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Ultrastructural characterization of tumor necrosis factor alpha receptor type 1 distribution in the hypothalamic paraventricular nucleus of the mouse

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Abstract

The immune/inflammatory signaling molecule tumor necrosis factor α (TNF α) is an important mediator of both constitutive and plastic signaling in the brain. In particular, TNF α is implicated in physiological processes, including fever, energy balance, and autonomic function, known to involve the hypothalamic paraventricular nucleus (PVN). Many critical actions of TNF α are transduced by the TNF α type 1 receptor (TNFR1), whose activation has been shown to potently modulate classical neural signaling. There is, however, little known about the cellular sites of action for TNFR1 in the PVN. In the present study, high-resolution electron microscopic immunocytochemistry was used to demonstrate the ultrastructural distribution of TNFR1 in the PVN. Labeling for TNFR1 was found in somata and dendrites, and to a lesser extent in axon terminals and glia in the PVN. In dendritic profiles, TNFR1 was mainly present in the cytoplasm, and in association with presumably functional sites on the plasma membrane. Dendritic profiles expressing TNFR1 were contacted by axon terminals, which formed non-synaptic appositions, as well as excitatory-type and inhibitory-type synaptic specializations. A smaller population of TNFR1-labeled axon terminals making non-synaptic appositions, and to a lesser extent synaptic contacts, with unlabeled dendrites was also identified. These findings indicate that TNFR1 is structurally positioned to modulate postsynaptic signaling in the PVN, suggesting a mechanism whereby TNFR1 activation contributes to cardiovascular and other autonomic functions.

Keywords

Autonomic function; Blood pressure; Cytokine; Dendrite; Excitatory synapse

INTRODUCTION

Tumor necrosis factor α , originally characterized as an inducer of cell death and apoptosis, is now known to be involved in proliferation, differentiation, and growth (Hayashi et al., 2013). More recently, TNF α has also been established to be an important signaling molecule

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involved in neural communication in the brain (Santello and Volterra, 2012). It is known that TNF α can act within the brain by several distinct routes including within circumventricular organs targeted by circulating TNF α , by infiltrating immune cells, particularly in the context of pathological states, or through local production by resident brain cells (Vezzani and Viviani, 2015).

Evidence that systemic administration of TNF α induces hypothalamic-pituitary-adrenal (HPA) axis activity (Bernardini et al., 1990, Matsuwaki et al., 2003), as well as increased monoamine utilization and increased neural activation (Tolchard et al., 1996, Zhang et al., 2003) in the hypothalamic PVN (Hayley et al., 2002), suggests that PVN neurons may be an important target of TNF α . This is supported by other data showing that local application of TNF α in the PVN influences autonomic function, including sympathetic activity and blood pressure (Bardgett et al., 2014, Shi et al., 2014).

Significantly, there appears to be an intrinsic functional TNF α system in the PVN itself, involving production by resident glia (Shi et al., 2010, Du et al., 2015), although there are reports of TNF α -like immunoreactivity in PVN neurons as well (Breder et al., 1993). Within the PVN, TNF α transcription is induced by challenges such as immunological stress (Kakizaki et al., 1999, Masson et al., 2015a). In addition, increased TNF α levels in the PVN are associated with increased local neural activity (Kang et al., 2008, Yu et al., 2015), as well as elevated systemic blood pressure (Sriramula et al., 2013, Dai et al., 2015, Dange et al., 2015) and sympathetic activity (Dange et al., 2015, Yu et al., 2015). All of these physiological responses accompany preclinical models of hypertension including the elevated blood pressure induced by angiotensin II (AngII), a critical blood pressure regulating molecule (Shi et al., 2010, Yu et al., 2015). Elevated PVN TNF α is also seen in spontaneously hypertensive rats (Masson et al., 2015b), as well as following models of heart failure (Guggilam et al., 2007, Wei et al., 2016) and acute myocardial infarction (Du et al., 2015). Importantly, inhibiting PVN TNF α attenuates the hypertension and cardiac hypertrophy seen in spontaneously hypertensive rats (Song et al., 2014).

Tumor necrosis factor is a trimeric type II transmembrane protein that exists in membranous or, following cleavage by the TNF α converting enzyme, soluble forms (Varfolomeev and Vucic, 2016). Functionally, TNF α transduces its intracellular effects by binding two receptors, the TNF α type 1 receptor [TNFR1; also known as p55 or CD120a] and the TNF α type 2 receptor (Varfolomeev and Vucic, 2016). The TNFR1 is able to bind both membranous and soluble TNF α , has a ubiquitous expression pattern, and is the source of much of what is known about TNF α signaling (Varfolomeev and Vucic, 2016). In the brain, TNFR1 is the major mediator of TNF α 's actions (Nadeau and Rivest, 1999, Bette et al., 2003), and is expressed in areas that participate in physiological and behavioral processes, including the PVN (Nadeau and Rivest, 1999, Rizk et al., 2001).

In terms of its signaling properties, TNF α is well known for its transcriptional actions (Varfolomeev and Vucic, 2016), however it can also modulate rapid neurotransmitter signaling. For example, TNF α has been reported to induce the rapid potentiation of glutamate-mediated excitatory currents in neurons (Grassi et al., 1994, Beattie et al., 2002), suggesting that TNFR1 acts as a pre- or postsynaptic modulator of excitatory transmission.

Alternatively, TNF α has also been shown to produce a fast and persistent decrease in inhibitory synaptic strength (Pribram and Stellwagen, 2013), suggesting that TNFR1 may serve as a modulator of inhibitory synaptic communication. Glial cells are another potentially important site where TNFR1 activation may modulate brain function, since there is evidence that TNFR1 activation in astrocytes can influence neuron-glia communication and long-term excitatory synaptic transmission (Habbas et al., 2015). Yet there is little evidence to distinguish these alternative models of TNFR1's actions, particularly in critical areas of neuroautonomic control like the PVN.

In the present study, immunoelectron microscopy, a method with the requisite spatial resolution to identify cellular and synaptic sites of protein localization critical for neural communication, was used to test these varying hypotheses concerning the cellular basis of TNFR1 signaling in the PVN.

METHODS

Subjects

The experimental subjects were five male C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME), weighing 20–25 grams, housed in groups of at least two animals per cage, and maintained on a 12-hr light/dark cycle (lights out 1800 hours) with unlimited access to water and rodent chow in their home cages. Two similarly housed and maintained male TNFR1 knockout (KO) mice on the C57BL/6 background (The Jackson Laboratory) were used to characterize the primary TNFR1 antiserum used in the anatomical studies. All experiments were approved by the Institutional Animal Care and Use Committees at Weill Cornell Medicine in accordance with guidelines established by the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize the number of animals used and their suffering.

Tissue preparation and immunocytochemical procedures

Following deep anesthesia with pentobarbital (150 mg/kg, i.p.), mouse brains were rapidly fixed via aortic arch perfusion at a flow rate of 20 ml/minute sequentially with: (a) 15 ml of 1000 units/ml of heparin in 0.9% saline, (b) 40 ml of a mixture of 3.75% acrolein/2% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB, pH 7.4), and (c) 100 ml of 2% PFA in PB. After dissection from the cranium, each brain was post-fixed in 2% PFA in PB for 60-minutes. For each animal, sections extending through the rostrocaudal extent of the PVN (~0.50 mm to 1.1 mm posterior to bregma) were coronally sectioned (40 μ m) according to the atlas of Hof et al. (Hof et al., 2000) using a vibrating microtome. Tissue sections were processed for immunocytochemical detection of an affinity-purified rabbit polyclonal antibody raised against a synthetic peptide sequence within the N-terminus of human tumor necrosis factor receptor superfamily Member 1A (TNFRSF1A PAB12277; Abnova Corporation, Walnut, CA). This was achieved by using previously described immunoperoxidase and immunogold-silver labeling methods (Milner et al., 2011). Sections were punch coded and pooled into single containers to ensure that tissue sections were identically exposed to reagents (Milner et al., 2011). To remove excess aldehydes, brain tissue was incubated in 1.0% sodium borohydride in PB, followed by washing in PB. After

this, brain sections were washed in 0.1 M Tris-buffered saline (TBS, pH 7.6) followed by a 30-minute incubation in 0.5% bovine serum albumin (BSA) to lessen nonspecific labeling. After rinsing in TBS, brain sections were then incubated for 48-hours in a primary rabbit anti-TNFR1 antiserum diluted in 0.1% BSA (immunoperoxidase (IP): 1:400; immunogold-silver (IGS): 1:100). Following primary antiserum incubation, sections were washed in TBS. For immunoperoxidase identification, sections were then incubated in anti-rabbit biotin-conjugated IgG in 0.1% BSA for 30-minutes. Sections were next rinsed in TBS followed by a 30-minute incubation in avidin-biotin-peroxidase complex (1:100, Vectastain Elite Kit, Vector Laboratories) in TBS. To visualize the bound peroxidase, brain sections were incubated for 5–6 minutes in a 0.2% solution of 3, 3'-diaminobenzidine (Sigma, St. Louis, MO) and 0.003% hydrogen peroxide in TBS, and then washed in TBS.

For immunogold-silver labeling, brain tissue was first rinsed in 0.01 M PBS (pH 7.4). Then, to reduce non-specific binding of gold particles, brain sections were incubated for 10-minutes in a blocking solution consisting of 0.8% BSA and 0.1% gelatin in PBS. After this blocking step, sections were incubated for 2-hours in anti-rabbit 1 nm gold particle-conjugated IgG (1:50, AuroProbeOne, Amersham, Arlington Heights, IL) diluted in the blocking solution. Following this, tissue was rinsed in the blocking solution followed by washing in PBS. Brain sections were then incubated in 2% glutaraldehyde in PBS for 10-minutes followed by rinsing in PBS. Next, the nano-gold particles were enlarged using a silver-enhancement solution for 6-minutes (IntenSE-M kit, Amersham, Arlington Heights, IL).

For electron microscopic analysis, both the immunoperoxidase and the immunogold-silver processed tissue were post-fixed for 1-hour in a solution of 2% osmium tetroxide in PB. Osmified brain sections were then dehydrated in a series of alcohols followed by propylene oxide. Sections were next incubated in a mixture of propylene oxide and EM BED 812 (EMS, Fort Washington, PA) overnight. After this, brain tissue was incubated in EM BED 812 for a minimum of 2-hours and then placed between 2 sheets of Aclar plastic for flat embedment.

Light microscopic assessment of primary antiserum specificity

To verify the specificity of the TNFR1 antiserum, subsets of brain sections from wild-type and TNFR1 KO mice were processed for immunoperoxidase detection of TNFR1 by light microscopy. In addition, in both the immunoperoxidase and immunogold experiments, non-specific labeling was evaluated by similarly processing tissue sections from wild-type mice except without incubating tissue in the primary antiserum. In all cases, sections were processed up until osmium tetroxide post-fixation, at which point they were mounted on gelatin-coated slides. Then the slide-mounted sections were dehydrated through an ascending series of alcohols followed by xylene, and coverslipped with DPX (Sigma-Aldrich). A Nikon Microphot light microscope was used to analyze these sections.

Electron Microscopy

The surface of each flat-embedded forebrain section containing the PVN (Fig. 1) was cut in 60–80 nm sections with a diamond knife using an ultramicrotome (Ultratome, NOVA, LKB,

Bromma, Sweden). The ultrathin sections were collected on 400-mesh, thin-bar copper grids (EMS) and counterstained with uranyl acetate and Reynold's lead citrate (Milner et al., 2011). Labeling for TNFR1 in the PVN was analyzed using a transmission electron microscope (Technai 12 BioTwin, FEI, Hillsboro, OR) interfaced to a digital camera (Advantage HR/HR-B CCD Camera System, Advanced Microscopy Techniques, Danvers, MA) that was used to collect digital images from the sampled tissue.

Ultrastructural analysis

To insure that tissue was sampled from regions of even reagent penetrance, electron micrographs were captured from the epon-tissue transition zone defined as an area where one edge of the sampling region was in contact with epon in a field of at least four grid squares. The classification of profiles was based on well-established guidelines for the ultrastructural identification of neuronal and glial elements (Peters et al., 1991). Profiles were defined as dendritic if they contained regular microtubule arrays, endomembranous organelles, and/or postsynaptic densities. Somata were distinguished by the presence of nuclei, Golgi bodies, as well as rough endoplasmic reticula. Structures that were at least 0.2 μm in diameter and that also contained numerous small synaptic vesicles were characterized as axon terminals, whereas profiles less than 0.2 μm and lacking small synaptic vesicles were designated as unmyelinated axons. Irregularly shaped profiles devoid of cytoplasmic organelles or containing arrays of filaments were considered to be astrocytes.

Structures were characterized as being immunoperoxidase labeled if they contained discrete or diffuse electron-dense precipitate that was considerably darker than that seen in comparable profiles in the same field. Profiles were determined to be immunogold-silver labeled if they contained at least one particle per small profile, or two particles for larger profiles, provided that structures not expected to be labeled for the primary antisera, such as myelin, were devoid of gold-silver deposits (Hara and Pickel, 2008).

To assess the ultrastructural distribution of TNFR1 in the PVN, a total of 18,150 μm^2 of tissue was sampled from ultrathin PVN sections. Electron microscopic analysis was performed on a total of 12,100 μm^2 (200 fields of 60.5 μm^2) of PVN tissue from forebrain sections processed for immunoperoxidase labeling of TNFR1, and 6,050 μm^2 (100 fields of 60.5 μm^2) of tissue processed for detection of TNFR1 by immunogold-silver. From these samples, the number of dendritic, axonal, axon terminal, and glial profiles expressing labeling for TNFR1 was tallied and then averaged for each animal. For graphical presentation of data, the number of each type of neuronal and glial profile were divided by the total number of labeled profiles and multiplied by 100 to obtain a percentage.

An estimate of the frequency with which TNFR1 labeled dendrites formed synaptic junctions was made by analyzing 150 TNFR1 labeled dendritic profiles, under the provision that each dendrite was contacted by at least one axon terminal. The analysis was performed in sections processed for immunoperoxidase labeling because the greater sensitivity of this approach maximized the detection of labeled dendritic profiles. When axonal and dendritic profiles had closely spaced plasma membranes without identifiable synaptic specializations, they were classified as forming non-synaptic contacts. Directly apposed axons and dendrites

with either thick or thin postsynaptic specializations were defined as either asymmetric or symmetric synapses, respectively.

An estimate of the frequency with which TNFR1 labeled axon terminals formed synaptic junctions was determined by analyzing 50 TNFR1 labeled terminal profiles, under the condition that each terminal contacted a dendritic profile. Non-synaptic contacts and synaptic junctions were determined as above. For graphical presentation, the number of TNFR1 labeled non-synaptic contacts or each type of synapse was summed, divided by the total number of labeled contacts, and multiplied by 100 to obtain a percentage.

An established procedure for the apportionment of particulate gold-silver labeling within subcellular compartments was used to estimate the distribution of TNFR1 in cytoplasmic and plasma membrane compartments of dendritic and terminal profiles (Glass et al., 2015, Marques-Lopes et al., 2016). A total of 200 dendritic profiles showing immunogold-silver labeling for TNFR1 were analyzed. The subcellular localization of TNFR1-immuno-gold silver particles was defined as either on the plasma membrane (i.e. directly touching the membrane), near the plasma membrane (within 70 nm of the plasma membrane), or cytoplasmic (greater than 70 nm from the plasma membrane). For the analysis of axon terminals, a total of 50 profiles showing labeling for TNFR1 were analyzed using the same guidelines for subcellular apportionment of immunogold-silver particles as described for dendrites.

Statistical analyses

Each distribution of means to be compared was tested for normality by the Shapiro-Wilk test and for equality of variance by the Levene test. Distributions meeting the criteria of normality and equality of variance were then analyzed by one or two-way ANOVA followed by post-hoc testing (Dunn's test) with a Bonferroni correction for multiple comparisons achieved by dividing the threshold significance level (0.05) by the number of comparisons made ($n = 3$) for a final p-value of 0.0167. In cases where distributions deviated from normality, or normality and equality of variance, data were analyzed by non-parametric tests. These tests included the Kruskal-Wallis test appropriate for three comparisons, followed by separate between-group tests of significance by individual Mann-Whitney tests in which the p-value (0.05) was adjusted by dividing it by the number of comparisons made ($n = 3$) as above. In the non-parametric tests, all reported values were corrected for ties.

Image preparation

Figures were prepared by adjusting images for contrast and/or brightness using Photoshop 11 software. These images were then imported into PowerPoint to add lettering and symbols. Prism 6 software was used to produce the graphical figures (GraphPad Software, La Jolla, CA).

RESULTS

TNFR1 expression in somata of PVN neurons

Immunoperoxidase labeling of TNFR1 was seen throughout the PVN and nearby dorsal hypothalamus by light microscopy (Fig 1). This labeling was absent in similarly processed sections from TNFR1 KO mice (Fig 1), or in immunoperoxidase and immunogold processed tissue from wild-type mice where the primary TNFR1 antiserum was omitted (not shown). Electron microscopic analysis of ultrathin PVN sections processed for TNFR1 immunolabeling revealed that neuronal somata are among the TNFR1-labeled structures seen by light microscopy (Fig 2). In these somata, TNFR1 labeling was associated with sites of protein synthesis, packaging, and transport. These included the Golgi complex that, when labeled by immunogold-silver, showed one or more particles associated with the outer cisternal membrane (Fig 2A, B). In addition, the endoplasmic reticulum was also labeled for TNFR1 (Fig 2C, inset). When labeled with immunoperoxidase, reaction product was readily apparent on the outer reticular membrane located between the nucleus and the plasma membrane. Small round vesicles in proximity to these structures also showed labeling for TNFR1 (Fig 2C inset).

Predominate TNFR1 labeling in dendritic and axonal profiles in the PVN

Labeling for TNFR1 also extended outside the neuronal cell body and was found in both dendritic (Fig 3A, B) and axonal profiles (Fig 3C). A small population of glia were also labeled for TNFR1 (not shown). In tissue processed for immunoperoxidase labeling of TNFR1, a total of 447 labeled profiles were sampled in 200 fields, and in tissue processed for immunogold-silver labeling of TNFR1, a total of 204 profiles were sampled in 100 fields. There were significant differences in the number of labeled dendritic, axonal, and glial profiles, and these differences were consistent when TNFR1 was labeled with each marker type (Fig. 4). A quantitative analysis of dendrites, axon terminals, and glia revealed that irrespective of immunoperoxidase or immunogold-silver labels 78–80% (IP: $n=358/447$; IGS: $n=159/204$) of all TNFR1-labeled profiles were dendrites (Fig. 4), demonstrating that TNFR1 was predominantly positioned for the postsynaptic modulation of PVN neurons. In dendrites, immunoreactivity for TNFR1 was present in large proximal compartments, where labeling was found near the endoplasmic reticulum and other intracellular membranes (Fig 3A and inset). Transport of TNFR1 into dendritic processes was further demonstrated by the frequent labeling of intermediate ($0.51 - 1.5 \mu\text{m}^2$) and small ($< 0.5 \mu\text{m}^2$), presumably distal, dendritic profiles. When labeled by immunoperoxidase, TNFR1 labeling in these profiles was punctate in nature, as seen in both longitudinally (Fig 3B) and coronally (Figs 5A, 5B) sectioned dendritic processes. Immunoperoxidase aggregates were present in the cytoplasm (Figs 3B, 5A, 5B), and labeling also lined the plasma membrane (Figs 5A, 5B). Similar discrete labeling was also seen when TNFR1 was labeled with the immunogold-silver marker (Figs 7A, B, C).

In addition to dendrites, TNFR1 immunoreactivity was also seen in axonal profiles (Fig 3C) that were typically small ($< 0.5 \mu\text{m}^2$) to intermediate ($0.51 - 1 \mu\text{m}^2$) in size and contained numerous small synaptic vesicles. TNFR1-labeled axonal profiles were less numerous than TNFR1-labeled dendrites. Approximately 14–15% (IP: $n = 67/447$; IGS: $n = 28/204$) of all

TNFR1-labeled profiles were axons or axon terminals (Fig 4). Similar to dendrites, immunoperoxidase labeling of TNFR1 in axon terminals formed punctate aggregates in the cytoplasm (Fig 3C). In these terminals, immunoperoxidase labeling was affiliated with vesicular organelles including small synaptic vesicles (Fig 3C). Immunogold-silver labeling for TNFR1 was also discretely located in axon terminals (Fig 7A). In addition to axon terminals, small unmyelinated axons were also labeled for TNFR1 (Fig 5A). These profiles were clearly distinguished when labeled with immunoperoxidase by dense punctate reaction product in the cytoplasm that also extended toward the plasma membrane.

TNFR1-labeled dendritic and axonal profiles form contacts and synapses with unlabeled neuronal profiles in the PVN

Dendritic profiles labeled for TNFR1 were contacted by axon terminals with, or without forming recognizable synapses (Figs 5A, 5B, 7A, 7B). In tissue processed for immunoperoxidase, TNFR1-labeled dendritic profiles were contacted by axon terminals that were themselves almost exclusively devoid of TNFR1 immunoreactivity. The number of synapses and non-synaptic contacts were counted in 150 TNFR1-labeled dendritic processes. The majority (48%; 72/150; Fig 6A) of TNFR1-labeled dendritic profiles were apposed to terminals that formed non-synaptic contacts. Another population of TNFR1-labeled dendritic profiles (35%; 53/150; Fig. 6A) received synapses that were characterized by asymmetric excitatory-type specializations (Figs 5A, 5B), and 17% (25/150) of the remaining TNFR1-labeled dendritic profiles formed symmetric inhibitory-type synaptic junctions (Fig 6A).

In TNFR1-labeled dendrites forming asymmetric synapses, punctate immunoperoxidase reaction product was found in the cytoplasm beneath the plasma membrane where the postsynaptic specialization was visible (Fig 5A). Immunoperoxidase aggregates for TNFR1 were also identified in the cytoplasm near the postsynaptic density (Fig 5A) or near the extrasynaptic plasma membrane (Fig 5B). In addition, immunoperoxidase TNFR1 labeling was seen on the plasma membrane and the postsynaptic density (Fig 5B).

The number of synapses and non-synaptic contacts were counted in total of 50 TNFR1-labeled axon terminals that formed contacts with one or more dendritic profile, the latter of which were observed to be almost exclusively devoid of TNFR1 immunoreactivity. Of all the TNFR1-labeled axon terminals contacting dendritic profiles, 78% (39/50) formed non-synaptic contacts, 8% (4/50) formed symmetric-type synaptic specializations, and 14% (7/50) formed asymmetric-type junctions (Figs 6B).

Cytoplasmic and plasma membrane affiliations of TNFR1 in dendritic and axonal profiles in the PVN

Using the non-diffusible immunogold method to label TNFR1, immunoreactivity was found in the cytoplasm of dendritic profiles of PVN neurons (Fig 7). Labeling was also observed on, or in proximity to, the plasma membrane of dendrites (Fig 7). A total of 341 immunogold-silver particles were sampled from a total of 150 labeled dendritic profiles. Approximately 72% (246/341) were located in the cytoplasm (Fig 8A), where TNFR1 labeling was associated with small tubulovesicular organelles (Fig 7A) as well as near other

organelles, including mitochondria (Fig 7B). Approximately 28% (95/341) of all observed immunogold-silver particles were associated with the plasma membrane (Fig 8A). These included 46 particles in direct contact with, and 47 near (within 70 nm) the plasma membrane, and 2 particles in contact with the postsynaptic density.

In terms of plasma membrane affiliation, immunogold-silver particles were distributed on the extrasynaptic plasma membrane of dendritic profiles receiving asymmetric (Fig 7A) or symmetric (Fig 7B) synaptic contacts. Immunogold-silver particles for TNFR1 were also present near the postsynaptic density of asymmetric-type synapses (Fig 7A). In TNFR1-labeled dendritic profiles forming symmetric-type synaptic junctions, TNFR1 labeling was affiliated with intracellular organelles and the extrasynaptic plasma membrane (Fig 7B). Immunogold-silver deposits for TNFR1 were also found both on and near the plasma membrane of dendrites that were not contacted by recognizable axon terminals (Fig 7C).

Axon terminals showing immunogold-silver labeling for TNFR1 were also seen in the neuropil (Fig 7A). A total of 83 particles were counted from 50 axon terminals. Approximately 71% (59/83) of all gold particles were found in the cytoplasm (Fig 8B), whereas approximately 29% (24/83) were affiliated with the plasma membrane (Fig 8B). The immunogold-silver particles in these terminals were present in cytoplasmic and plasma membrane compartments that were distal from areas of synaptic contact (Fig 7A).

DISCUSSION

This is the first report demonstrating the ultrastructural distribution of TNFR1 in the PVN, a key central coordinator of autonomic function. Using high-resolution immunoelectron microscopy, in the PVN TNFR1 was largely expressed in neuronal profiles, but was also detected in a small population of glia. Although TNFR1 immunoreactivity was present in diverse neuronal compartments, the majority of labeling was seen in dendritic profiles. Many TNFR1-labeled dendritic profiles were contacted by axon terminals, which formed symmetric and, more frequently, asymmetric synapses. This finding indicates that TNF α modulates inhibitory and excitatory transmission by acting at postsynaptically located TNFR1 in PVN neurons.

The present characterization of TNFR1 in many neuronal as well as in a few glial profiles in the PVN of normal animals is consistent with prior evidence that TNF α is not only an inducer of cell death and apoptosis, but also a signaling molecule in diverse cell types and participates in non-pathological physiological and behavioral processes (Wajant et al., 2003). The present findings also support the now accepted view that TNF α acts through TNFR1 receptors in the brain (Santello and Volterra, 2012).

Activation of TNFR1 has been shown to participate in fast neuronal transmission mediated by classical neurotransmitters like glutamate (Santello and Volterra, 2012). In the present study, TNFR1 immunoreactivity was identified in dendritic profiles receiving excitatory-type synapses characteristic of glutamate inputs (Peters et al., 1991). Glutamate activity in the PVN plays a critical role in homeostasis by influencing behavioral (Hettes et al., 2003), metabolic (Amir, 1990, Kalsbeek et al., 2008), neuroendocrine (Hattori et al., 1992, Feldman

and Weidenfeld, 1997, Israel et al., 2010), and autonomic (Yeh et al., 1997, Zhang and Fogel, 2002, Crestani et al., 2010) processes. In particular, glutamate signaling in the PVN is an established central coordinator of sympathetic outflow critically involved in cardiovascular regulation (Ferguson et al., 2008, Kc and Dick, 2010). Dysregulated glutamate signaling in the PVN is also known to accompany the elevated blood pressure associated with genetic models (Li and Pan, 2007a, Li et al., 2014), slow-pressor angiotensin (Glass et al., 2015), sodium-sensitivity (Gabor and Leenen, 2012), and heart failure (Li et al., 2003).

Significantly, activation of TNFR1 has been shown to modulate the actions of glutamate. For example, TNFR1 potently potentiates glutamate signaling, in part by regulating the plasma membrane and synaptic transport of AMPA-type glutamate receptors (Rainey-Smith et al., 2010, Zhang et al., 2011, He et al., 2012). Like glutamate, TNF α activity in the PVN is implicated in blood pressure control. Increased TNF α levels in the PVN, originating from a microglial source (Shi et al., 2010), are associated with the increased local neural activity (Kang et al., 2008, Yu et al., 2015), as well as elevated systemic blood pressure (Sriramula et al., 2013, Dai et al., 2015, Dange et al., 2015) and sympathetic activity (Dange et al., 2015, Yu et al., 2015) that accompany experimental hypertension (Shi et al., 2010, Yu et al., 2015). In addition, acute PVN TNF α administration increases sympathetic activity and blood pressure (Bardgett et al., 2014, Shi et al., 2014), and inhibiting central TNF α has been shown to block forms of experimental hypertension (Song et al., 2014) including the increase in blood pressure occurring with AngII administration (Sriramula et al., 2013). Therefore, the activation of TNFR1 and glutamate may interact in the regulation of blood pressure, and the elevated sympathetic activation associated with hypertension.

In addition to receiving excitatory inputs, TNFR1 labeled dendrites were also associated with a smaller population of symmetric inhibitory-type synapses. The affiliation of TNFR1 with presumptive GABA inhibitory synapses is consistent with functional findings in other systems showing that TNF α application results in a rapid and long-lasting TNFR1-dependent decrease in inhibitory synaptic signaling mediated by an increase in the removal of plasma membrane GABA-A receptors (Stellwagen et al., 2005, Pribrag and Stellwagen, 2013). Within the context of neural signaling in the PVN, GABA plays a key role in regulating the excitatory state of PVN neurons (Schlenker et al., 2001, Chen et al., 2003, Li and Pan, 2007a), particularly by exerting tonic inhibition of glutamate signaling (Li et al., 2006). This inhibitory control provides an important mechanism for regulating sympathetic outflow and blood pressure (Martin et al., 1991, Chen et al., 2003, Li and Pan, 2007a, Hsu et al., 2011, Martins-Pinge et al., 2012). Significantly, dysfunctional inhibitory signaling in the PVN is seen in heart failure (Zhang et al., 2002), renal (Martin and Haywood, 1998), genetic (Li and Pan, 2007b), and AngII-mediated (LaGrange et al., 2003) hypertension. Importantly, both the decrease in PVN GABA signaling and the increase in sympathetic activity associated with hypertension are dependent on local TNF α signaling (Kang et al., 2010). Therefore, a convergent decrease in inhibitory communication and potentiated glutamate signaling linked to TNFR1 activation may contribute to both the heightened activity of PVN sympathoexcitatory circuits and blood pressure dysregulation during hypertensive states.

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- In the PVN, TNFR1 was largely expressed in neuronal profiles, but was also detected in a small population of glia
- Within PVN neurons TNFR1 was prominently found in dendrites
- TNFR1 labeled dendrites were contacted by axon terminals forming excitatory and inhibitory synapses
- TNFR1 is strategically positioned for modulation of excitatory and inhibitory transmission in the PVN

Fig 1. Immunolabeling for TNFR1 is present in the PVN

Light micrographs of the dorsal medial hypothalamus from vibratome sections processed for immunoperoxidase labeling of TNFR1 in a wild-type (A) and a TNFR1 KO mouse (B). In the wild-type mouse, TNFR1 immunoreactivity is present throughout the PVN, which is shown in the area bounded by the dashed oval. This labeling is not apparent in the brain section from the KO mouse. III = third ventricle, d: dorsal, m: medial. Scale bars: 0.1 mm (A), 0.2 mm (B).

Fig 2. TNFR1 is localized to sites of protein synthesis, packaging, and transport in somata of PVN neurons

(A–B) Serial ultrathin sections showing immunogold-silver (IGS) particles for TNFR1 (thick arrows) near a Golgi complex (Gc) in the soma of a PVN neuron (TNFR1-s). In B, SIG particles are also found on membranes near the nucleus (nuc). (C) Immunoperoxidase labeling of TNFR1 is present near the endoplasmic reticulum (er) in the soma (TNFR1-s) of a PVN neuron. A labeled endoplasmic reticulum and small vesicular organelles (vo) can be seen between the nucleus and the plasma membrane at a higher magnification in the area bounded by the dashed box shown in the **inset**. Scale bars: 1 μ m.

Fig 3. TNFR1 is found in large proximal and smaller distal dendrites as well as axon terminals in the PVN

(A) A large proximal dendritic profile (TNFR1-d) shows immunogold-silver particles for TNFR1 (thick arrows) near an endoplasmic reticulum (er) and cytoplasmic membranes (mo). The latter are demarcated in the area enclosed by the dashed box, which is shown at a higher magnification in the **inset**. (B) A longitudinally sectioned dendritic profile (TNFR1-d) displays punctate immunoperoxidase labeling for TNFR1 (thick arrows) in the cytoplasm. (C) An axon terminal profile (TNFR1-t) shows immunoperoxidase labeling (thick arrows) in the cytoplasm near the plasma membrane. An unlabeled axon terminal (ut) is present in the adjacent neuropil. Scale Bars: 500 nm.

Fig 4. TNFR1 is predominantly found in dendritic profiles of PVN neurons

Histogram showing the distribution of TNFR1 labeling in dendritic, axonal (axons and axon terminals), and glial profiles detected with either immunoperoxidase (IP; presented on left side of the figure) or immunogold-silver (IGS; presented on the right side of the figure). Profile type [$F(2, 24) = 59.1$, $p = 0.0001$], but not marker [$F(1, 24) = 1.9$, $p = 0.18$], was a significant factor influencing the distribution of TNFR1. There was no marker by profile type interaction [$F(2, 24) = 0.37$, $p = 0.697$]. Under both labeling conditions, there were significantly more TNFR1 labeled dendritic profiles compared to either axons (immunoperoxidase: $p = 0.0001$; immunogold: $p = 0.0003$) or glia (immunoperoxidase: $p = 0.0001$; immunogold: $p = 0.0001$), however, there were no significant differences in the number of axons and glia (immunoperoxidase: $p = 0.41$; immunogold: $p = 0.16$). * $p = 0.0001$ dendrites relative to axons (IP); # $p = 0.0001$ dendrites relative to glia (IP); ♦ $p = 0.0003$ dendrites relative to axons (IGS); @ $p = 0.0001$ dendrites relative to glia (IGS).

Fig 5. TNFR1 labeling is seen in dendritic profiles apposed to unlabeled axon terminals some of which form asymmetric excitatory-type junctions

(A) A dendritic profile (TNFR1-d) with cytosolic immunoperoxidase reaction-product for TNFR1 forms an asymmetric excitatory-type synapse with an unlabeled axon terminal (ut). The punctate immunoperoxidase labeling for TNFR1 (thick arrow) is in the vicinity of the postsynaptic density (arrow head). A small unmyelinated axon (TNFR1-a) is present in the nearby neuropil. The lumen (lu) of a blood vessel is seen at the lower right. (B) A small dendritic profile (TNFR1-d) displays discrete immunoperoxidase labeling for TNFR1. Labeling is visible on the outer membrane of a tubulovesicular organelle (to), indicated by the thick arrow, and along the postsynaptic membrane specialization (chevron) of an asymmetric synapse formed by an unlabeled axon terminal (ut). Scale Bars: 500 nm.

Fig 6. TNFR1 expressing dendritic and axon terminal profiles have synaptic and non-synaptic contacts with other neurons in the PVN

Frequencies of appositions (asymmetric synapses, symmetric synapses, or non-synaptic contacts) formed between TNFR1 labeled dendritic profiles and unlabeled axon terminals (**in A**) and between TNFR1 labeled axon terminals and unlabeled dendritic profiles (**in B**). Contact type affected the distribution of TNFR1 in dendrites [$F(1, 15) = 48.8, p = 0.0001$]. In TNFR1 containing dendrites contacted by axon terminals, there were more contacts compared to asymmetric ($p = 0.019$) and symmetric ($p = 0.022$) type synapses, although these were just below the threshold for significance. There was no difference in the number of TNFR1 labeled dendritic profiles receiving asymmetric or symmetric synaptic specializations ($p = 0.9$). There were also differences in contacts formed by TNFR1 axon terminals contacting dendritic profiles [$H = 7.1, p = 0.028$]. Axon terminals labeled for

TNFR1 formed more contacts relative to asymmetric ($U = 2$, $p = 0.035$) and symmetric ($U = 1.5$, $p = 0.02$) type synapses, however only the latter reached statistical significance. There was no significant difference in asymmetric and symmetric synapses ($U = 12$, $p > 0.9$). The horizontal lines represent the median values in **B**. # $p < 0.05$.

Fig 7. TNFR1 is expressed in diverse subcellular sites in dendrites and axon terminals in the PVN

(A). Immunogold-silver particles for TNFR1 are found near a tubulovesicular organelle (to, thick arrow), the extrasynaptic plasma membrane (thin arrow), and beneath the postsynaptic density (chevron) of a dendritic profile (TNFR1-d) that receives an asymmetric-type synapse (black arrow head) from an unlabeled axon terminal (ut). In the nearby neuropil, an axon terminal (TNFR1-t) shows immunogold-silver particles for TNFR1 in the cytoplasm (thick arrow) and on the plasma membrane (thin arrow). This terminal contacts an unlabeled dendritic profile (ud). (B) A dendritic profile (TNFR1-d) shows immunogold-silver labeling for TNFR1 near a mitochondrion (m, thick arrow) and on the extrasynaptic plasma

membrane (thin arrow) adjacent to a symmetric synapse (white arrow head) formed by an unlabeled axon terminal (ut). (C) A dendritic profile (TNFR1-d) shows immunogold-silver particles for TNFR1 near the plasma membrane (thick arrow) and on the extrasynaptic plasma membrane (thin arrow). This profile is ensheathed by thin unlabeled glia (asterisks). Scale bars: 500 nm

Fig 8. TNFR1 is expressed in cytoplasmic and plasmalemmal compartments of dendrites and axon terminals in the PVN

Graphs showing the distribution of TNFR1 in the cytoplasm and on/near the plasma membrane of either dendritic (**in A**) or axon terminal (**in B**) profiles. Labeling for TNFR1 in the PVN was impacted by subcellular compartment (i.e. cytoplasm, near plasma membrane, on plasma membrane) in dendritic profiles ($H = 10.9$, $p = 0.0045$). There were more gold particles for TNFR1 in the cytoplasm compared to near ($U = 0$, $p = 0.009$) or on ($U = 0$, $p = 0.0088$) the plasma membrane. There was no significant difference in TNFR1 labeling near or on the plasma membrane ($U = 4$, $p = 0.07$). With respect to axon terminals, the subcellular compartment also influenced TNFR1 labeling ($H = 10.1$, $p = 0.0063$). In terminals, immunogold TNFR1 labeling was also greater in the cytoplasm than either near ($U = 0$, $p = 0.008$) or on ($U = 0$, $p = 0.008$) the plasma membrane. There was no difference in TNFR1 labeling near and on the plasma membrane of axon terminals ($U = 7$, $p = 0.24$).

Since there was no difference in IGS labeling near and on the plasma membrane these two groups are combined and presented as a single plasma membrane categories in each figure. @ $p = 0.008$ cytoplasm relative to plasma membrane (in **A**); # $p = 0.008$ cytoplasm relative to plasma membrane (In **B**). The horizontal lines represent the median values.