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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 (TNFα) is an important mediator of both constitutive and plastic signaling in the brain. In particular, TNFα is implicated in physiological processes, including fever, energy balance, and autonomic function, known to involve the hypothalamic paraventricular nucleus (PVN). Many critical actions of TNFα are transduced by the TNFα 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.** **© 2017 IBRO. Published by Elsevier Ltd. All rights reserved.**

**Key words: 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, TNFα has also been established to be an important signaling molecule involved in neural communication in the brain (Santello and Volterra, 2012). It is known that TNFα can act within the brain by several distinct routes including within circumventricular organs targeted by circulating TNFα, 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 TNFα 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 TNFα. This is supported by other data showing that local application of TNFα 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 TNFα system in the PVN itself, involving production by resident glia (Shi et al., 2010; Du et al., 2015), although there are reports of TNFα-like immunoreactivity in PVN neurons as well (Breder et al., 1993). Within the PVN, TNFα transcription is induced by challenges such as immunological stress (Kakizaki et al., 1999; Masson et al., 2015a). In addition, increased TNFα 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 TNFα 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 TNFα attenuates the

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*Abbreviations:* BSA, bovine serum albumin; HPA, hypothalamic–pituitary–adrenal; IGS, immunogold-silver; IP, immunoperoxidase; KO, knockout; PB, phosphate buffer; PBS, phosphate buffered saline; PFA, paraformaldehyde; PVN, paraventricular nucleus; TBS, Tris-buffered saline; TNFα, tumor necrosis factor α.

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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 TNFα converting enzyme, soluble forms (Varfolomeev and Vucic, 2016). Functionally, TNFα transduces its intracellular effects by binding two receptors, the TNFα type 1 receptor [TNFR1; (also known as p55 or CD120a)] and the TNFα type 2 receptor (Varfolomeev and Vucic, 2016). The TNFR1 is able to bind both membranous and soluble TNFα, has a ubiquitous expression pattern, and is the source of much of what is known about TNFα signaling (Varfolomeev and Vucic, 2016). In the brain, TNFR1 is the major mediator of TNFα'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, TNFα is well known for its transcriptional actions (Varfolomeev and Vucic, 2016), however it can also modulate rapid neurotransmitter signaling. For example, TNFα 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, TNFα has also been shown to produce a fast and persistent decrease in inhibitory synaptic strength (Pribiag 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-glial 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.

## EXPERIMENTAL PROCEDURES

### Subjects

The experimental subjects were five male C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME), weighing 20–25 g, housed in groups of at least two animals per cage, and maintained on a 12-hr light/dark cycle (lights out 1800 h) 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/min 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-min. 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 μm) 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 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-min incubation in 0.5% bovine serum albumin (BSA) to lessen nonspecific labeling. After rinsing in TBS, brain sections were then incubated for 48-h 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-min. Sections were next rinsed in TBS followed by a 30-min 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 min 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 phosphate buffered saline (PBS; pH 7.4). Then, to reduce non-specific binding of gold particles, brain sections were incubated for 10-min in a blocking solution consisting of 0.8% BSA and 0.1% gelatin in PBS. After this blocking step, sections were incubated for 2-h 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-min

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