynthesis of catecholamines and elevated expression of catecholamine biosynthetic enzymes in the adrenal medulla [reviewed in (Sabban and Kvetnansky 2001; Wong and Tank 2007; Kvetnansky et al. 2009)]. Although the stress-triggered changes of catecholaminergic enzymes in the adrenal medulla are well studied, little is known regarding regulation of VMATs and whether they can participate in the response of the adrenal medulla to stress.

The present study examined the expression of VMAT isoforms in the rat adrenal medulla under basal conditions and following exposure of the animals to stress. Our findings reveal, for the first time, that immobilization stress (IMO) triggers increased expression of VMAT2 at the mRNA and protein levels. Following repeated IMO, expression of VMAT2 is no longer restricted to the noradrenergic, but also evident in adrenergic chromaffin cells. The findings suggest an additional mechanism of adaptation to repeated stress with increased catecholamine storage enabling enhanced adrenergic neurosecretory capacity.

Materials and Methods

Animals

All animal experiments were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996) and approved by the Institutional Animal Care and Use Committee. Male, Sprague-Dawley rats (280–320 g) were obtained from Taconic Farms (Germantown, NY). They were maintained under controlled conditions (23 ± 2°C, 12-h light/dark cycle, lights on from 6 AM) with food and water ad libitum.

Immobilization was performed as previously (Nankova et al. 1994; Liu et al. 2005) on a metal board by taping the limbs with surgical tape and restricting the motion of the head exactly as originally described by Kvetnansky and Mikulaj (1970). For a single IMO (1× IMO), rats were restrained once for 2 h. For repeated IMO, they were immobilized for 2 h daily for six consecutive days (6× IMO). The IMO was performed at the same time of the day (between 8 AM and noon). Rats were killed immediately after IMO stress for RNA isolation and 3 h after IMO stress for protein isolation. Control animals were not exposed to stress.

The right and left adrenal medulla were removed from each individual animal, frozen separately in liquid nitrogen, and kept at −80°C. In some experiments, adrenals were fixed in paraformaldehyde solution and processed for immunofluorescence.

Western Blot

For the Western blot analysis, the right adrenal medulla from individual animals was homogenized in SDS extraction buffer (2% SDS; 62.5 mM Tris–HCl, pH 6.8) and denatured for 5 min at 95°C. After centrifugation (10,000g, 10 min at 4°C), the supernatant was taken and the protein concentration determined with a detergent compatible protein assay (DC Protein Assay; Bio-Rad). Western blot analysis was carried out as described previously (Liu et al. 2005), with primary antibody to VMAT2 (sc-15314; Santa Cruz Biotechnology) diluted 1:500 in 5% non-fat dry milk in TBS-T. As a control of loading, the membranes were stripped and re-probed for β-actin (A5441; Sigma). Films were scanned with Bio-Rad G-800 Calibrated Densitometer, and the optical density of individual bands was evaluated within the linear range by PCBAS 2.08e software (Raytest).

Quantitative Real-Time RT-PCR

Total RNA was isolated from the left adrenal medulla using RNA-Stat-60 (Tel-Test, Inc.) according to manufacturer's instructions. The concentration of total RNA from each sample was quantified using Quant-iT RiboGreen RNA Kit (Invitrogen). The RT reactions were performed with 5′-CAA TGAGATAAGAGATGCTC-3′ for VMAT2 or 5′-CCT ACTGCCACCATCCCAAC-3′ for VMAT1, in 5-μl reaction mixture (1× AMV buffer, 10 mM dNTP, 8 units RNAse inhibitor, 1.25 units AMV polymerase, 10 μM reverse primer, and 100 ng of template RNA). Quantitative analysis of mRNA levels was performed by real-time RT-PCR with SYBR Green using the Light Cycler (Roche). PCR reactions were carried out in 20 μl with a final concentration of 1× LightCycler FastStart DNA Master SYBR Green I mix (Roche), 0.5 μM of each of the forward and reverse primers and 2 μl of the cDNA. A standard curve plotted using serial dilutions from 2 ng to 0.2 pg of VMATs cDNA was used for the quantification by Fit Points Method. The following primers were used: forward 5′-TCTTGGATGGGGCTAT TCAG-3′, reverse 5′-CTTTCGGGAACACATGGTCT-3′

for VMAT2 and forward 5′-GCCTTCGAAAGTGTCT CCTG-3′, and reverse 5′-GCCAACACACCAAAGAGGT T-3′ for VMAT1. The presence of the specific target was confirmed with melting curve analysis by comparing its melting temperature to the melting temperatures of the standards as a positive control.

Immunofluorescence

For immunofluorescence, the adrenals were fixed overnight in 4% paraformaldehyde, 0.05 M sodium orthovanadate in 0.1 M sodium phosphate buffer pH 7.4 at 4°C. The next day for cryoprotection, the adrenals were transferred into 15% and then 30% sucrose, both in 0.1 M sodium phosphate buffer pH 7.4 at 4°C. The adrenals were embedded in OCT (Ted Pella) and kept at −70°C and sectioned (10 μm) on a cryostat (Leica) and mounted onto glass slides coated with silicone adhesive. The slides were treated with 50% ethanol for 30 min, washed in Tris-Buffered Saline (TBS) and TBS-Tx (0.05% Triton X-100), then pre-blocked with 10% normal donkey serum (Jackson Immunoresearch Lab) in 3% BSA in TBS-Tx for 1 h at room temperature and washed in TBS-Tx. The sections were incubated with primary rabbit antibodies against VMAT1 or VMAT2 (H-V002 and H-V004; Phoenix Pharmaceuticals) diluted 1:200 for VMAT1 and 1:100 for VMAT2 in 10% normal donkey serum in 3% BSA, in TBS-Tx for 24 h at 4°C. After washing in TBS-Tx, slides were incubated in either mouse antibody to tyrosine hydroxylase (TH) (ImmunoStar, cat. #22941) or sheep antibodies to phenylethanolamine N-methyltransferase (PNMT) (Chemicon; cat. #AB146) each diluted 1:500 in 3% BSA, 10% normal donkey serum in TBS-Tx at 4°C overnight. After washing in TBS-Tx, the sections were incubated in Alexa Fluor (Molecular Probes) secondary antibodies to rabbit (Alexa Fluor Donkey Anti-Rabbit 594; cat. #A21207) and mouse (Alexa Fluor donkey anti-mouse 488; cat. # A21202) or sheep (Alexa Fluor donkey anti-sheep 488; cat. # A11015) diluted 1:200 in 1% normal donkey serum in 3% BSA, TBS-Tx for 1 h in the dark at room temperature. The slides were subsequently washed in TBS several times and then coverslipped in an anti-fading medium (Vectashield Hard Set, Vector Laboratories). The sections were visualized using a Zeiss AxioImager Microscope with an Axiocam Digital Camera.

Statistical Analysis

Results are presented as mean ± SEM. Statistical differences among groups were determined by one-way analysis of variance (ANOVA) followed by Bonferroni comparisons. Values of $P \leq 0.05$ were considered to be significant.

Results

VMAT1 and VMAT2 Localization in Adrenal Medulla

First, basal expression of VMAT1 and VMAT2 in rat adrenals was examined using immunofluorescence. VMAT 1 (Fig. 1a, g) showed widespread expression in the adrenal medulla, while expression of VMAT2 was more restricted (Fig. 1d, j). We did not detect VMAT1 nor VMAT2 in the adrenal cortex (data not shown). Double labeling with specific antibodies for tyrosine hydroxylase (TH), as a marker for NE and Epi synthesizing cells, and with antibody for phenylethanolamine N-mehtyltransferase (PNMT), for Epi synthesizing cells in the adrenal were used to determine which chromaffin cells express VMAT1 and VMAT2. VMAT1 is widely expressed in the adrenal medulla in all, or nearly all, TH positive chromaffin cells (Fig. 1c) and also in PNMT positive cells (Fig. 1i). In contrast, VMAT2 is expressed in only a subset of TH positive cells (Fig. 1f). There was no co-localization of VMAT2 and PNMT in adrenal medullary chromaffin cells (Fig. 1l), indicating that VMAT2 is expressed exclusively in NE, and not Epi, synthesizing cells in the adrenal medulla.

Effect of Stress on Expression of VMATS in the Adrenal Medulla

The effect of IMO stress on expression of VMAT1 and VMAT2 mRNA was examined by quantitative real-time RT-PCR in the adrenal medulla of rats exposed to single (1× IMO) and repeated IMO (6× IMO). Levels of VMAT1 mRNA were unchanged after either single (1.13 ± 0.08 vs. 1.00 ± 0.04 control) or repeated (0.85 ± 0.07 vs. 1.00 ± 0.04 control) exposure to IMO stress (Fig. 2a). However, IMO triggered a significant rise in expression of VMAT2 mRNA. Exposure to single (1.83 ± 0.13 vs. 1.00 ± 0.08 control, $P \leq 0.001$) as well as repeated (1.54 ± 0.06 vs. 1.00 ± 0.08 control, $P \leq 0.01$) IMO elicited an elevation in VMAT2 mRNA levels (Fig. 2b).

To determine if these changes in mRNA levels are accompanied by increased VMAT2 protein levels, western blots were performed (Fig. 3A). VMAT2 protein levels were not significantly changed following 1× IMO (1.75 ± 0.49 vs. 1.00 ± 0.17 control). However, following repeated IMO (6× IMO) VMAT2 protein levels were about 2.5-fold higher than in controls (2.55 ± 0.30 vs. 1.00 ± 0.17 control, $P \leq 0.05$).

The localization of VMAT2 in the adrenal medulla was examined following exposure to stress using immunofluorescence (Fig. 3B). It revealed the presence of more VMAT2 expressing cells in the adrenal medulla, especially following 6× IMO (Fig. 3B, c) compared to control

Fig. 1 Localization of VMAT1, VMAT2, TH and PNMT in rat adrenal medulla under basal conditions. Immunostaining for VMAT1 (**a**, **g**), TH (**b**, **e**) or PNMT (**h**, **k**) and its co-localization with TH (**c**) and PNMT (**i**) and VMAT2 staining (**d**, **j**) and its co-localization with TH (**f**) and PNMT (**l**) are shown
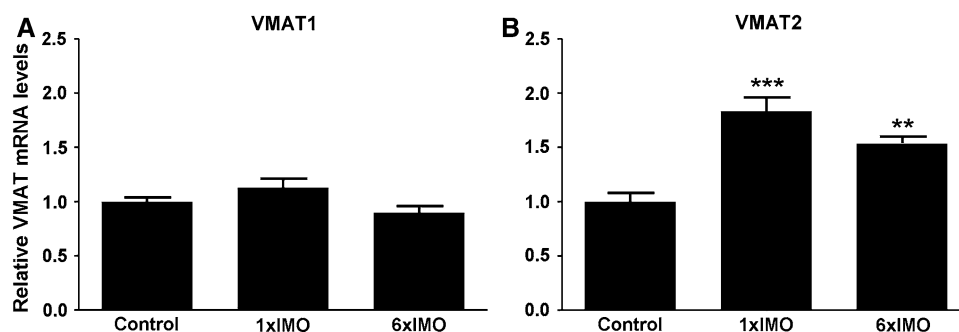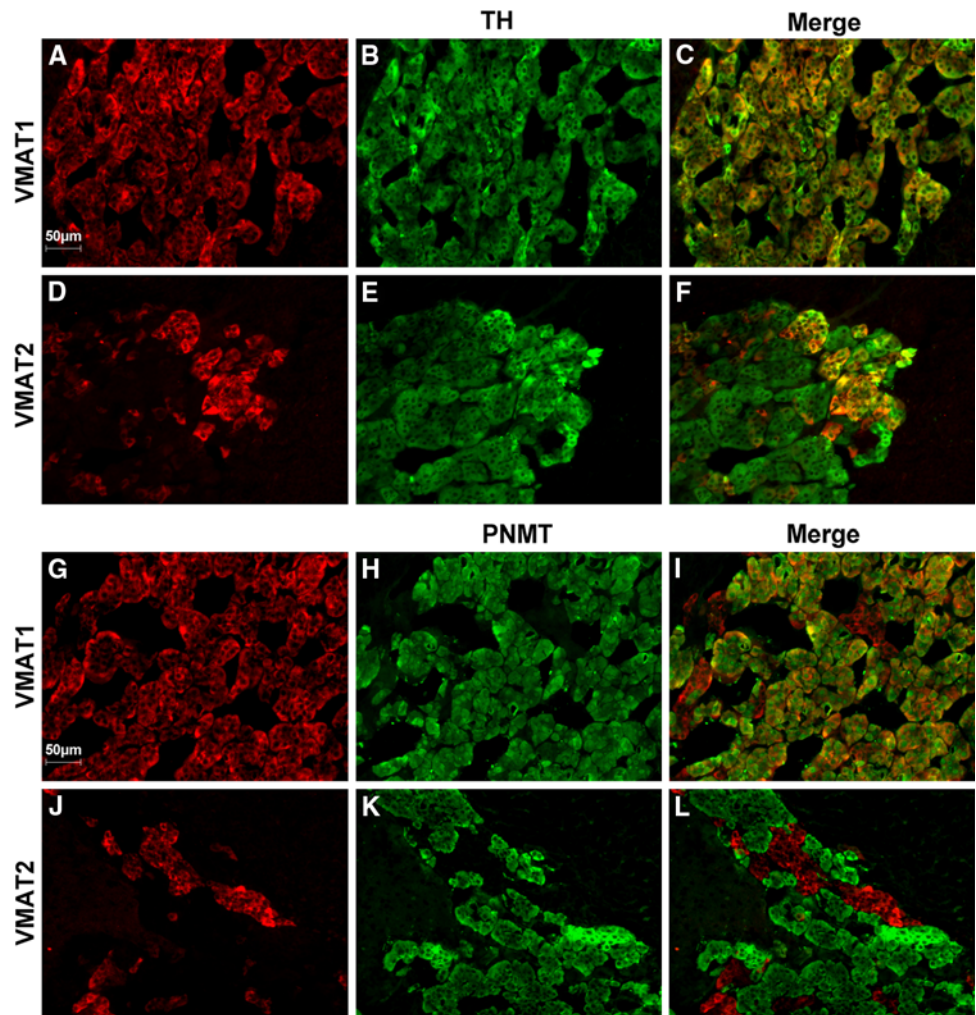

Fig. 2 Effect of single and repeated IMO on VMAT1 and VMAT2 mRNA levels in rat adrenal medulla. The relative levels of VMAT1 (**a**) and VMAT2 (**b**) mRNAs were determined by quantitative real-time RT-PCR. Data are expressed as mean ± SEM (6–8 animals) relative to control unstressed levels taken as 1 (** $P \leq$ 0.01, *** $P \leq$ 0.001 vs. control)

conditions (Fig. 3B, a). Double labeling showed, in contrast to the adrenals of unstressed controls (Fig. 4a–c), or immediately after a 2 h IMO (1× IMO, Fig. 4d–f) where there was no co-localization of VMAT2 with PNMT, after 6× IMO there were not only more cells expressing VMAT2 but also that there was expression in many of the PNMT positive chromaffin cells (Fig. 4g–i).

Discussion

Our results show that VMAT2 expression in the adrenal medulla is localized in the NE synthesizing chromaffin cells but is not detectable in the Epi synthesizing cells under basal conditions, while VMAT1 is expressed widely in both types of chromaffin cells. Following six

Fig. 3 Effect of single and repeated immobilization stress on VMAT2 protein levels in rat adrenal medulla. **A** Western blot analysis of VMAT2 in adrenal medulla of animals exposed to 2 h IMO stress and decapitated 3 h after the last IMO. Results are presented as a mean ± SEM and each value represents an average of 5–7 animals (* $P < 0.05$ vs. controls). **B** Immunofluorescence for VMAT2 in rat adrenal medulla under basal conditions (control, **a**) or after single (1× IMO, **b**) or repeated (6× IMO, **c**) stress
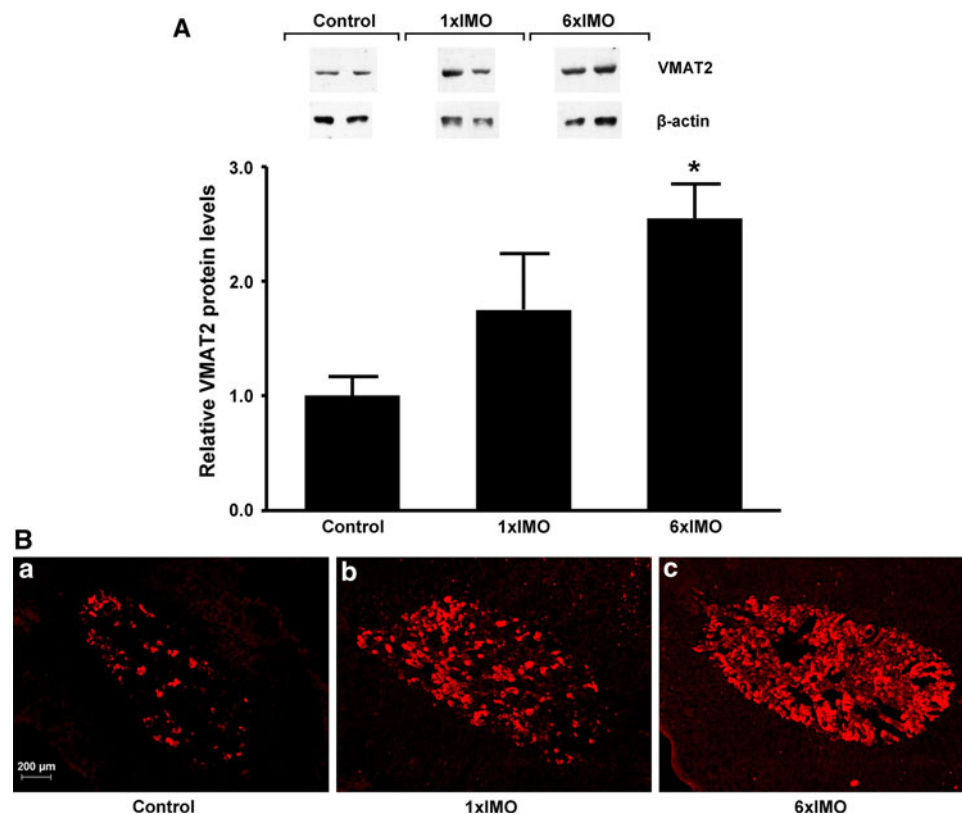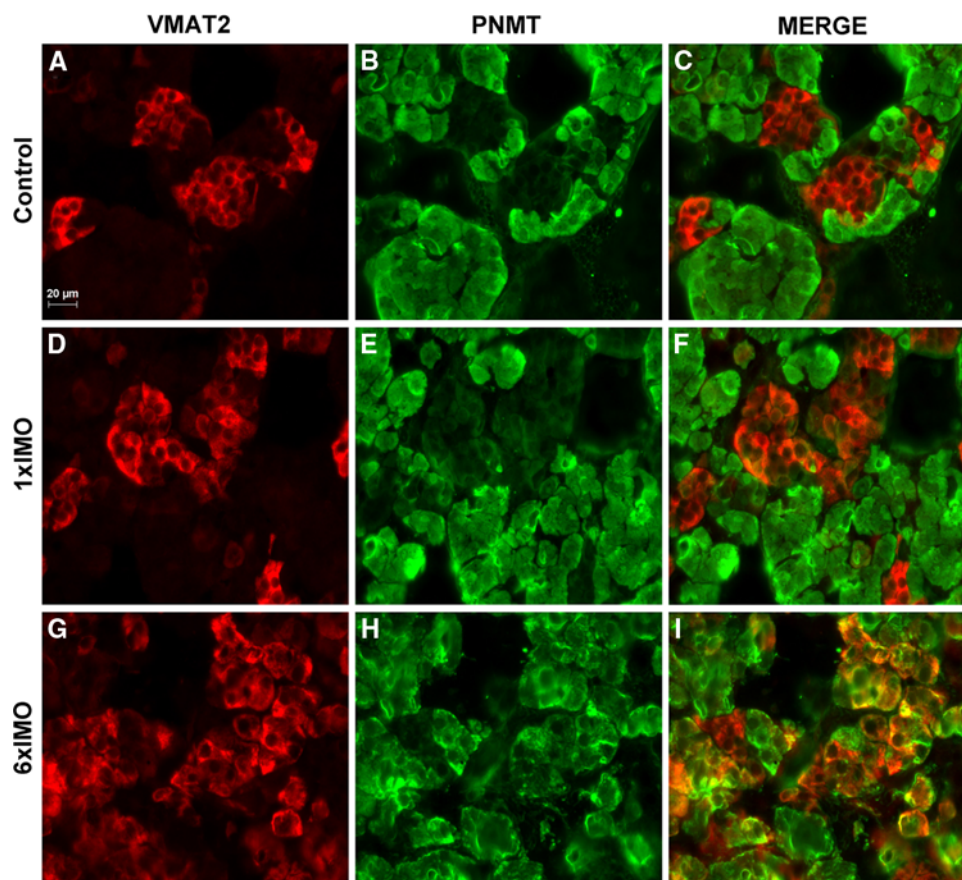

Fig. 4 Expression of VMAT2 and PNMT in response to single and repeated IMO stress. Immunofluorescence is shown for VMAT2, PNMT and their co-localization in rat AM under basal conditions (control, **a–c**), after single (1× IMO, **d–f**) or repeated (6× IMO, **g–i**) stress

consecutive days of IMO, levels of VMAT2 protein and mRNA are robustly increased. Moreover, the expression of VMAT2 is detected also in many PNMT positive, Epi synthesizing, chromaffin cells.

This is the first study to demonstrate that VMAT2 expression in the adrenal medulla can be regulated by stress. There are limited reports of regulation of VMAT2 expression and only a few observations regarding its response to stress. These relate to changes in VMAT2 expression in the brain. IMO is reported to increase VMAT2 mRNA levels in noradrenergic neurons in A2 and A1 brain nuclei, but not in the locus coeruleus (Rusnak et al. 2001). Exposure to repeated swim stress significantly reduced VMAT2 density in dopaminergic neurons of the nucleus accumbens and striatum (Zucker et al. 2005). In gastric enterochromaffin-like histamine producing cells, gastrin enhances VMAT2 expression by way of MAPK-activated phosphorylation of CREB and binding to a gastrin-response element in the VMAT2 promoter (Gerhard et al. 2001; Catlow et al. 2007).

Here, we show increased VMAT2 mRNA expression in the adrenal medulla after single and repeated exposure to IMO, and higher VMAT2 protein expression following repeated IMO. In addition to elevation in levels of VMAT2 mRNA and protein, the study revealed more VMAT2 expressing adrenomedullary chromaffin cells following repeated IMO, with expression in many of the Epi synthesizing chromaffin cells not observed under basal conditions. The localization of VMAT1 and VMAT2 in the adrenal medulla under unstressed conditions is in good agreement with previous studies. VMAT1 is reported to be expressed in virtually all chromaffin cells in the adrenal medulla from adult rats with restricted expression of VMAT2 to a subpopulation of chromaffin cells (Peter et al. 1995; Erickson et al. 1996; Hansson et al. 1998; Schutz et al. 1998; Weihe and Eiden 2000).

Repeated IMO has been shown to elevate expression and activity of catecholamine biosynthetic enzymes in the adrenal medulla [reviewed in (Sabban and Kvetnansky 2001; Kvetnansky et al. 2009)]. The increase in VMAT2 observed here, and the expression in Epi synthesizing chromaffin cells, may facilitate more efficient use of the elevated catecholamine biosynthesis. The greater affinity for monoamines and higher turnover number of VMAT2 compared to VMAT1 (Peter et al. 1994) may enable chromaffin cells to better respond to increased demand for catecholamines with repeated IMO.

In this regard, an early study (Kvetnansky et al. 1970) of the adrenal NE and Epi contents after various repetitions of daily IMO showed that while after acute exposure to IMO stress NE and Epi were partially depleted, with further daily IMO, levels of NE and Epi not only restored to initial levels but there was an overcompensation to levels 60–100% higher than in unstressed controls. This would suggest that with repeated exposure to IMO the adrenal is primed to respond to an additional stress input with even greater intensity. Thus, the finding of increased expression of VMAT2 with repeated IMO would be consistent with increased capacity or strength of catecholamine storage, as an important adaptation to repeated stress.

In conclusion, VMAT2 is expressed by a subpopulation of rat chromaffin cells. Under basal conditions, these are exclusively NE synthesizing cells. Repeated IMO stress increased expression of VMAT2 levels also in many Epi synthesizing cells, indicating an adaptation of the adrenal medulla with prolonged exposure to a strong stressor which could provide a mechanism to facilitate utilization of the enhanced catecholaminergic capacity.

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