**INCREASED EXPRESSION OF AQUAPORIN 4 IN THE RAT HIPPOCAMPUS AND CORTEX DURING TRIMETHYLtin-INDUCED NEURODEGENERATION**


Abstract—Trimethyltin chloride (TMT) is a neurotoxicant producing neuronal degeneration and reactive astrogliosis in the mammalian central nervous system, especially the hippocampus. A previous magnetic resonance imaging investigation in TMT-treated rats evidenced dilation of lateral ventricles, also suggesting alterations in blood–brain barrier permeability and brain edema. Aquaporin 4 (AQP4), a glial water channel protein expressed mainly in the nervous system, is considered a specific marker of vascular permeability and thought to play an important role in brain edema (conditions). We studied AQP4 expression in the hippocampus and cerebral cortex of TMT-treated rats in order to explore the molecular mechanisms involved in brain edema occurring in these experimental conditions. Real-time PCR and western blotting data showed significant up-regulation of both AQP4 mRNA and protein levels starting 14 days after TMT treatment in the hippocampus and cortex. Parallel immunofluorescence studies indicated intense astrogliosis and AQP4 immunoreactivity diffusely pronounced in the hippocampal and cortex areas starting 14 days after TMT intoxication. In order to study the effects of TMT on vascular integrity, double-label immunofluorescence experiments for rat immunoglobulin G (IgG) and rat endothelial cell antigen-1 (RECA-1) or neuronal nuclei (NeuN) (endothelial and neuronal markers respectively) were performed. The results indicated, at 21 and 35 days after treatment, the presence of rat IgG in paravascular parenchyma and in some neuronal cells of the hippocampus and cortex. The extravasated IgG staining was temporally correlated with over-expression of neuronal vascular endothelial growth factor (VEGF) and the active phosphorylated form of its neuronal receptor (VEGFR-2P), suggesting that these factors may cooperate in mediating vascular leakage.

Key words: trimethyltin, hippocampus, cortex, Aquaporin 4, vascular endothelium growth factor, neurodegeneration.

**INTRODUCTION**

Trimethyltin chloride (TMT) is a well characterized neurotoxicant. It is a by-product in the manufacture of dimethyltin chloride, commonly used in both industrial and agricultural settings. TMT intoxication produces significant, selective neuronal death in the rodent limbic system, especially in the CA1 and CA3/CA4 subfields of the hippocampus, but also in layer II of the piriform/entorhinal cortex and in the pyramidal cells of the neocortex (Chang and Dyer, 1985; Aschner and Aschner, 1992; Koczyk, 1996; McCann et al., 1996; Nilserth et al., 2002; GeloSO et al., 2011; Kuramoto et al., 2011; Lattanzi et al., 2013).

In animal models TMT treatment induces behavioral alterations such as hyperactivity, hyperexcitability, aggression and cognitive deficits that include memory loss and learning impairment (Dyer et al., 1982a,b; Chang and Dyer, 1983; Balaban et al., 1988).

The molecular mechanisms that mediate TMT-induced toxicity have not yet been completely elucidated, although many hypotheses have been formulated (Patel et al., 1990; PavlakoviC et al., 1995; Gunasekar et al., 2001; GeloSO et al., 2002). The neurotoxicant action has nonetheless been shown to be mediated by intracellular Ca2+ concentration overload in cultured rat hippocampal neurons (PiAcentiNi et al., 2008), in HeLa and neuroblastoma cells (Florea et al., 2005a,b) and also in spiral ganglion cells (Fechter and Liu, 1995). In this respect, it is noteworthy that rat hippocampal neurons expressing the Ca2+-binding proteins calretinin and parvalbumin are selectively spared by the neurotoxicant (GeloSO et al., 1996, 1997, 1998; Businaro et al., 2002; Gangitano et al., 2006), possibly through a calcium-buffering effect. Finally, we have previously demonstrated that lysosomal protease cathepsin D

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Abbreviations: AQP4, Aquaporin 4; BBB, blood–brain barrier; FC, fold change; GFAP, glial acidic fibriillary protein; i.p., intraperitoneal; IgG, immunoglobulin G; MRI, magnetic resonance imaging; NDS, normal donkey serum; NeuN, neuronal nuclei; PB, phosphate buffer; PBS, phosphate-buffered saline; PFA, paraformaldehyde; PVDF, polyvinylidene fluoride; RECA-1, rat endothelial cell antigen-1; RT, room temperature; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TBS-T, Tris–buffered saline–Tween; TMT, trimethyltin; VEGF, vascular endothelial growth factor; VEGFR-2, vascular endothelial growth factor receptor-2; VEGFR-2P, vascular endothelial growth factor receptor-2 phosphate.

http://dx.doi.org/10.1016/j.neuroscience.2014.05.047
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plays a crucial role in the TMT-induced hippocampal neurodegeneration process in a calcium-dependent manner (Ceccariglia et al., 2011).

TMT neurodegeneration is also accompanied by an increased expression of astrocytes and activation of microglial cells (Koczyk and Oderfeld-Nowak, 2000; Geloso et al., 2004; Reali et al., 2005; Röhl and Sievers, 2005; Little et al., 2012). There is also increasing evidence that astrocytic cells play a crucial role in neurological disorders and may be considered a potentially useful target to address brain injury (Steinhäuser and Seifert, 2002; Tian et al., 2005; Binder and Steinhäuser, 2006; Seifert et al., 2006).

Aquaporins (AQPs) are a family of plasma membrane water-channel proteins expressed in many tissues and cell types (Verkman, 2002, 2005). In particular, Aquaporin 4 (AQP4), the most studied member of this family, is expressed in the brain and is localized mainly in pericapillary astrocytic end-feet (Nielsen et al., 1997; Rash et al., 1998; lacovetta et al., 2012). This pattern of distribution suggests an active role for AQP4 in the control of water flow into and out of the brain, although this protein has also been proposed to be involved in astrocyte migration and in the regulation of neuronal activity (e.g. synaptic plasticity) (Nagelhus et al., 2004; Tait et al., 2008; lacovetta et al., 2012; Nagelhus and Ottersen, 2013; Scharman and Binder, 2013). Essentially, AQP4 is regarded as a specific marker of vascular permeability and, in general, its deregulation is believed to be potentially associated with damage to the blood–brain barrier (BBB) and to play a role in brain edema (Vizuete et al., 1999; Ke et al., 2001; Nico et al., 2003; Warth et al., 2007; Nico and Ribatti, 2012). An altered expression of AQP4 has been studied in several, different diseases such as cerebral ischemia, brain traumatic injury, brain tumors, hydrocephalus and kainic acid-induced status epilepticus (Tangichi et al., 2000; Saadoun et al., 2002; Nesic et al., 2006; Aghayev et al., 2012; Lee et al., 2012).

To date, the role of AQP4 protein in TMT neurotoxicity has not been studied, though a magnetic resonance imaging (MRI) investigation, in TMT-treated rats, evidenced dilation of lateral ventricles also suggesting alterations in blood–brain barrier permeability and brain edema (Andjus et al., 2009).

Vascular endothelial growth factor A (VEGF-A, abbreviated as VEGF) and its main receptor-2 (VEGFR-2) are known for their essential roles in blood vessel growth, neurogenesis and neuroprotection (Morin-Brureau et al., 2011; Mackenzie and Ruhrberg, 2012; Licht and Keshet, 2013). Most importantly, previous studies have shown that the VEGF/VEGFR-2 pathway can also increase vascular permeability, thereby disrupting the BBB, and promote inflammatory cell infiltration in the nervous system (Froescholdt et al., 1999; van Bruggen et al., 1999; Zhang et al., 2000; Klic et al., 2006; Lafuente et al., 2006).

The present study aimed to investigate AQP4 expression, vascular leakage and VEGF/VEGFR-2 signaling pathway in the rat hippocampus and cortex during TMT-induced neurodegeneration, in order to explore possible molecular mechanisms involved in alterations to vascular permeability and brain edema occurring in this experimental model of neurodegeneration.

**EXPERIMENTAL PROCEDURES**

**Animals and treatment**

Experiments were performed using adult female Wistar rats (200–250 g body weight, age 2 months). Animals were housed in an air-conditioned environment with constant temperature (25 °C) and a standardized light/dark schedule, food and water being supplied ad libitum. The rats were treated with a single intraperitoneal (i.p.) injection of TMT (Sigma, St. Louis, MO, USA) dissolved in saline 0.9% NaCl at a dose of 8 mg/kg body weight in a volume of 1 ml/kg body weight. Control rats received the same volume of saline solution. The animals were anesthetized by i.p. injection with 2 mg/100 g body weight of diazepam (Biologici Italia Laboratories, Milano) followed by 4 mg/100 g body weight of ketamine (Intervet International, GmbH, Germany) and then sacrificed 7, 14, 21 or 35 days after treatment. All the animal protocols were approved by the Animal Experimentation Committee of Catholic University, Rome. In particular, animal experiments were performed in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC). Every effort was made to minimize the number of animals used and their suffering.

**AQP4 and glial acidic fibrillary protein (GFAP) real-time PCR analyses**

AQP4 and GFAP expression levels were analyzed by quantitative real-time PCR (qPCR) in the rat hippocampus and cortex. Total RNA was isolated both from the entire hippocampus and from the cortex for each control and each 7-, 14-, 21- and 35-day TMT-treated animal (3 animals/group, sacrificed by decapitation after deep anesthesia) using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and purified using the RNeasy MiniElute Cleanup Kit (Qiagen, Valencia, CA, USA), according to the manufacturer’s instructions. An additional on-column DNase incubation step was performed to allow the selective removal of genomic DNA during the isolation process. Thereafter, two-step reverse transcription and qPCR were carried out as previously described (Barba et al., 2012). The oligonucleotide primers were designed using Primer 3 software (http://bioinfo.ut.ee/primer3-0.4.0/), primer sequences are reported in Table 1.

Real-time PCR was performed in at least three independent experiments.

**Western blotting analyses**

For western blotting analyses, the entire hippocampus or cortex of control and TMT-treated (7, 14, 21, 35 days) rats (3 animals/group, sacrificed by decapitation after deep anesthesia) were dissected out and mechanically homogenized in radio immune precipitation assay
(RIPA) buffer (50 mM Tris–HCL, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS and 0.5% sodium deoxycholate) with a protease inhibitor cocktail (Sigma–Aldrich, St Louis, MO, USA) added just before use. Homogenized tissues were incubated on ice for 30 min and then centrifuged at 14,000 rpm for 30 min at 4 °C. The supernatants were collected and protein concentrations were determined using Lowry’s method (Lowry et al., 1951).

Equal amounts of protein (40 μg/lane) from each sample were electrophoresed on 8–12% SDS–PAGE and western blotted at 100 V for 2 h to Hybond polyvinylidene fluoride (PVDF) membranes (Amersham Biosciences, Uppsala, Sweden). Bovine serum albumin (5% w/v) in Tris–buffered saline (50 mM Tris–HCL, pH 7.4, 150 mM NaCl, 0.1% Tween 20) (TBS-T) was used to block the membranes for 1 h at room temperature (RT). After washing with TBS-T the membranes were incubated overnight at 4 °C with appropriate primary antibody: goat anti-AQP4, or rabbit anti-VEGF, or mouse anti-VEGFR-2 (1:100, Santa Cruz Biotechnology, CA, USA), or rabbit anti-VEGFR-2P (phosphorylation sites on tyrosine 1054 and tyrosine 1059) (1:200, Abcam, Cambridge, UK) and mouse anti-β-actin (used to normalize each protein loading) (1:1000, Sigma–Aldrich). After extensive washing with TBS-T, PVDF membranes were incubated for 1 h at RT with adequate horseradish peroxidase-conjugated secondary antibody IgG (1:2000, Santa Cruz Biotechnology). After three washes, bands were visualized using Kodak BioMax light autoradiography. A GS-800 Calibrated Densitometer (Bio-Rad Hercules, CA, USA) was used to scan and store images of the immunoblotting. Densitometry was performed using Quantity One (Bio-Rad) software.

Western blotting analysis to determine the mature form (42 kDa) of VEGF factor was performed in non-reducing conditions.

For immunoglobulin G (IgG) analysis, the entire hippocampus or cortex of control and TMT-treated (14, 21, 35 days) rats (3 animals/group) was dissected out and processed as described above. After incubation with biotinylated goat anti rat-IgG secondary antibody (1:200, Vector Laboratories, Burlingame, CA, USA) and with an avidin–biotin peroxidase complex (ABC method, 1:500, Vector Laboratories), antibody reactivity was detected using 3,3’-diaminobenzidine tetrahydrochloride (Sigma–Aldrich) and 0.01% v/v hydrogen peroxide in phosphate-buffered saline (PBS). Monoclonal antibody against β-actin (1:20,000, Sigma–Aldrich) was used to normalize protein loading. The optical density of the heavy chain of IgG (~55 kDa) was analyzed by Bio-Rad Gel doc Bio-Rad Multi-analyser software.

All western blotting analyses were performed in at least three independent experiments.

**Immunohistochemical localization of AQP4, VEGF and VEGFR-2**

Under deep anesthesia the animals were perfused by a peristaltic pump through the aorta with 250 ml of saline solution, followed by 250 ml of 4% (w/v) paraformaldehyde (PFA) at a rate of 4–5 ml/min. About 30 min after perfusion brains were removed from the skull, post-fixed in 4% PFA overnight at 4 °C and transferred in phosphate buffer (PB) 0.1 M pH 7.4. Serial 40-μm-thick coronal sections were cut on a vibratome (Leica VT 1000S) and collected in a free-floating manner.

Tissue sections of control and 7-, 14-, 21- and 35-day TMT-treated animals (3 animals/group) were processed for triple and double immunofluorescence labeling. Non-specific binding sites were blocked with 5% normal donkey serum (NDS) in PBS 0.01 M pH 7.4 for 1 h at RT. One first series of sections was incubated with the following primary antibodies: goat anti-AQP4 (1:200, Santa Cruz Biotechnology), rabbit anti-vascular endothelial growth factor (VEGF) (1:100, Santa Cruz Biotechnology) and mouse anti-GFAP for astrocytes (1:500, Chemicon, Temecula, CA, USA) or mouse anti-Neu N antibody for neurons (1:500, Chemicon). A second series of slices was incubated with the following primary antibodies: mouse anti-VEGF receptor-2 (VEGFR-2 also named Flk-1, 1:100, Santa Cruz Biotechnology) and mouse anti-GFAP for astrocytes (1:500, Chemicon, Temecula, CA, USA) or mouse anti-Neu N antibody for neurons (1:500, Chemicon). A second series of slices was incubated with the following primary antibodies: mouse anti-VEGF receptor-2 (VEGFR-2 also named Fik-1, 1:100, Santa Cruz Biotechnology) and rabbit anti-VEGF (1:100, Santa Cruz Biotechnology). After incubation in primary antisera overnight at 4 °C, sections were rinsed three times with PBS and then exposed to the appropriate donkey secondary antibodies: Cy3 anti-goat for AQP4 (1:400, Jackson Immunoresearch Laboratories, West Grove, PA, USA), Alexa Fluor 647 (1:200, Jackson Immunoresearch Laboratories) and Cy2 (1:250, Jackson Immunoresearch Laboratories) anti-rabbit for VEGF, Alexa Fluor 488 anti-mouse for GFAP and neuronal nuclei (NeuN) (1:250, Jackson Immunoresearch Laboratories) and Cy3 anti-mouse for VEGF-2 (1:400, Jackson Immunoresearch Laboratories), for 1 h at RT. After further washing, sections were mounted on glass slides, cover-slipped with gel-mount anti-fading mounting medium (Sigma–Aldrich), examined and photographed under a Zeiss LSM 510 confocal laser scanning microscopy system.

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**Table 1. List of primer sequences used in this study**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Symbol</th>
<th>Sense</th>
<th>5′–3′ sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin-beta</td>
<td>ACTB</td>
<td>F</td>
<td>ATGGTGCGATGGTATGGTCAGAAA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>GGTCTCATTTTCCACGTTGGG</td>
</tr>
<tr>
<td>Aquaporin 4</td>
<td>AQP4</td>
<td>F</td>
<td>TGGCAGTTTTGTGTCCTTGGA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>CCGCTTGGTCTGTAGGTTTC</td>
</tr>
<tr>
<td>Glial acidic fibrillary protein</td>
<td>GFAP</td>
<td>F</td>
<td>ACATCGAGATGGCCACCTAC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>ATGGTGATGCCGTTTTCTTC</td>
</tr>
</tbody>
</table>

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**List of primer sequences used in this study**

- **GFAP** F: 5′-ATGGTGATGGCGGTTTTCTTC-3′
- **AQP4** F: 5′-TGGCAGTTTTGTGTCCTTGGA-3′
- **ACTB** F: 5′-ATGGTGATGGCGGTTTTCTTC-3′

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The specificity of the labeling was confirmed by incubation of sections with only the secondary antibodies, omitting the primary antisera. No immunoreactivity was observed under these conditions. Five non-consecutive sections were immunostained from each animal/time point.

**IgG immunofluorescence labeling**

In order to investigate possible TMT-induced damage of the BBB, we assessed the degree of endogenous IgG leakage in the rat hippocampus and cerebral cortex using immunofluorescence labeling with fluorescein anti-rat IgG antibody. Because IgGs are not normally present in brain parenchyma (but are restricted to the inside of vessels), immunoreactivity of these molecules in brain parenchyma is regarded as a reliable indicator of BBB alteration (Rigau et al., 2007; Sun et al., 2012).

IgG immunofluorescence was investigated in the hippocampus and cortex of controls and 14-, 21- and 35-day TMT-treated rats (3 animals/group). The animals were perfused as described above.

Serial 30-μm-thick coronal sections were cut on a vibratome and processed for double label immunofluorescence.

After several rinses with PBS, tissue sections were blocked with 5% NDS in PBS for 1 h at RT, followed by incubation overnight at 4 °C with the primary antibodies against: mouse rat endothelial cell antigen-1 (RECA-1) (1:250, AbD Serotec, Kidlington, Oxford, UK), for some sections, and mouse anti-NeuN (1:500, Chemicon) for the others. After incubation in primary antisera, sections were rinsed with PBS, exposed to the appropriate secondary antibodies: donkey Cy3 anti-mouse (1:400, Jackson Immunoresearch Laboratories) for 1 h at RT and then incubated with rabbit fluorescein anti-rat IgG secondary antibody (1:250, Vector Laboratories) overnight at 4 °C. After further washing, sections were processed, observed and photographed as described above.

Background staining was not observed in negative control sections, when the primary antisera were omitted.

Five non-consecutive sections were immunostained from each animal/time point.

**Statistical analysis**

The real-time PCR results are expressed as fold changes (FC), calculated according to the ΔΔCt method, and presented as mean ± SEM. For statistical analyses, group differences were evaluated using a one-way analysis of variance [Analysis of Variance (ANOVA) Calculator-One-Way ANOVA from Summary Data-Software. Available from http://www.danielsoper.com/statcalc] and the Bonferroni post-hoc test, assuming the level of probability *P < 0.05* as significant. For western blotting results, data are expressed as mean ± SEM. Statistical analyses were performed by a one-way ANOVA followed by Fisher’s test, assuming the levels of probability *P < 0.05* and **P < 0.001** as significant.

**RESULTS**

**AQP4 protein is over-expressed after TMT treatment and is localized in astrocytic cells**

We analyzed AQP4 and GFAP gene expression at different intervals after TMT treatment (7, 14, 21 and 35 days). Real-time PCR analysis revealed a significant up-regulation (*P < 0.05*) of the AQP4 gene in the hippocampus, starting 14 days after treatment (FC = 4.1); this up-regulation remained significantly high at 21 (FC = 3.8) and 35 days (FC = 4.6) (Fig. 1A). The level of AQP4 in the cortex was also significantly up-regulated (*P < 0.05*) starting from 14 days after treatment (FC = 3.7), but decreased slightly after 21 (FC = 2.9) and 35 days (FC = 2.7) (Fig. 1B) while remaining statistically significant. GFAP gene expression showed significant up-regulation (*P < 0.05*) already 7 days after treatment in both the hippocampus (FC = 6.8) and the cortex (FC = 3.9). The GFAP expression level was higher 14 days after treatment in both the hippocampus (FC = 14.1) and the cortex (FC = 9.7). After 21 and 35 days of intoxication the GFAP gene expression decreased in both tissues but remained statistically significant (hippocampus at 21 and 35 days: FC = 13.2 and 11.6 respectively; cortex at 21 and 35 days: FC = 5.4 and 5 respectively) (Fig. 1C,D).

In order to analyze AQP4 protein expression, western blotting analysis was carried out 7, 14, 21 and 35 days after toxin administration. AQP4 protein was significantly over-expressed (*P < 0.001*) from 14 to 35 days in the hippocampus (Fig. 2A). Similarly, in the cortex we observed a significant albeit less marked increase (*P < 0.05*) at 14 days in AQP4 levels, which were further increased (*P < 0.001*) 21 and 35 days after treatment (Fig. 2B).

In order to visualize the distribution and cellular localization of the AQP4 immunoreactivity in the hippocampus and cortex of control and TMT-treated rats, immunofluorescence experiments using an antibody to AQP4 in combination with GFAP and NeuN markers, were performed.

In all hippocampal regions and in the cerebral cortex areas, we observed moderate AQP4 co-localization with GFAP-positive astrocytic cells 7 days after TMT intoxication (Fig. 3H). From 14 to 35 days after treatment, AQP4 immunolabeling in the activated astrocytes exhibited a diffuse, marked increase, in accordance with PCR and blotting data, in both the hippocampus (Fig. 3J,N,R) and cortex areas (Fig. 4J,N,R), where an intense and reactive astrocytosis was observed (Figs. 3 and 4I,M,Q). AQP4 immunoreactivity was localized in astrocyte end-feet surrounding blood vessels, as expected. We also observed that AQP4 immunolabeling was not completely co-localized with GFAP staining in either the hippocampus or the cortex at any of the time-points analyzed. This is in accordance with the observation of Simard and Nedergaard (2004) that many fine leaflet astrocytic processes are not labeled with anti-GFAP antibodies.
All the experiments performed on the control animals evidenced only weak AQP4 labeling (Figs. 3 and 4B) co-localized with GFAP (Figs. 3 and 4A). AQP4 protein did not co-localize with NeuN-positive neuronal cells of either the hippocampus or the cortex at any time after TMT treatment (Fig. 7D,H).

IgG is extravasated after TMT treatment

In order to investigate the effects of TMT on BBB integrity, we first measured endogenous IgG levels by western blotting analysis in the hippocampus and cortex homogenates of control and toxin-treated (14, 21 and 35 days) rats. Protein levels increased significantly \((P < 0.05)\) at 21 days in the hippocampus and decreased at 35 days, while remaining higher than in the controls (Fig. 2G). The levels of cortex IgG increased moderately at 21 and 35 days after toxin administration (Fig. 2H). No IgG increase (compared with control values) was observed 14 days after treatment in either the hippocampus or the cortex (data not shown).

We then evaluated extravasation of IgG by double label immunofluorescence experiments using an anti-rat IgG fluorescein antibody in combination with a vascular endothelium (RECA-1) or neuronal marker (NeuN), because of the importance of endothelial and neuronal components in the context of BBB alteration.

We observed partial immunolabeling of both IgG and RECA-1 in the CA1 hippocampal subfield (Fig. 5F) and in some cortical areas (Fig. 6F) 21 days after TMT treatment. In addition, at the same time after treatment (21 days), we found, in the CA3 and in some cortical regions, extra-vascular IgG staining (green staining) diffused in the paravascular parenchyma (Figs. 5K and 6H). It is also interesting to note that 35 days after toxicant administration the immunofluorescence images showed a selective accumulation of IgG in the cytoplasm of a few residual neurons scattered in the hippocampus (Fig. 5R). Similarly, at the same treatment time (35 days), some IgG-positive neurons were clearly visible in the cortex (Fig. 6O). Some neuronal cells were faintly IgG-stained, whereas others were strongly IgG-labeled. Notably, many damaged IgG-positive neurons lost their NeuN immunogenicity.

The IgG extravasation was not seen 14 days after TMT treatment. As expected, no IgG staining was observed in control samples (Figs. 5B,H,N and 6B,K).

TMT induces VEGF/VEGFR-2 pathway over-expression and VEGFR-2 activation in neuronal cells

To examine the possible implications of VEGF and VEGFR-2 in the event of BBB impairment during TMT intoxication, we analyzed the expression and cellular localization of these proteins in both the hippocampus and cortex of control and TMT-treated rats.

We first measured the levels of VEGF and VEGFR-2 by immunoblotting at 7, 14, 21, 35 days after TMT treatment. VEGF protein levels were significantly increased \((P < 0.001)\) starting from 7 days in both the hippocampus and the cortex and remained persistently over-expressed at later treatment times (14, 21, 35 days) (Fig. 2C,D).

To confirm further the role of VEGF protein, we evaluated the expression levels of its receptor
Fig. 2. TMT-induced increased expression of AQP4, VEGF, VEGFR-2, VEGFR-2P and IgG proteins in the rat hippocampus and frontal–parietal cortex. (A–H) Graphic presentations and western blotting images of AQP4, VEGF, VEGFR-2, VEGFR-2P proteins and IgG accumulation are shown. Values are presented as mean ± SEM for each group: the control rats (n = 3) and 7-, 14-, 21-, 35-day TMT-treated rats (n = 3/group), *P < 0.05 and **P < 0.001 compared with controls, Fisher's-test. Ctrl: control sample.
VEGFR-2. The receptor expression was transiently and significantly increased ($P < 0.001$) at 7 days after TMT treatment in the hippocampus and at 7–14 days in the cortex, then gradually decreased to control values at later treatment times in both analyzed areas (Fig. 2E, F).

In addition, we measured a characteristic feature of VEGFR-2 activation: the trans-autophosphorylation of

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**Fig. 3.** Increased expression of AQP4 and VEGF in the rat hippocampus after TMT treatment. Sections of the CA3 region at different treatment times (7, 14, 21, 35 days) are triple-labeled for GFAP (green), AQP4 (red), VEGF (blue) and GFAP/AQP4/VEGF (merge). Control sections (A–D). After 7 days of toxicant administration AQP4 immunoreactivity (F) is faintly observed in GFAP-positive cells (E) and VEGF staining (G) is more pronounced compared with controls. At 14, 21 and 35 days after treatment a marked increase in both AQP4 immunoreactivity (J, N, R) in GFAP-labeled astrocytes (I, M, Q) and VEGF staining (K, O, S) is observed. Note the gradual increase in the number of GFAP-positive cells in TMT-treated rats (7, 14, 21, 35 days). Scale bar = 100 μm. Ctrl: control sample, d: days. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
Fig. 4. Increased expression of AQP4 and VEGF in the rat cortex after TMT treatment. Sections of the frontal–parietal cortex at different treatment times (7, 14, 21, 35 days) are triple-labeled for GFAP (green), AQP4 (red), VEGF (blue) and GFAP/AQP4/VEGF (merge). Control sections (A–D). After 7 days of toxicant administration AQP4 immunoreactivity (F) is faintly observed in GFAP-positive cells (E) and VEGF staining (G) is more pronounced compared with controls. At 14, 21 and 35 days after treatment a marked and notable increase in both AQP4 immunoreactivity (J,N,R) in GFAP-labeled astrocytes (I,M,Q) and VEGF staining (K,O,S) is observed. Note the gradual increase in the number of GFAP-positive cells in TMT-treated rats (7, 14, 21, 35 days). Scale bar = 100 μm. Ctrl: control sample, d: days. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
Fig. 5. Immunohistochemical localization of extravasated IgG in the rat hippocampus after TMT treatment. Double label immunofluorescence for endothelial cells (RECA-1, red, A,D,G,J) or neuronal cells (NeuN, red, M,P), IgG (green) and RECA-1/IgG or NeuN/IgG (merge) in control and TMT-treated rats (21, 35 days) is observed. IgG immunoreactivity is shown in CA1 (E) and CA3 (K) areas at 21 days and in CA4 area (Q) at 35 days after treatment. The merged images demonstrate co-localization of RECA-1/IgG (F), IgG extravasation in paravascular parenchyma (L) at 21 days of treatment and co-localization of NeuN/IgG (R) at 35 days. C,F,I,L merged images are overlapped with differential interference contrast images. No IgG staining is observed in control sections of CA1 (B), CA3 (H) and CA4 (N) fields. Scale bar = 100 µm. Ctrl: control sample, d: days. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
Fig. 6. Immunohistochemical localization of extravasated IgG in the rat frontal–parietal cortex after TMT treatment. Double label immunofluorescence for endothelial cells (RECA-1, red, A,D,G) or neuronal cells (NeuN, red, J,M), IgG (green), and RECA-1/IgG or NeuN/IgG (merge) in control and TMT-treated rats (21, 35 days) is observed. IgG immunoreactivity is shown in images (E,H) at 21 days and in image (N) at 35 days of treatment. The merged images show co-localization RECA-1/IgG (F), IgG extravasation in paravasal parenchyma (I) at 21 days of treatment and co-localization of NeuN/IgG (O) at 35 days. No IgG staining is observed in control sections (B,K). Scale bar = 100 μm. Ctrl: control sample, d: days. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
tyrosine residues Y1054/Y1059 in the cytoplasmatic domain following receptor activation by VEGF (Dougher and Terman, 1999). The immunoblotting analysis showed that the levels of phospho Y1054/Y1059 (i.e. VEGFR-2P) were significantly increased \( (P < 0.001) \) on day 7 and this increase persisted to 35 days in both the hippocampus (Fig. 2E) and the cortex (Fig. 2F).

Finally, we also performed a series of immunolabeling experiments to identify the cell type(s) expressing VEGF and VEGFR-2 factors during TMT treatment. VEGF immunoreactivity was localized only in neuronal cells (by VEGF/NeuN co-localization), mainly in the soma of hippocampal (Fig. 7D) and cortical neurons (Fig. 7H), and VEGFR-2 staining co-localized with the VEGF-positive neuronal cells in both the hippocampus (Fig. 8F) and the cortex (Fig. 8L).

**DISCUSSION**

The present results show that AQP4 is up-regulated in both the rat hippocampus and the cortex during neurodegenerative processes triggered by TMT.

AQP4 water channels are known to be primarily expressed in astrocytes surrounding blood vessels in the brain (Vizuete et al., 1999; Ke et al., 2001; Papadopoulos and Verkman, 2007; Saadoun and Papadopoulos, 2010; Iacovetta et al., 2012). Our data show AQP4 immunoreactivity to be increased and diffuse in perivascular astrocytes between 14 and 35 days after treatment in all hippocampal (Fig. 3J,N,R) and (frontal–parietal) cerebral cortex areas (Fig. 4J,N,R). RT-PCR and western blotting analyses also show significant up-regulation respectively of AQP4 mRNA (Fig. 1A,B) and protein expression (Fig. 2A,B) in all the areas analyzed, at the same time points (14–35 days) after toxicant treatment. Both in the hippocampus and in the cortex the over-expression of AQP4 appears to be related to TMT-induced reactive astrociosis, as evidenced by immunofluorescence (Figs. 3 and 4E,I,M,Q). Activation of astrocytes in response to TMT, which is a well-known phenomenon, is also confirmed by the increased mRNA level of GFAP, which is a recognized hallmark of reactive astrocytes (Koczyk and Oderfeld-Nowak, 2000; Jahnke et al., 2001; Pekny and Nilsson, 2005). In addition, AQP4 and GFAP gene expression analysis during toxicant treatment revealed a direct correlation between AQP4 and GFAP mRNA up-regulation (between 14 and 35 days after treatment) (Fig. 1), the increase in GFAP mRNA being higher and earlier (7 days) than that in AQP4 mRNA. This might suggest that the delayed induction of AQP4 does not participate in the initiation of astrogliosis and the up-regulation of protein is linked to an increased glial reaction rather than to an increase in the protein in each individual astrocyte. It is possible that AQP4 participates in an apparent dilation of lateral ventricles as suggested by Andjus and collaborators (2009). It is also noteworthy, in this respect, that TMT treatment also reduces the wet weight of the hippocampus in a time- and dose-dependent fashion (Brock and O’Callaghan, 1987). The possibility that this reduction in hippocampal weight would participate to the appearance of dilated ventricles should also be taken into account, although the TMT-induced effects do not produce an extended and massive brain atrophy, being essentially restricted to the hippocampus.

At present it is unclear which signaling pathway may lead to AQP4 expression changes in astrocytes following TMT neurodegeneration. Many conditions have been reported to induce modifications in the expression of AQP4. In this respect, it has been
reported that exposure of the brain to hypo-osmotic shock increases AQP4 protein levels (Vajda et al., 2000), and that endothelin-1, which increases after stroke, induces AQP4 expression and edema (Lo et al., 2005). Interestingly, Arima et al. (2003) showed that hyperosmotic stress induced by mannitol increased the expression of AQP4 in cultured rat astrocytes, suggesting that oxidative stress mechanisms are involved in AQP4 expression regulation. It is interesting, in this respect, that several authors have demonstrated that
TMT-mediated neurotoxicity is associated with an increase in oxidative stress in both animal and cell models (Shin et al., 2005; Qu et al., 2011; Kaur and Nehru, 2013).

Considering the potential roles of AQP4 in brain edema (Papadopoulos and Verkman, 2007; Saadoun and Papadopoulos, 2010), we also evaluated the level of BBB integrity by immunostaining extravasated endogenous rat IgG, which is regarded as a marker of BBB injury (Rigau et al., 2007; Sun et al., 2012), in both the rat hippocampus and cortex, 21–35 days following TMT treatment. The IgG levels increased significantly 21 days after treatment in the hippocampus (Fig. 2G), as already observed in conditions of BBB impairment (Chen et al., 2008; Jalal et al., 2012; Sun et al., 2012). In fact, the extravasation of IgG in cases of BBB impairment has been described in several diseases of the central nervous system (Ryu and McLarnon, 2009; Ndode-Ekane et al., 2010; Michalak et al., 2012, 2013). In our study, we found moderate IgG immunolabeling in endothelial capillary cells and/or in the brain parenchyma outside blood vessels, as evidenced by double-labeling studies using the endothelial marker RECA-1 (Figs. 5F, L and 6F, I). In the areas investigated, some neuronal cells also appeared to be IgG-labeled (Figs. 5R and 6O). Based on the assumption that neurons double-labeled by anti-NeuN and anti-IgG antibodies have ingested IgG, this finding could be interpreted as TMT-induced cellular damage, as has been shown in ischemic brain tissue (Michalski et al., 2010). In addition, we observed IgG-positive neurons (35 days after intoxication) that displayed marked evidence of degeneration and loss of NeuN staining.

Our findings on IgG leakage are not consistent with those obtained by a previous study (Little et al., 2002), which found that exposure of rats to TMT did not result in extravascular immunostaining of IgG in the hippocampus 21 days after intoxication. This discrepancy could reasonably be due to different IgG staining methods used and to their different sensitivity (avidin/biotin complex system versus immunofluorescence used in the present study). However, our data on the quantification of IgG levels are in agreement with those of the same Authors, who also found a significant increase in IgG 21 days after treatment. In any case, it is noteworthy that we observed both AQP4 increase and IgG leakage in the same areas (hippocampus and frontal/parietal cortex) in which edema has been observed using MRI investigation during TMT-induced neurodegeneration (Andjus et al., 2009).

In addition, we analyzed cellular localization and expression of VEGF growth factor and its VEGFR-2 receptor, thought to be involved in alterations in vascular permeability, in both the hippocampus and the cortex. We observed an over-expression of VEGF (Fig. 2C,D) and its VEGFR-2 receptor, as well as receptor activation by its phosphorylation (VEGFR-2P) (Fig. 2E,F), in both hippocampal (Figs. 7D and 8F) and cortical neurons (Figs. 7H and 8L).

In the present study, VEGF and VEGFR-2 expression was restricted to neurons both of the hippocampus and cortex in control and TMT-treated rats, while in some neurological disorders (Carmeliet and Storkebaum, 2002), and in seizure disorders (Croll et al., 2004) VEGF and VEGFR-2 have been reported to be expressed both in neurons and glial cells. This finding suggests that glial cells, differently from neurons, are not directly involved in TMT-induced VEGF/VEGFR-2 signaling pathway. This finding is consistent with data observed during ischemic preconditioning (Lee et al., 2009), while the biological meaning of the exclusive involvement of neurons in VEGF/VEGFR-2 signaling pathway regulation in these brain injury models deserves further investigation.

Interestingly, an involvement of the neuronal VEGF/VEGFR-2 system in BBB integrity has been suggested in several neuroinflammatory pathologies such as experimental autoimmune encephalomyelitis, Alzheimer’s disease and kainate-induced epilepsy (Ballabh et al., 2004; Zlokovic, 2008; Argaw et al., 2009; Morin-Brureau et al., 2011). It is also interesting, in this respect, that VEGF-2 activation precedes IgG extravasation, as well as continuing during the latter phenomenon. These findings might hint at a pivotal role for the VEGF/VEGFR-2 system in initiating TMT-induced BBB leakage in the analyzed areas. This is in accordance with a previous study (Klic et al., 2006) demonstrating that these factors are related to BBB permeability after focal cerebral ischemia.

Taken together, our data show that the expression of AQP4 protein during the development of the TMT-derived degenerative process, as analyzed by western blotting and immunofluorescence, is correlated with AQP4 mRNA findings; AQP4 expression also appears to be correlated with reactive astrocytosis, as evidenced by GFAP immunofluorescence and mRNA analysis. On the basis of these findings, the early and long-lasting induction of AQP4 may suggest a relevant role of this water channel in the formation/clearance of brain edema in the TMT neurodegeneration model.

Finally, these observations offer useful information that may better elucidate the critical role of this water channel protein, which is currently regarded as a useful marker in neurological disorders such as brain edema, seizure and brain tumors (Papadopoulos et al., 2004; Binder and Steinhäuser, 2006; Papadopoulos and Verkman, 2007) and also as an important tool to identify new therapeutic approaches in these disorders.

CONCLUSIONS

We show AQP4 up-regulation and over-expression in perivascular astrocytes of the rat hippocampus and cortex, suggesting the possible involvement of this water channel protein in brain edema, as outlined by previous MRI investigations during TMT-induced neurodegeneration. We also show IgG leakage into the hippocampal and cortical paravasal parenchyma, as well as enhanced neuronal VEGF/VEGFR-2 production and VEGFR-2 activation (VEGFR-2P), both phenomena being regarded as being related to the increase in vascular leakage in a series of neurological diseases.

Acknowledgments—This work was supported by funds from Catholic University, Rome to C.G. and F.M. We would also like to thank Roberto Passalacqua, Klein Baltazar Del Carmen and Enrico Guadagni for their skilful technical support. We want also
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(Accepted 21 May 2014)
(Available online 6 June 2014)