# NEUROPATHOGENESIS OF THE ACUTE PHASE RESPONSE TO

# INFLUENZA VIRUS IN MICE

By

VICTOR HUGO LEYVA GRADO

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       The members of the Committee appointed to examine the thesis of VICTOR

HUGO LEYVA-GRADO find it satisfactory and recommend that it be accepted.


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Chair


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University of Mexico.

To my wife, Lupita Leyva that has helped me in a lot of ways: she has made me a

better student, a better scientist and a better person.

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## Abstract

By Victor Hugo Leyva Grado PhD

Washington State University

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Chair:  James M. Krueger

Influenza virus infection causes severe systemic clinical signs in infected mice. Non-neurotropic human strains of influenza virus are believed to be confined to the respiratory tract following intranasal (IN) infection. Characteristic symptoms of influenza infection include changes in body temperature, locomotor activity and sleep patterns. These symptoms are part of the acute phase response (APR), or 'flu' syndrome. Such symptoms are in part regulated by cytokines such as tumor necrosis factor alpha (TNFα) and interleukin 1 beta (IL1β). However, it remains to be established whether the cytokines that act on the brain to induce the APR are produced in the brain or if they are made systemically and then reach the brain through the blood or other routes.

To better understand the pathophysiology of the APR, we have characterized the presence of extrapulmonary virus in the brain and its effect on cytokine up-regulation. Furthermore, we characterized the role of the olfactory pathway in the ontogenesis of the

APR after intranasal inoculation with influenza virus. We used for all our experiments a human mouse adapted strain of influenza virus named PR8. Virus was found in the mouse olfactory bulb (OB) as early as 4 h post-challenge where the virus appeared to go into partial replication. The virus co-localized in microglia and astrocytes but not in neurons. An increase in TNFα, IL1β and interferon-induced enzymes was also observed in the OB after viral challenge. Cytokines were produced by microglia, astrocytes and neurons in the OB. Surgical transection of the olfactory nerve (ONT) prior to the viral challenge delayed the virus-induced hypothermia. Additionally, the number of viral antigen-, TNFα− and IL1β− immunoreactive (IR) cells was reduced in the OBs of mice that received the ONT. We also examined brain regions that have direct and indirect connections with the OB. No viral antigen-IR was observed in any of these regions; however, an increase in the number of TNFα− and IL1β− IR cells was observed in selected regions along the olfactory pathway. Taken together, these data elucidate in part some of the possible mechanisms involved in the ontogenesis of the influenza-induced APR in infected mice.

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# LIST OF FREQUENTLY USED ABBREVIATIONS

APR (acute phase response)

Arc (hypothalamic arcuate nucleus)

ATP (adenosine triphosphate)

BLA (basolateral amygdala)

CeA (central amygdala)

CNS (central nervous system)

CVO (circumventricular organs)

DAB (diaminobenzidine)

EPL (external plexiform layer)

GFAP (glial fibrillary acidic protein)

GL (glomerular layer)

HA (hemagglutinin)

HT (hypothalamus)

ICV (Intracerebroventricular)

IFN (interferon)

IHC (immunohistochemistry)

IL1β (interleukin 1 beta)

IL1R (IL1 receptor)

IN (intranasal)

IP (intraperitoneal)

IR (immunoreactive)

KO (knock out)

LPS (lipopolysaccharide)

ML (mitral cell layer)

NA (neuraminidase)

NF-kappa B (nuclear factor-kappa B)

NP (nucleoprotein)

NREMS (non-rapid eye movement sleep)

OAS (2'-5' oligoadenylate synthetase)

OB (olfactory bulb)

OEC (olfactory ensheathing cells)

ON (olfactory nerve)

ONT (olfactory nerve transection)

ORN (olfactory receptor neurons)

PI (post-infection)

Pir (piriform cortex)

POA (pre-optic area)

PR8 (influenza A virus PR/8/38 H1N1)

REMS (rapid eye movement sleep)

RNA (ribonucleic acid)

Tb (body temperature)

TLR (toll-like receptors)

TNF$\alpha$ (tumor necrosis factor-alpha)

TNFR (TNF receptor)

Tu (olfactory tubercle)

## DEDICATION

This thesis is dedicated to my beautiful and loving wife Lupita Leyva.

I love you!

# CHAPTER I


# INTRODUCTION

## Influenza virus infection

Influenza viruses are members of the *Orthomyxoviridae* family which includes 5 genera: Influenza A, Influenza B, Influenza C, Isavirus and Thogovirus (Wright and Webster, 2001). This thesis is limited to influenza A virus. Influenza particles bear a lipid envelope from which two different types of glycoprotein spikes radially project: hemagglutinin (HA) and neuraminidase (NA) (Smith, 1952; White and Fenner, 1994). The viral genome consist of single-stranded, negative-sense ribonucleic acid (RNA) divided in eight segments that encode for ten different viral proteins (Baigent and McCauley, 2003, Steinhauer and Skehel, 2002, Wright and Webster, 2001) (Table 1.1).


Table 1.1 Influenza A virus gene segments

| Segment | Size | Weight | Protein | Function(s) |
|---|---|---|---|---|
| | | | | |
| 1 | 2341 | 96 kD | Polymerase (PB2) | Cap binding /Proapoptosis |
| 2 | 2341 | 87 kD | Polymerase (PB1) | Elongation |
| 3 | 2233 | 85.5 kD | Polymerase (PA) | Genome RNA synthesis |
| 4 | 1778 | 220 kD | Hemagglutinin (HA) | Binding to cell/Main viral antigen |
| 5 | 1565 | 55 kD | Nucleoprotein (NP) | Encapsidates RNA / Transport signaling |
| 6 | 1413 | 240 kD | Neuraminidase (NA) | Release of virus particle |
| 7 | 1027 | 28 kD | Matrix (M1) | Structure/Virus assembly |
| | | 15 kD | Integral membrane (M2) | Ion channel |
| 8 | 890 | 25kd | Non structural (NS1) | Splicing/ Response to interferon |
| | | 14 kD | Non structural (NS2) | Nuclear export protein |

Adapted from Steinhauer and Skehel, 2002.

The replication process starts when the virus binds through its HA proteins to the sialic-acid containing receptors on the target cells and then penetrates the cell via clathrin-dependent receptor-mediated endocytosis (Sidorenko and Reichl, 2004). Once in the nucleus, the minus-strand viral RNA (vRNA) is copied by the viral polymerase complex to produce three types of RNAs: plus-strand viral messenger RNA (mRNA), minus-strand vRNA, and plus-strand complementary RNA (cRNA) that serves as template for new vRNA synthesis. Viral mRNAs are produced using the 5' end cap and the poly A tail from host mRNA as primers for their synthesis and then are exported to the cytoplasm to be translated in the ribosomes with previous splicing of M and NS mRNAs. Envelope proteins (HA, NA, and M2) are directed to the endoplasmic reticulum where HA and NA are glycosylated before migrating to the cell surface. Meanwhile, the rest of the synthesized proteins are imported to the nucleus, where they participate in the synthesis of full length vRNA (-) and cRNA (+) strands. The new viral ribonucleoproteins (vRNPs) are formed in the nucleus as a result of binding of PB1, PB2, PA, NP, M1 and NS2 proteins with the newly synthesized vRNAs, and are exported to the cell membrane to interact with HA, NA and M2. This complex forms the viral particles which are released by budding from the plasma membrane by cleavage of HA by the NA protein.

One of the more remarkable characteristics of these viruses is their antigenic variability which in turn is associated with epidemics almost every year and the more sporadic appearance of pandemics (Oxford, 2000; Zambon, 2001). Changes in the structural proteins of the virus produce an antigenic variation that prevent the specific antibodies produced in previous infections to bind to their antigen, enabling the virus to

evade the immune response despite pre-existing specific neutralizing antibodies (Wright and Webster, 2001; Zambon, 2001). Two mechanisms of antigenic variation have been identified for Influenza: Antigenic drift and antigenic shift.

Antigenic drift is the consequence of accumulation of individual point mutations leading to amino acid substitutions in structural glycoproteins on the surface of the virion, specifically HA and NA (Naeve et al., 1984). Such structural changes are associated with influenza epidemics because the pre-existed antibodies are not able to control the virus after the changes in epitopes (Steinhauer and Skehel, 2002; Treanor, 2004).

Antigenic shift implies a major change in the surface proteins of the virus due to acquisition of novel genes that encode completely new surface glycoproteins and results in the replacement of HA and sometimes NA, originating new virus subtypes (Flint et al., 2004; Treanor, 2004). These genotypic changes are facilitated by the segmented nature of the influenza genome that enables the exchange of genetic information between two different influenza viruses while co-infecting a host, a genetic process known as reassortment. Due to the nature of changes in the antigenic structures of the virus, antigenic shifts have been associated with the presentation of global pandemics (Wright and Webster, 2001; Zambon, 2001; Flint et al., 2004).

At least 15 non-overlapping subtypes of hemagglutinin (H1-H15) and 9 subtypes of neuraminidase (N1-N9) have been identified for influenza viruses (Steinhauer and Skehel, 2002). Only 3 HA subtypes (H1, H2 and H3) and 2 NA subtypes (N1 and N2) have been prevalent infecting humans since 1918 (Flint et al., 2004, Nicholson et al., 2003, Wright and Webster, 2001).

Infection with influenza virus produces a highly contagious respiratory disease that can cause mild to severe illness, but seldom leads to death unless pneumonia (viral or bacterial) ensues (Centers for Disease Control and Prevention, 2005). Clinical manifestations of infection are associated with the pneumotropic (affinity for the respiratory tract tissues) nature of the virus in humans and in animals such as chickens, pigs, and horses (Baigent and McCauley, 2003, Nicholson et al., 2003, Zambon, 2001). In humans, infection can be asymptomatic or can be a mild to severe respiratory illness characterized by fever, fatigue, dry cough, sore throat, anorexia and myalgia (Studahl, 2003). Mice are not naturally infected by influenza virus (Ward, 1997); however both human and avian strains can be adapted to replicate in the mouse and produce clinical signs of infection and death. When a human-derived strain is adapted to mice by serial lung passage, some of the clinical signs in the infected mice include pneumonia, hypothermia, decreased locomotor activity, decreased rapid eye movement sleep (REMS) and increased non-REMS (Conn et al., 1995; Fang et al., 1995; Toth et al., 1995; Alt et al., 2003).

**Neurotropic strains versus non-neurotropic strains of influenza**

Viruses that have a selective affinity for nervous tissue and exert their main effect on the nervous system are termed neurotropic or neuroinvasive viruses (Flint et al., 2004, Johnson, 1998). Generally, neurotropic viruses are also neurovirulent (i.e., the virus replicates in neurons and produces cell death), a classic example being the rabies virus. Such viruses can spread from neuron to neuron within the brain and cause encephalitis. Viruses that lack the capacity to invade the brain are termed non-neurotropic viruses or

strains. Influenza viruses are not generally recognized as neurotropic in humans. However, in the last decade influenza virus has been increasingly associated in children with both occasional cases of post-infection encephalitis and, more frequently, an often-lethal encephalopathy. Most of these neurological diseases have been reported in Japanese children (Kawada et al., 2003, Okumura et al., 2005, Sugaya, 2002), but recently influenza-associated encephalopathy was reported in American children as well (Maricich et al., 2004), indicating that a tougher screening of pediatric cases of influenza will probably demonstrate a higher incidence of this clinical manifestation.

In mice, replication of most mouse-adapted human strains of influenza is thought to be restricted to the respiratory tract (Hennet et al., 1992). Notable exceptions are the influenza strains A/WSN/33 and A/NWS/33, which have been sequentially passaged in the brains of mice and which are neurovirulent when inoculated intracerebrally into adult mice. These strains are also neurotropic when intranasally inoculated in neonatal mice (Schlesinger et al, 1998). For example, in 7 day-old mice after intranasal (IN) inoculation with influenza A/WSN/33, the viral antigen-immunoreactivity in the olfactory bulb (OB) is restricted to neurons and widely distributed through the different layers of the OB (Aronsson et al., 2003). Avian strains of influenza are generally neurotropic and neurovirulent in animals (chickens, ferrets, and mice) but have not demonstrated neurotropism in recent human outbreaks (Maines et al., 2005). After IN inoculation of mice with an avian strain of influenza, the viral antigen-immunoreactivity is distributed into different regions of the brain including the OB (Iwasaki et al., 2004) and the viral antigen is detected mainly in neurons and occasionally in glial cells surrounding areas of inflammation (Shinya et al., 2000; Iwasaki et al., 2004).

**Influenza A/ PR/8/34 H1N1 (PR8)**

PR8 is a human isolate adapted to produce pneumonitis in mice following IN

inoculation (Johnson and Mims, 1968). Inoculation of mice with this influenza strain will

produce a lethal pneumonitis marked by hypothermia, somnolence and anorexia (Alt et

al., 2003, Fang et al., 1995, Toth et al., 1995, Chen et al., 2004). Furthermore, virological

and pathological studies with this and other H1N1 strains reveal that when the virus is

inoculated IN, the infection is restricted to the respiratory epithelium with no detectable

pathology in the central nervous system (Iwasaki et al., 2004). When PR8 is used to

infect mouse brain cell cultures (21 days old) the results are similar, with no production

of infectious virions when evaluated by plaque-forming unit measurements. However, a

transient increase in HA and NA proteins is seen, suggesting that the virus goes through a

partial replication (Bradshaw et al., 1989).  Viral protein (NP and M1) immunoreactivity

is observed in cultured neurons and astrocytes (Bradshaw et al., 1989).


**Detection of infection and cytokine production**

Cytokines are a diverse group of proteins that are secreted by most nucleated cells

(Dinarello, 2000). Since cytokines have a key role in the regulation of the immune and

inflammatory responses, many cytokines have been discovered and characterized in

association with these pathophysiological events (Opp, 2005). Cytokines such as IL1 and

TNF$\alpha$ promote their own synthesis, stimulate the synthesis of other cytokines including

some anti-inflammatory cytokines, and stimulate the production of glucocorticoids in

autocrine and paracrine manners (Vitkovic et al., 2000; Silverman et al., 2005).

The presence of a virus or any other pathogenic microorganism is detected by immune system cells that initiate the process to control and limit virus replication as well as to activate the adaptive immune response. A similar mechanism takes place when the infection targets the CNS where the microglia and astrocytes detect the presence of the microorganism and are activated to produce an immune response (Konsman et al., 2002, Owens et al., 2005). Detection of the presence of influenza virus is accomplished through cell receptors like the mannose receptor and the Toll-like receptors.

The mannose receptor (MR) is a cell membrane-bound receptor expressed in macrophages, immature dendritic cells, microglia, and astrocytes (Reading et al., 2000; Regnier-Vigouroux, 2003) that mediates the uptake of glycoproteins containing a terminal mannose, fucose, or N-acetylglusosamine (Janeway et al., 2001, Olson and Miller, 2004, Zimmer et al., 2003). These MRs bind both sugar molecules expressed on the surface of different microorganisms and certain host proteins such as myeloperoxidases and hydrolases. As mentioned previously, both influenza envelope proteins HA and NA are glycosylated proteins that can function as ligands for this receptor (Reading et al., 2000). The mannose-binding protein is a defense mechanism against influenza virus because it blocks binding of virus to the MR and acts as an opsonin. Opzonization of the virus by this protein facilitates the antiviral activity of neutrophils (Hartshorn et al., 1993), one of the non-specific immune defense mechanisms in the nasal airway (Fokkens and Scheeren, 2000).

The toll-like receptors (TLR) are a family of type I integral transmembrane glycoproteins located on the cellular membrane or in intracytoplasmic compartments of various cell types including macrophages, dendritic cells, microglia, and astrocytes. TLR

recognize common molecular motifs or pathogen-associated molecular patterns (PAMP) from different groups of microorganism such as bacteria, fungi, and viruses. In particular, the TLR associated with viral ligands include TLR3, that recognizes double stranded RNA (dsRNA), TLR7 in mouse and TLR8 in humans that both recognize single stranded RNA (ssRNA), and TLR9 which recognizes unmethylated motifs (CpG) in dsDNA viruses (Prehaud et al., 2005, Kawai and Akira, 2006).

Almost all viruses produce dsRNA during their replication cycle (Applequist et al., 2002; Majde, 2000; Guillot et al., 2005); recognition of this PAMP by the intracellular TLR3 appears to be important in the innate immune response against viral infections. After TLR3 binds its ligand, it uses the Toll-IL-1 receptor resistance (TIR) domain-containing adaptor inducing IFN-β (TRIF) and culminates with the activation of transcription factors NF-κB and IFN regulatory element-3 (IRF3). These transcription factors play a central role in the innate immune response by regulating the expression of genes for pro-inflammatory cytokines and type I interferons, respectively (Kawai and Akira, 2006). Mouse microglial cells and astrocytes express TLR3, suggesting that these cells can be activated by influenza A virus and synthetic dsRNA (Bsibsi et al., 2002; Scumpia et al., 2005).

Cytokines can be divided in pro- and anti-inflammatory cytokines according to their function. Some cytokines, such as type I interferons, have both, pro-inflammatory and anti-inflammatory functions (Taylor and Grossberg, 1998). Pro-inflammatory cytokines include TNFα, IL1, IL6, and IL12 (Janeway et al., 2001, Kaufmann et al., 2002, Schmitz et al., 2005). Inflammatory processes are triggered in part by cytokines and are aimed to localize and control the infection as well as to amplify and target the immune response.

The biological activities of these cytokines are usually synergistic and are associated with the up-regulation of genes coding for molecules that regulate the inflammatory response (Dinarello, 2000). Additionally, the pro-inflammatory cytokines are associated with the up-regulation of the acute phase response (APR) observed in response to infection. Pro-inflammatory cytokines can act in the hypothalamus (HT) to induce fever, sleep, sickness behavior, and the production of the APR-proteins (e.g. c-reactive protein, ceruloplasmin, and metallothionine) and complement (Basset et al., 2003, Fang et al., 1995; Krueger and Majde, 2003).

Anti-inflammatory cytokines include IL4, IL5, IL10, IL13, and transforming growth factor beta (TGF β). These cytokines participate in the regulation of the inflammatory process by suppressing the production of pro-inflammatory cytokines, such as IL1 and TNF. In addition, they also inhibit the synthesis of integrins in the vascular endothelium (Dinarello, 2000). Anti-inflammatory cytokines act to dampen the pro-inflammatory-induced response in an adaptive, time-dependent manner (Akaike et al., 1996, Kawada et al., 2003, Schmitz et al., 2005). They also induce the production of glucocorticoids that function as immunomodulatory hormones (Silverman et al, 2005). Consequently, during acute infections the time course for anti-inflammatory genes or the production of their proteins often lags behind the pro-inflammatory cytokine response signals.


**Tumor necrosis factor-α**

Tumor necrosis factor (TNF) also known as TNFα is a prototypical inflammatory cytokine that was identified in 1975 as an endotoxin-induced serum glycoprotein that caused necrosis of tumor cells (Carswell et al., 1975). TNFα has now been associated

with a plethora of physiological functions both in the normal and diseased body (Perry et al., 2002; Bertazza and Mocellin, 2008; Bradley, 2008). Many different cells in the body can express TNFα, including microglia, astrocytes, and neurons in the central nervous system (Breder et al., 1993; Ignatowski et al., 1997; Ohtori et al., 2004; Yan et al., 2007; Juliet et al., 2008). TNFα is synthesized as a membrane-bound homotrimer (formed of 26 kD monomers), pro-TNF, that is cleaved by the TNFα-converting enzyme (Solomon et al., 1999; Tracey et al., 2008). After cleavage, the soluble cytokine is released as the 17 kD form. Both trimeric forms are bioactive and may have different activities (Solomon et al., 1999; Bradley, 2008). Membrane-bound TNFα can function as both a receptor and as a ligand (Tracey et al., 2008).

The biological responses to TNFα are mediated by signaling through two distinct receptors designated as TNFR1 (also known as p55) and TNFR2 (also known as p75) (Vandenabeele et al., 1995). Although both receptors are structurally related, they differ in their cellular expression, affinity for ligands, and signaling mechanisms (Palin et al., 2007; Tracey et al., 2008). TNFR1 is expressed on virtually all cell types whereas TNFR2 is usually inducible and expressed only in endothelial and immune cells (Bertazza and Mocellin, 2008). Signaling is mediated by adapter proteins in the cell cytoplasm that attach to the intracellular domains of the receptors. One major TNFα activated signaling pathway leads to the activation of nuclear factor kappa-B (NF-κB), a family of transcription factors that activates new gene transcription, whereas another distinct signaling pathway leads to programmed cell death (Palin et al., 2008; Tracey et al., 2008).

In the brain, TNFα serves as a key regulator of several pathological effects during infectious diseases of the CNS as well as neurological, neurodegenerative, and neurotoxic conditions (Sriram and O'Callaghan, 2007). TNFα is upregulated in the brain after immune and inflammatory responses including those caused by infection and may function as a neurotoxic factor or as a neuroprotector (Breder et al., 1994; Ghoshal et al., 2007; Sergerie et al., 2007; Sriram and O'Callaghan, 2007; Tonelli et al., 2008). Neuroprotection is exerted on neurons through both TNF receptors (Cheng et al., 1994) and may be achieved by sustaining activation of NF-κB (Tracey et al., 2008). For example, TNFR1 is required for the protective effects of erythropoietin in neurons of mice subjected to ischemic injuries (Taoufik et al., 2008). Furthermore, the neuroprotective role of TNFα has been demonstrated during viral infections of the central nervous system such as herpes virus (Sergerie et al., 2007) and rabies (Faber et al., 2005).

In the normal brain, evidence suggests a physiological role for TNFα (Perry et al., 2002, for review). TNFα and its receptors are expressed in different regions of the brain including the cortex, thalamus, hypothalamus, amygdala, bed nucleus of the stria terminalis, hippocampus, cerebellum, brainstem, and basal ganglia (Breder et al., 1993; Gahring et al., 1996; Vitkovic et al., 2000), suggesting a role in endogenous brain function. Furthermore, TNFα shows a diurnal biorhythm with highest concentrations when sleep propensity is higher (discussed below), suggesting a role for TNFα in the neuromodulation of autonomic functions (Perry et al., 2002).

**Interleukin 1β**

Interleukin 1β (IL1β) is a pro-inflammatory cytokine originally described as an endogenous pyrogen that induced fever in rabbits (Atkins and Wood; 1955). IL1β is a member of the interleukin 1 family (Gibson et al., 2004). The IL1 family now comprises at least eleven different proteins (IL1F1-IL1F11) on the basis of their sequence homology, structure and receptors they use and includes IL1α (IL1F1) IL1β (IL1F2) and the IL1 receptor antagonist or IL1ra (IL1F3) (Allan et al., 2005; Barksby et al., 2007). IL1β transcription may be induced by different pro-inflammatory stimuli such as microorganisms or their products (Konsman et al., 2002) and by pro-inflammatory cytokines including type I interferons (Brandwein, 1986), TNFα (Williams et al., 2000), and IL1β itself (Granowitz et al., 1992; Churchill et al., 2006). Almost all nucleated cells, including cells of the hematopoietic lineage, produce IL1β (Brough and Rothwell, 2007). IL1β is produced as a large ~ 36 kD inactive precursor protein (pro-IL1β) that needs to be cleaved for biological activity (Fogal and Hewett, 2008). Processing of the pro-IL1β to its active form (17 kD protein) requires caspase-1, an enzyme that is activated by a multiprotein complex called inflammasome (Church et al., 2008). Only a fraction of the active cytokine is released into the extracellular space (Dinarello, 1998; Solle et al., 2001). The release process is enhanced by the secretory stimulus of ATP signaling via the P2X7 receptor (Solle et al., 2001; Ferrari et al., 2006). The active IL1β binds to a specific 80 kD membrane-bound receptor named IL1-receptor (IL1-R) type 1 or IL1-R1. This complex interacts with the cytoplasm protein IL1 receptor accessory protein to form another complex leading to the recruitment of adaptor molecules such as MyD88 and IL1-R associated kinases (IRAK) (Fitzgerald and O'Neill, 2000). Phosphorylation of

IRAK mediates the recruitment of the TNF receptor-associated factor 6 (TRAF6), in turn, this complex along with the tumor growth factor β-activated kinase (TAK1) and the TAK binding protein 2 (TAB2) allows the activation (phosphorylation) of the inhibitor of κB (I-κB) kinase or IKK (Barksby et al., 2007; O'Neill and Greene, 1998). IKK activation produces the phosphorylation and degradation of I-κB, leading to the release of NF-κB, which translocates into the nucleus (O'Neill and Greene, 1998; Trinchieri and Sher, 2007). IL1β also can bind to the IL1-R2, a receptor that lacks an intracellular signaling domain and consequently no signaling is activated. The IL1R2 functions as a decoy receptor (Colotta et al., 1993); i.e., it binds the ligand and prevents it from associating with the signaling receptor (Allan et al., 2005).

IL1β functions as an important neuromodulator in both the normal brain (Kronfol and Remick, 2000; Vitkovic et al., 2000) and in pathological conditions (Gibson et al., 2004; Allan et al., 2005; Simi et al., 2007). IL1β is constitutively expressed in the central nervous system (Tabarean et al., 2006) by microglia, astrocytes, oligodendrocytes, endothelial cells, and neurons at both mRNA and protein levels (Gibson et al., 2004; Simi et al., 2007; Fogal and Hewett, 2008). The expression of IL1β in the brain is upregulated in response to experimental or clinical insults, such as injuries (Allan et al., 2005), neurodegeneration (Simi et al., 2007), and infections (Lundkvist et al., 1999; Bluthe et al., 2000; Sergerie et al., 2007).

**Production of cytokines in influenza-induced infection**

Production of cytokines is also part of the murine immune response against influenza virus infections (Hennet et al., 1992; Conn et al., 1995). Bronchoalveolar lavages (BAL)

from mice infected with PR8 showed the presence of IL1 and TNFα at 2 days PI; furthermore, similar results are observed *in vitro* after viral challenge of alveolar macrophages (Vacheron et al., 1990). Kinetic studies of cytokine production after influenza infection demonstrate that, in BAL from mice infected with PR8, TNFα increases from 24h PI and reaches the peak of production at 36 h PI. IL1 increases are observed from 24h with the peak of production at 48h (Hennet et al., 1992). An increase of IL1α and antiviral activity (perhaps type I IFN) is observed in serum of mice infected IN with influenza (Kurokawa et al., 1996). Survivorship is improved and lesion development in the lungs is attenuated in mice that receive a single dose of an anti-TNFα polyclonal antibody at the time of virus challenge suggesting a detrimental role for this cytokine in response to infection (Peper and Van Campen, 1995). However, studies using IL1R1 deficient mice infected with influenza PR8 demonstrate that IL1α and/or IL1β activity is necessary to reduce the mortality rate after infection (Schmitz et al., 2005). Intranasal inoculation with influenza in IL1β-KO mice also demonstrates a protective role for IL1β that is evident by an increased survival rate after the challenge in the wild type compared to the transgenic mice (Kozak et al., 1995).

The CNS is able to establish an innate immune response and to produce pro-inflammatory cytokines in response to both viral and synthetic dsRNA (Rempel et al., 2005). By 48 h PI, IL1β mRNA increases in the brain stem after intranasal inoculation with influenza (Chen et al., 2004). Also, in mice IN infected with PR8, levels of mRNA for IL1β and TNFα are up regulated in the HT 38 h after inoculation (Alt et al., 2007). Furthermore, after in vitro challenge of mouse microglia and astrocytes with a human strain of H1N1 influenza virus an increase expression of IL1β, IL6 and TNFα mRNA

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and proteins is observed at 6 h post challenge. Finally, the production of cytokines is significantly higher when the cells are stimulated with a neurotropic avian-derived strain of influenza (Wang et al., 2008).

**Cytokines and the thermoregulatory response**

Thermoregulatory responses to systemic inflammation are often determined by the development of either fever or hypothermia (Romanovsky et al., 2005), or both (Leon, 2004). Fever is part of the APR and can be induced by a large number of compounds, including bacterial and viral antigens (Luheshi, 1998; Cartmell et al., 1999; Deak et al., 2005). Cytokines are produced in response to immunological stimulus and they have an important role as endogenous pyrogens (Conti et al., 2004). The history of cytokines and fever started about 50 years ago with the demonstration of a neutrophil-released protein, or leukocyte pyrogen, which induced fever in rabbits and circulated in animals during fevers of different etiology (Atkins and Wood, 1955; Dinarello, 1996). The heat-labile protein was then called an "endogenous pyrogen" by Atkins and Wood (1955). This endogenous pyrogen was later purified, characterized and cloned (Auron et al., 1984). It consists of two polypeptides with the same molecular weight but different electrical charges that were then renamed as IL1β and IL1α (Dinarello, 1996). Since then many studies have been completed that demonstrate the role of IL1β during fever. For example, intraperitoneal and intracerebroventricular (ICV) injection of recombinant IL1β in rats induces a significant increase in body temperature in a dose-dependent manner (Anforth et al., 1998). Furthermore, fever responses after LPS injection are attenuated in IL1β knockout (KO) compared to wild type mice (Kozak et al., 1995). In an experiment with

rats using adenovirus vectors, fever responses are reduced if animals are pretreated with a recombinant IL1ra (Cartmell et al., 1999), which functions as a competitive receptor antagonist to block binding of IL1$\alpha$ and IL1$\beta$ to the IL1-R1, thus preventing IL1RI activation and inhibiting the biological actions of IL1 (Hallegua and Weisman, 2002).

TNF$\alpha$ is detectable early in the circulation after LPS injection and it is also considered an endogenous pyrogen (Dinarello et al., 1986; Zetterstrom et al., 1998). Intraperitoneal injection of mice with TNF$\alpha$ increases temperature for at least 4 h (Zetterstrom et al., 1998; Chida and Iwakura, 2007). Similar results are observed in rabbits after ICV injection with human recombinant TNF$\alpha$ (hrTNF$\alpha$) (Kapas et al., 1992). However, TNF$\alpha$ also has cryogenic or antipyretic properties (Gourine et al., 2000; Leon 2004). Intraperitoneal injection of rats with hrTNF$\alpha$ reduces LPS-induced fever, an antipyretic effect that is abrogated when the animals are pretreated with hrTNF soluble receptor (Klir et al., 1994). Similar results are observed in TNF$\alpha$ double receptor-KO mice studies where the wild type group has a reduced febrile response to LPS compared to the KO group (Leon et al., 1997). These results suggest that the thermoregulatory response to immunological stimuli such as LPS is biphasic in nature and that cytokines modulate such responses by acting as endogenous pyrogens or cryogens (Leon, 2004).

In contrast to influenza infection in humans and other mammals, in mice the observed thermoregulatory response is hypothermia instead of hyperthermia (Kluger et al., 1991; Klein et al., 1992; Fang et al., 1995). Hypothermia during influenza infection is a regulated response (Klein et al., 1992) that may function as a survival mechanism to reduce the metabolic demands of the response to the infection (Leon, 2004). When given access to thermal gradients, influenza infected mice seek cooler temperatures during the

later days of infection when hypothermia is more dramatic (Klein et al., 1992).

Furthermore, influenza-infected mice show hypothermia even in warm environments

(30$^{\circ}$C) (Jhaveri et al., 2007).


**Cytokines and sleep**

Cytokines have an important role in normal physiological functions, such as in sleep

regulation (Krueger et al., 2001). Several cytokines have the capacity to enhance non-

rapid eye movement sleep (NREMS); e.g. the pro-inflammatory cytokines IL1α, IL1β,

IL6, IFNα, IFNγ, TNFα, and TNFβ (for review; Krueger et al., 2001; Krueger and Majde,

2003). Two cytokines studied extensively in their relationship with sleep are IL1β and

TNFα (Opp, 2005). IL1β and TNFα are constitutively expressed in brain and in rats they

have a diurnal variation in their mRNA and protein levels with highest concentrations

correlating with highest sleep propensity (Bredow et al., 1997; Taishi et al., 1998).

Furthermore, after sleep deprivation the increase in NREMS is associated with an

increase in IL1β and TNFα expression (Takahashi et al., 1997; Taishi et al., 1998;

Krueger et al., 2001).

The sleep promoting actions of IL1β were initially demonstrated in rabbits. Central

administration of IL1β enhanced NREMS in this species (Krueger et al., 1984). Further,

ICV injection with an IL1R fragment, an IL1β inhibitor, reduces sleep (Takahashi et al.,

1995). In rats enhanced NREMS occurs after ICV injection of low doses of IL1β (Opp et

al., 1991). In contrast, anti-IL1β antibodies or the IL1ra reduces spontaneous sleep (Obal

et al., 1990). Mice display a robust increase in NREMS and suppression of REMS after

intraperitoneal injection with IL1β (Fang et al., 1998). The somnogenic effects of IL1β

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are abrogated in IL1-R1 knock out mice (Fang et al., 1998). Exogenous administration of IL1β also increases NREMS in other species such as cats (Susic and Totic, 1989) and monkeys (Friedman et al., 1995). These results suggest a role for IL1β in the regulation of physiological sleep (Obal et al., 1990).

The somnogenic effects of TNFα were first described after ICV inoculation with recombinant TNFα in rabbits (Shoham et al., 1987). Intravenous or ICV administration of exogenous TNFα enhances duration and intensity of NREMS and decreases REMS in this species (Shoham et al., 1987; Kapas and Krueger, 1992). The use of anti-TNFα antibodies or the TNFα soluble receptor attenuates spontaneous sleep and reduces the sleep rebound after sleep deprivation (Takahashi et al., 1996; Krueger et al., 2001). In rats, peripheral administration of TNFα increases NREMS (Kubota et al., 2001). Unilateral microinjection of TNFα into the preoptic area of the anterior HT increased NREMS and brain temperature in a dose-dependent manner (Kubota et al., 2002). After intraperitoneal injection with TNFα, mice show a dose-dependent increase in NREMS. This effect is not observed in TNFR1-KO mice that have significantly less baseline sleep than the controls (Fang et al., 1997). Finally, an increase in NREMS is observed in sheep after ICV injection of TNFα (Dickstein et al., 1999). These results suggest that TNFα also has a role in the regulation of physiological sleep (Krueger and Majde, 2003).

The sleep regulatory roles of IL1β and TNFα are closely related to each other (Baracchi and Opp, 2008). For example, IL1-type 1 receptor/TNFR1- double KO mice have a reduced NREMS rebound (compared to wild type) and do not exhibit a REMS rebound in response to sleep deprivation (Baracchi and Opp, 2008). In TNFR1 KO mice that are non-responsive to TNFα, the injection of IL1β increases the NREMS response

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suggesting a degree of independence in their somnogenic actions (Fang et al., 1997).

Similarly, the IL1- type 1 receptor KO mice increase NREMS and decrease REMS after

administration of TNFα (Fang et al., 1998). Downstream effectors responsible for IL1β

and TNFα-induced sleep include adenosine, nitric oxide (NO), nerve growth factor and

growth hormone releasing hormone (GHRH), suggesting that both cytokines can promote

sleep via similar mechanisms (Krueger et al., 2001).

**Murine olfactory bulb organization and its possible interaction with the influenza virus**

One potential pathway for influenza virus access to the CNS is via the olfactory bulb

(OB) after viral IN inoculation (Park et al., 2002, Studahl, 2003). The olfactory system

begins at the olfactory epithelium, a pseudostratified epithelium that contains cilia

embedded in the mucus of the nasal cavity, peripheral processes (olfactory rods),

sustentacular cells and receptor cell bodies. The olfactory epithelium is the only place in

the body where unmyelinated nerve terminals are in direct contact with the environment

(Brodal, 2004, Iwasaki et al., 2004, Mori et al., 1999). These primary olfactory neurons

send unmyelinated axons that form the olfactory nerve (ON), the first cranial nerve,

which passes through the cribriform plate to the OB where the neurons synapse with

second order neurons. The olfactory receptor neurons (ORN) contain surface

glycoproteins that include D-galactosyl and sialic acid components (Allen and Akeson,

1985) that are thought to be recognition sites for the influenza HA protein and necessary

to initiate cell infection (Smith, 1952, Steinhauer and Skehel, 2002). This suggests that

the ORN may be capable of viral uptake.

Another possibility is that the virus found in the ON is located in the specialized cells called olfactory ensheathing cells (OEC). Bundles of the olfactory nerve axons are wrapped by these OEC in place of Schwann cells. In adult mice the OEC are localized in the olfactory nerve (ON) and the GL of the OB (Heredia et al., 1998). These cells are immunoreactive (IR) to neuropeptide Y, protein S100 and vimectin. These cells also contain the polysialic acid containing molecule as well as the neural-cell adhesion molecule or NCAM (Ramon-Cueto and Avila, 1998, Hisaoka et al., 2004). Therefore these cells might be able to capture the viral antigen by endocytosis and transport it along the ON. The ON is a possible route for different viruses to reach the CNS and produce infection (Iwasaki et al., 2004, Reiss et al., 1998, Mori et al., 2005).

Selective removal of the OB using surgical bulbectomy or chemical deafferentation prior to intranasal inoculation with mouse hepatitis virus prevents the spread of this neurotropic virus into the brain (Barnett and Perlman, 1993). Using a recombinant construct of the rhabdovirus, vesicular stomatitis virus, expressing the reporter gene green fluorescent protein (GFP) demonstrates the virus presence as early as 2 days post IN infection in the olfactory nerves within the OB, particularly in the axons that terminated in the glomeruli of the OB (van den Pol et al., 2002). These data provide evidence that the olfactory nerve pathway is used by different viruses to reach the CNS.

Neurotropic strains of influenza virus, such as the recombinant influenza A WSN/33, were used to study the olfactory route of neuroinvasion (Mori et al., 2005). Intranasal inoculation of 2 day old mice with a recombinant strain of influenza (avian-derived H7N1 X human-derived H3N2) results in virus spread to the brain using the olfactory and the trigeminal pathways as evidenced by the presence of viral antigens in the OBs and in

fibers and neurons of the trigeminal nerve and ganglion. This effect is not abrogated even in the presence of passive neutralizing anti-influenza antibodies (Reinacher et al., 1983). After IN inoculation of mice with an H5N1 strain of the virus isolated from a human case after the outbreak of chicken and human influenza in Hong Kong in 1997, the viral antigen is observed in the OB, the vagus and the trigeminal ganglia, suggesting that the virus reaches the brain using the afferent fiber of such nerves following replication in the respiratory epithelium (Park et al., 2002). Mori et al. (1999) suggested that after the virus is injected into the OB, it replicates in OB neurons and from there spreads to different structures within the brain including the anterior olfactory nucleus, medial habenular nucleus, paraventricular thalamic nucleus, dorsal raphe and locus coeruleus. Within 4 days after the IN inoculation of 7 day old mice with a neurotropic strain of the virus, the viral antigen was observed in the ORN and extended into the ON layer of the OB (Aronsson et al., 2003). When immunodeficient mice (which lack the recombination activating gene 1 involved in the recombination process to generate antibodies and T cell receptors) of the same age are used, the presence of viral antigen is found along the olfactory pathway, including the anterior olfactory nucleus, the piriform cortex (Pir), the taenia tecta and groups of neurons in the hypothalamus and the upper brainstem. Wild type mice survive the infection and the virus antigen is present only in the ORN and the OB. These results demonstrate that the immune system is important in controlling the infection and limiting the virus spread to the rest of the brain.

**The olfactory pathway**

In the GL, processes from the ORN establish synaptic contacts with dendrites of the

mitral cell neurons and tufted cells. These cells send their axons along the lateral

olfactory tract to the olfactory cortex, including the Pir and the olfactory tubercle (Tu)

(Price et al; 1991; Brodal, 2004; Suzuki and Bekkers, 2007). The Pir, along with the

accessory olfactory nucleus, sends projections to the feeding regulatory area of the

posterolateral hypothalamus (Price et al., 1991; Josephson et al; 1997; Russell et al.,

2001). Projections from the OB also reach the anterior and the posterolateral nuclei of the

amygdala (Price, 2003; Ubeda-Banon et al., 2007). The information received from the

olfactory input is relayed to the basal (BLA) and then to the central (CeA) nuclei of the

amygdala (LeDoux, 2007). CeA connects with the lateral hypothalamus through the stria

terminalis and sends projections to the reticular formation, vagal nuclei and to the medial

preoptic area (Price, 2003; Wang and Swann., 2006), a site involved in both thermo- and

sleep regulation (Roth et al., 2006; Baker et al., 2005; Saper et al., 2005). Indirect

connections to the hypothalamus are suggested by the specific increase in the number of

Fos IR cells in this brain region in response to olfactory stimuli (Hurtazo and Paredes,

2006). The hypothalamic arcuate nucleus (Arc) lies along the ventrolateral border of the

third ventricle and above to the median eminence and plays an important role in the

regulation of food intake, energy balance and body weight (Bouret et al., 2004). The Arc

receives projections from the medial preoptic area (Magoul et al., 1993) and sends

projections to almost all the nuclei in the hypothalamus as well as to the brain stem (Cone

et al., 2001). Arc projections include the lateral hypothalamus, the medial preoptic area

and posterolateral hypothalamus all of which receive direct or indirect projections from

the olfactory cortex (Price et al., 1991; Price, 2003).

**Propagation of the cytokine signals through the brain**

Cytokines such as TNFα or IL1β often induce the production of each other and themselves as well as diffusible second messengers, including NO, prostaglandins and adenosine. These messengers are induced in part via the activation of the transcription factor NF-κB (Grilli and Memo, 1999; Vitkovic et al., 2000). For instance, one of the major signaling pathways involved in NO production in response to treatment with TNFα is mediated through serine/threonine protein kinases, such as protein kinase A, protein kinase C, and calcium/calmodulin-dependent protein kinase (Tripathi and Sodhi, 2008).

Activation of neurons to express TNFα and IL1β in different parts of the brain in response to an initial central stimulus (disease or lesions) may involve cell to cell communication through the release of nucleotides (Inoue et al., 2007). Neurons express both ATP receptors (Koizumi et al., 2005) and adenosine receptors (Liu and Gao, 2006). Activation of microglia and astrocytes induces the secretion of ATP that serves as a gliotransmitter that binds to the P2 receptors on the neurons and modulates neuronal activity (Zhang et al., 2007). This mechanism has been observed for astrocyte-neuron, astrocyte-microglia and microglia-neuron communication (Inoue et al., 2007). In particular this mechanism is very important for IL1 processing and release from cells (Solle et al., 2001; Ferrari et al., 2006). IL1β secretion is greater when glial cells are stimulated with LPS and ATP compared to untreated controls (controls using only LPS or only ATP), suggesting a possible synergism between the immune stimuli and ATP (Mingam et al., 2008). Furthermore, P2X7 KO mice have attenuated LPS-induced expression of IL1β and TNFα mRNA expression in the hypothalamus (Mingam et al., 2008). Stimulation of microglia with ATP through the P2X7 receptor increases the

production of TNFα and increases the neuroprotective effect of TNF in neuron-microglia cocultures treated with glutamate (Suzuki et al., 2004).

Adenosine, a purine nucleoside, has a role in the control of specific functions in the CNS in both physiological and pathophysiological conditions (Hasko et al., 2005). Interaction of adenosine and its receptors is involved in brain regulated functions such as sleep and arousal, locomotion, cognition and memory, neuroprotection, neuronal degeneration, pain and neuronal maturation (Ribeiro et al., 2002). Adenosine is also involved in the regulation of the cerebral blood flow, especially during neuronal activation (Jakovcevic and Harder, 2007; Shi et al., 2008). In astrocytes, adenosine stimulates their proliferation (astrogliosis) and increases their secretory functions (Hasko et al., 2005). Activation of the adenosine receptor 1 (A1) in astrocytes enhances the secretion of nerve growth factor and S-100β protein both involved in neuronal differentiation and survival (Ciccarelli et al., 1999). Furthermore, adenosine stimulates astrocytes through the A2 receptor to produce IL6, serving as a mechanism of damage control for the brain (Schwaninger et al., 2000). In neurons, IL1β reduces glutamate transmission, an effect that is inhibited when the A1 is blocked, suggesting that specific effects of IL1β on neurons are regulated via adenosine-dependent pathways (Luk et al., 1999).

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# CHAPTER II

# DETECTION OF MOUSE-ADAPTED HUMAN INFLUENZA VIRUS IN THE OLFACTORY BULBS OF MICE WITHIN HOURS AFTER INTRANASAL INFECTION

Jeannine A. Majde[1], Stewart G. Bohnet[1], Georgeann A. Ellis[2], Lynn Churchill[1], Victor Leyva-Grado[1], Melissa Wu[1], Eva Szentirmai[1], Abdur Rehman[1] and James M. Krueger[1]

[1]Department of Veterinary and Comparative Anatomy, Pharmacology and Physiology, Washington State University, Pullman, WA 99164; [2]Department of Biological Sciences, College of Sciences and Mathematics, Auburn University, Auburn, AL 36849

**Abstract**

Influenza pneumonitis causes severe systemic symptoms in mice, including hypothermia and excess sleep. The association of extrapulmonary virus, particularly virus in the brain, with the onset of such disease symptoms has not been investigated. Mature C57BL/6 male mice were infected intranasally with mouse-adapted human influenza viruses (PR8 or X-31) under inhalation, systemic or no anesthesia. Core body temperatures were monitored continuously by radio-telemetry, and tissues (lung, brain, olfactory bulb, spleen and blood) were harvested at the time of onset of hypothermia (13-24 h post-infection) or at 4 or 7 h post-infection (PI). Whole RNA from all tissues was examined by one or more of three RT-PCR procedures using H1N1 nucleoprotein (NP) primers for minus polarity RNA (genomic or vRNA) or plus polarity RNA (replication intermediates). Selected cytokines were assayed at 4, 7 and 15 h in the olfactory bulb (OB). Minus and plus RNA strands were readily detected in OBs as early as 4 h PI by nested RT-PCR. Anesthesia was not required for viral invasion of the OB. Cytokine mRNAs were also significantly elevated in the OB at 7 and 15 h PI in infected mice. In contrast, controls receiving boiled virus expressed only input vRNA and that only in lung. Immunohistochemistry demonstrated localization of H1N1 and NP antigens in olfactory nerves and the glomerular layer of the OB. Therefore a mouse-adapted human influenza viral strain, not known to be neurotropic, was detected in the mouse OB within 4 h PI where it appeared to induce replication intermediates and cytokines.

**Introduction**

Many respiratory and intestinal viral infections are marked by abrupt onset of fever, somnolence and malaise, commonly termed the 'flu,' within 2-3 days following infection. There is little evidence in the majority of cases that such viruses replicate outside the mucosal surfaces that they target, although their systemic symptoms can be quite severe. It is assumed that acute viral symptoms are a consequence of cytokine release from infected target tissues acting upon the brain, although evidence for this assumption is minimal.

Influenza virus is among the respiratory viruses thought to be restricted to the upper respiratory tract in classical human 'flu' and to the entire respiratory tract in more severe influenzal pneumonitis. However viremia does occur in mild human influenza, although it can only be detected prior to onset of clinical symptoms using classical virus isolation techniques (Stanley and Jackson, 1966). Furthermore, clinical observations made during the influenza pandemics of 1918 (Kristensson, 2006) and 1957-58 [reviewed (Schlesinger *et al.,* 1998; Ward, 1996)] indicate that influenza virus can invade the brain in certain lethal infections. Psychiatric sequellae to influenza are also seen (Kristensson, 2006). In the last ten years, influenza-associated encephalitis/encephalopathy has been diagnosed relatively frequently in Japanese children (Kristensson, 2006; Sugaya, 2002), but no particular viral strain has been implicated. A review of 84 hospitalized children in Taiwan with documented influenza A reported that 31% had neurological symptoms (Wang *et al.,* 2003); influenza A is more commonly seen in children with neurological involvement than is influenza B (Romero and Newland, 2003). Viral RNA is

occasionally detected in cerebral spinal fluid by the reverse transcriptase polymerase chain reaction (RT-PCR) in encephalopathy patients (Fujimoto *et al.,* 1998; Steininger *et al.,* 2003). Japanese case reports have led to a greater awareness of these neurological complications of influenza, with the consequence that adult and pediatric cases in Europe (Rantalaiho *et al.,* 2001; Steininger *et al.,* 2003) and pediatric cases in the United States (Maricich *et al.,* 2004; Weitkamp *et al.,* 2004) have been recognized. More recent studies have detected influenza-associated neurological complications, primarily seizures, in 4 pediatric cases per 100,000 person-years; risk factors include neurological or neuromuscular diseases (Newland *et al.,* 2007). Studies in Finland suggest that 4-7% of human viral encephalitides are associated with influenza infections (Rantalaiho *et al.,* 2001).

Infections of mice with mouse-adapted human strains of influenza virus by the intranasal (IN) route are also assumed to be restricted to the respiratory tract, although viremia with the most commonly employed human strain, mouse-adapted A/PR/8/34 H1N1 (PR8), can be detected with sensitive methods within the first 24 h post-infection (PI) (Frankova and Rychterova, 1975; Ishida *et al.,* 1959; Mori *et al.,* 1995). Mouse central nervous system (CNS) infections with human strains have been primarily studied using the neurovirulent WSN or NWS strains derived by serial intracerebral (IC) passages in mice (Ward, 1996). These strains are studied using the IC route in adult mice or the IN route in neonates (Schlesinger *et al.,* 1998); WSN does not appear to invade the brain when inoculated by the IN route in immunocompetent adult mice (Garcia-Sastre *et al.,* 1998a). Other studies of influenza infections of the mouse brain have employed avian strains or avian recombinants that are intrinsically neurotropic as well as

neurovirulent in susceptible birds and mammals (Schlesinger *et al.,* 1998). Neurotropism

in mice is also a notable feature of human isolates of avian H5N1 viruses from Hong

Kong in 1997 (Tanaka *et al.,* 2003) and Southeast Asia in 2004 (Maines *et al.,* 2005).

In order to clarify the role of extrapulmonary virus in the viral 'flu' syndrome (or

acute phase response), we have employed one-step RT-PCR, or the more sensitive two-

step nested RT-PCR (nPCR) or real-time quantitative PCR (qPCR) techniques to detect

both minus polarity (genomic vRNA) and plus polarity (replication intermediates) PR8

nucleoprotein (NP) RNA. RNA was extracted from tissues collected from mature male

C57BL/6 mice infected IN with PR8 or X-31 mouse-adapted human H1N1 influenza A

strains, with or without anesthesia. Tissues were harvested at or near the onset of

hypothermia, the earliest quantifiable manifestation of systemic influenzal disease in

mice (Fang *et al.,* 1995), or at 4 or 7 h PI when no symptoms were perceptible. PCR

analyses were performed in lung, whole brain minus intact olfactory bulb (OB), isolated

intact OB, spleen and blood from infected mice or from controls inoculated with heat-

inactivated virus. In addition, cytokine mRNA expression was examined by qPCR in

selected 4, 7 and 15 h OB samples. Following detection of viral RNA in the OB by

nPCR, we employed immunohistochemistry (IHC) to detect viral antigens in the OB at

15 h PI. To our knowledge this study represents the first demonstration of neurotropism

of a human influenza virus in immunologically mature mice within the first 15 h PI. The

results reported here may provide a biological basis for the severe systemic symptoms

associated with influenza infections as well as influenza-associated neuropathologies.

# Materials and methods

*Mice*

Specific pathogen-free male C57BL/6 mice (Taconic Farms, Germantown, NY, or Jackson Laboratory, Bar Harbor, ME), 10 to 14 weeks of age at the time of inoculation, were maintained in AAALAC-approved animal quarters under veterinary supervision. The procedures employed were approved by the Washington State University Institutional Animal Use and Care Committee. After two weeks quarantine, mice were housed in standard plastic cages with filter tops and maintained at $29 \pm 2°C$ [the thermoneutral zone for mice that allows full expression of stimulus-induced temperature changes (Hoffman-Goetz and Keir, 1985)] in a closed environmental chamber on a 12:12 hr light/dark cycle.

*Viruses and virus preparation*

PR8 influenza virus was purified from specific pathogen-free (SPF) egg allantoic fluid using endotoxin-free reagents and titered as described in (Chen *et al.*, 2004). The virus stock was shown to be free of detectable endotoxin or mycoplasma contamination (Chen *et al.*, 2004). In one experiment the X-31 strain of influenza A [a reassortant between PR8 and A/Aichi/68 (H3N2) (Lee *et al.*, 2001)] in allantoic fluid from SPF eggs was employed. X-31 expresses H3N2 surface genes but contains the internal genome segments of PR8, including the NP. X-31 requires about 10-fold more virus to kill mice in the same time-frame as PR8 (Price *et al.*, 2000).

*Inoculation procedures*

PR8-infected mice received 2.5 x $10^6$ TCID$_{50}$ purified PR8 (high dose, lethal in 4-5 days PI) in Dulbecco's PBS, or two 10-fold dilutions of this virus inoculum. X-31 infected mice virus received 1.5 x $10^6$ TCID$_{50}$ of crude virus.  All control mice received virus that was heat-inactivated using one of two incubation techniques: a 56°C water bath for 30 min or submersion in a boiling water bath for 15-25 min (boiled virus). Inoculations were performed within an hour of light onset.

For IN inoculations under anesthesia, either light methoxyflurane (Metofane, Schering-Plough Animal Health, Union, NJ) inhalation (INH) anesthesia or intraperitoneal (IP) ketamine/xylazine (K/X) (Chen *et al.,* 2004) were used. Anesthetized mice were hand-held in a semi-recumbent position and inoculated with 50 μl volumes delivered slowly to the nostrils (25 μl per nostril) with a 100 μl micropipette. Unanesthetized mice were restrained in a 50 ml conical plastic tube with the tip removed to provide access to the nose and inoculated as described above.  After inoculation unanesthetized mice were held in a recumbent position for one min.  Control animals received the same volume of high dose virus that was heat inactivated by one of the two methods described above, except in the body temperature studies where infected mice were compared against untreated mouse baseline data.

*Body temperature measurements*

At least one week prior to infection, mice (6/group) were implanted intraperitoneally with 0.5 g VM-FM radio transmitters (Mini-Mitter, Inc., Sunriver, OR) capable of monitoring body temperature and locomotor activity through receivers under the

individual cages.  Temperature data from individually housed mice were collected at 6

min intervals and processed using the VitalView data acquisition system (Mini-Mitter,

Inc.); each data point in Fig. 1 is an average of 10 values collected over 1 h.


*Tissue sampling*

A total of 127 boiled virus controls and 131 live PR8 inoculated mice were sampled

for RT-PCR analysis.  Tissues (lung, blood, spleen, intact OB, or whole brain with only

the caudal root of the OB[*]) were harvested at the time of onset of hypothermia (drop in

body temperature of 1°C or more of at least two animals in the group compared to

baseline temperatures at that time of day, cf. Fig. 1).  In mice not monitored for

temperature change, tissues were harvested at 14 or 15 hr PI.  Lungs and OBs from high

dose PR8-infected mice were also harvested at 4 and 7 hr PI prior to onset of

hypothermia.  For molecular analysis mice were exsanguinated by open cardiac puncture

under Metofane anesthesia and tissues were harvested into liquid nitrogen (taking care to

clean the dissecting tools in dilute sodium hydroxide between each organ dissection), and

stored at -80°C for subsequent RNA extraction.

For IHC analysis OBs were harvested from whole brains taken at 15 hr after IN

inoculation under light Metofane anesthesia with high dose live PR8 or boiled PR8.

Mice under deep Metofane anesthesia were perfused transcardially with 20 ml of warm

normal saline followed by 40-60 ml of 4% Para formaldehyde in phosphate-buffered

saline (PBS).  Control mice unexposed to either inoculum were perfused in a similar

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[*] Our dissection methods for removing the brain or OB changed over time; early experiments where whole
brain was examined were not dissected in a manner that reliably removed the rostral OB with olfactory
nerve roots together with the caudal regions of the brain from cortex through the brain stem.  Studies
involving the OB *per se* were performed with intact OBs where the olfactory nerves were dissected away
from the cribriform plate.

manner.  Whole brains, including intact OBs, were removed, allowed to post-fix for 2 hr, and then immersed in 20% sucrose overnight.  The brains were then frozen in crushed dry ice and stored at -80 ºC until sectioned.

*RNA isolation for RT-PCR procedures*

Total RNA was extracted from frozen tissues except blood for RT-PCR and nPCR using TRIzol reagent (Invitrogen, Carlsbad, CA, Cat. No. 15596-018) according to the manufacturer's recommended protocol.  Total RNA was extracted from whole blood samples (approximately 0.7 ml) using the SV Total RNA Isolation System (Promega, Cat. No. Z3100, Madison, WI) following the manufacturer's protocol.  Alternatively, RNA from blood was extracted using the RiboPure-Blood kit (Ambion, Austin, TX) according to the manufacturer's instruction.

*Primers Employed*

Primers used for NP RT-PCR, nPCR, and qPCR studies are listed in Table 3.  Primer sequences used for qPCR analysis of IL1β, TNFα, OAS-1A and Mx1 are reported in (Traynor *et al.,* 2004).   Our NP primers were examined to determine if their products would include the human protein that shares sequence homology with influenza NP (Cooper, Jr. *et al.,* 1996); the PCR products of our NP primers do not include this potentially confounding region.

*Detection of viral genes by RT-PCR and nPCR analysis*

One-step RT-PCR and two-step nPCR procedures were adapted from the methods of Mori, *et al.*, 1995.  Two nPCR methods were employed, which differed primarily with respect to the amount of cDNA template used.  Method 1 used ten-fold more cDNA template for the first step and five-fold less for the second step than Method 2.

*Copy number analysis*

Viral RNA was isolated from a sucrose-gradient-purified PR8 preparation using Trizol (Invitrogen). Viral RNA (0.25 μg) was reverse transcribed with primers complementary to the 5' or 3' ends using superscript II (Invitrogen).   Additional cysteines were ligated to the 5' primer region to increase the $T_m$ in order to make the primers useful for PCR amplification. cDNA was PCR amplified with the sense and anti-sense primers and the products cloned into pCR 4.2 TOPO vector (Invitrogen Catalog # K4575-01).  Plasmids were linearized with Not-I or Spe-I restriction enzymes.  Plus and minus GS-5 RNA was amplified with MEGAscript® T7 or MEGAscript® T3 kits (Ambion) using the manufacturer's instructions.  RNA was purified with MEGAclear™ Kit (Ambion).  Approximately 100 μg of RNA was obtained per 1 μg of linearized plasmid.  RNA was mixed with yeast tRNA (0.2 μg/ml in 10 mM Tris, 1 mM EDTA buffer, pH = 7.6) prior to 10-fold serial dilutions to establish a standard curve.  Nested PCR Method 1 was shown to detect as few as 18 copies of viral RNA/μg tRNA, both minus and plus strands.  Method 2 detected 1800 copies of plus and 18,000 copies of minus strand RNA/μg tRNA.

*Quantification of RNA transcripts by qPCR*

Five µL of cDNA (12.5 ng of total lung RNA) or 5 µL of cDNA (25 ng of total brain RNA) were amplified by PCR using NP and cyclophilin primers provided in Table 1 or cytokine primers provided in (Traynor *et al.*, 2004). The total reaction volume was 25 µL containing 0.2 µM sense and anti-sense primers, 12.5 µL Platinum qPCR Supermix-UDG (Invitrogen, Carlsbad, CA), a 1:100,000 dilution of SYBR green (Molecular Probes, Eugene, OR), and a 1:100,000 dilution of Fluorescein Calibration Dye (Bio-Rad Laboratories, Hercules, CA). QPCR was performed and analyzed as described in (Bohnet *et al.*, 2004). Data are expressed as fold-increase of experimental over control ± the standard error of the mean (SEM). The Student's t-test was used for statistical analysis of the fold-increase data and $p < 0.05$ was considered to indicate a statistically significant difference.

*Olfactory bulb immunohistochemistry*

Coronal 30 µm frozen sections of the OB were cut and stained by the immunoperoxidase procedures described in (Churchill *et al.*, 2005) using diaminobenzidine as a chromophore. Viral antigen studies were performed with either the mouse monoclonal PR8-reactive anti-influenza virus A H1N1 antibody (Chemicon, Temecula, CA, catalog # MAB8261, lot # 24050638, dilution 1:100) or the mouse monoclonal anti-influenza N1 NP antibody (Chemicon catalog # MAB8257F-5, lot #0506002204, dilution 1:100) as the first antibody. The second antibody for viral antigen studies was biotinylated horse anti-mouse IgG (1:500; Vector Labs, catalog # BA2001, lot # N0109, Burlingame, CA) with 2% normal horse serum as a blocking agent. Control

sections were treated with the antibody to mouse IgG but no primary antibody.  No

specific immunoreactivity was observed in the control sections where the primary

antibody was omitted.  Rostral portions of the OB were examined at various

magnifications and photographed using a Leica DMLB microscope and a Spot camera.  .

**Results**

*Body temperature response to PR8*

We and others have demonstrated that a rapid fall in body temperature occurs early after lethal influenza infection of mice (Conn *et al.,* 1995; Fang *et al.,* 1995). In our model the time of onset of hypothermia was observed in order to ascertain the time at which the mice manifested influenza symptoms following infection with various doses of PR8. Precipitous hypothermia began at 11-13 h PI (three experiments, n = 20) in mice given 2.5 x $10^6$ median tissue culture infection doses ($TCID_{50}$) of PR8 (the highest dose of PR8 employed) inoculated IN under Metofane anesthesia (representative experiment Fig. 2.1). The body temperature continued to decline steadily until it reached about 32°C on day 4 (data not shown), at which point the animals began to die (Wong *et al.,* 1997). Ten-fold dilutions of virus induced a qualitatively similar pattern of hypothermia beginning at 23.5 h and 25.2 h, respectively (data not shown). Based on these observations, mice infected with 2.5 x $10^6$ $TCID_{50}$ PR8 were harvested at 15 h to assure that all animals were manifesting hypothermia, or at 4 or 7 h PI prior to the onset of hypothermia. Mice infected with 1.5 x $10^6$ $TCID_{50}$ X-31 under Metofane anesthesia were harvested at 14 h PI after onset of hypothermia.

*Viral RNA detection using heat-inactivated virus*

The standard procedure for inactivating virus at 56°C for 30 min did not reliably eliminate detection of hemagglutinin (HA) plus strand RNA in lung as determined by one-step RT-PCR (data not shown). Thus heat inactivation in subsequent experiments

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was conducted by suspending the virus preparation in a boiling water bath for 15-25 min. The more intense heat inactivation procedure eliminated the detection by nPCR Method 1 or Method 2 of nucleoprotein (NP) plus strand in all lung samples regardless of whether lungs were harvested at 4, 7, or 15 h PI (data not shown). Neither RNA strand was detected by any PCR method in the OBs of 66 mice inoculated with boiled virus (e.g., Table 2.1) nor was either RNA strand detected in spleen, blood or whole brain (data not shown) in mice challenged with the boiled virus. In contrast, the HA minus strand was detected by RT-PCR in 5/6 infected lungs at 13 h PI and the NP minus strand was detected in 2/3 infected lungs using nPCR Method 1 at 12 h PI in mice challenged with $2.5 \times 10^6$ TCID$_{50}$ of PR8 under Metofane anesthesia (data not shown). There was no detection by nPCR Method 1 of NP minus or plus strand RNA in any tissue obtained from 6/6 mice unexposed to the virus, boiled or live (data not shown).

*Viral RNA detection by nPCR in whole brain (lacking an intact OB)*

An early experiment using one-step RT-PCR and primers for the PR8 hemagglutinin (Table 2.3) detected minus strand in 2/6 whole brain samples harvested at 13 h (data not shown). Subsequent experiments with whole brains employed the more sensitive two-step nPCR procedure (Method 1) and established primers for PR8 NP (Table 2.3). Whole brain was positive at hypothermia onset for both NP RNA strands in a majority of samples from mice receiving high dose PR8 using nPCR Method 1 (10/12 minus strand; 8/12 plus strand). Both NP plus and minus strands were detected sporadically using the lower PR8 doses as well (3/12 minus strand; 4/12 plus strand) in mice sampled at the

time of hypothermia onset.  X-31 NP minus and plus strand RNA were also detectable by nPCR Method 1 in 3/3 samples of whole brain harvested at 14 h PI.


*Viral RNA detection in the intact OB*

We examined the OB for NP expression in a series of mice infected with high dose PR8.  OBs yielded positive nPCR Method 2 signals (e.g., Tables 2.1 and 2.2) in most mice sampled at 4, 7 or 15 h PI, regardless of the anesthesia protocol employed.  The NP minus strand was more consistently detected than NP plus strand using either method of nPCR (Tables 2.1 and 2.2); NP plus strand was never detected in the absence of NP minus strand in the OB.  Nested PCR Method 1 increased the frequency of NP detection in OB over Method 2 (Table 2.2), especially at 4 h PI.  In qPCR analyses, a few mice expressed 100-fold the NP level (both strands) seen in other mice in the same experiment, suggesting highly variable invasion of the OB by PR8 in quantitative terms (data not shown).


*Effects of different anesthesia protocols on OB invasion by virus*

The frequency of viral NP expression in the intact OB using nPCR Method 2 for mice anesthetized or unanesthetized during infection is shown in Table 2.1.  These nPCR data obtained from OBs sampled at 4, 7 or 15 h PI, indicate that OB invasion by PR8 (minus strand) is not substantially enhanced by inhalation anesthesia, though plus strand expression is less consistent in the IP-anesthetized mice (Table 2.1).  In unanesthetized mice, 5/6 OB samples obtained at 7 h PI were positive for viral RNA (Table 2.1), indicating that anesthesia was not required for viral invasion of the OB.

*Comparison of viral RNA detection in OB and somatosensory cortex*

Somatosensory cortex (SCtx), which lacks direct projections from the olfactory bulb, was examined for viral RNA by nPCR Method 2 (Table 2.2). Positive signal (minus strand only) was detected in only 1/6 mice at 4 h PI and no other animals at 7 or 15 h PI. In contrast, the same method applied to the OBs of the same mice detected minus strand in 12/16 mice and plus strand in 5/16 mice at the various time periods PI (Table 2.2).

*Viral RNA detection by nPCR in lung, blood and spleen*

In mice challenged IN with live PR8, both NP strands were detected at 4 h PI by nPCR Method 2 in 16/16 lungs infected under either inhalation (n=6), IP ketamine/xylazine (K/X) (n=6) anesthesia, or no anesthesia (n=4) (data not shown but same mice as used for Tables 2.1 and 2.2). Similarly, in another experiment, lungs in 12/12 mice challenged with live PR8 were positive using PCR Method 1 for both RNA strands when lungs were harvested at the time of hypothermia onset (data not shown). In yet another experiment, mice infected with live X-31 were also positive for both strands in 3/3 lung samples harvested at 14 h PI.

In the mice represented in Table 2.1, whole blood and spleens were negative for viral NP RNA at all time periods using nPCR Method 2 with the exception of one spleen sampled at 15 h PI (Table 2.2). However, when PCR Method 1 was used, viral NP RNA was sporadically detected in both blood and spleen at the time of hypothermia onset, regardless of the dose of virus employed (data not shown). Detection frequency was similar at 4, 7 and 15 h PI in a second experiment where nPCR Method 1 was used

(Table 2.2).   Mice positive for NP in blood did not correlate with the mice positive for

NP in spleen.  NP minus strand detection was more frequent in the OB than in blood,

spleen or SCtx (Table 2.2).

Using copy number analysis, nPCR Method 1 was shown to detect as few as 18

copies of viral RNA/µg tRNA, both minus and plus strands, while the lowest level of

detection by nPCR Method 2 was 1,800 copies of plus and 18,000 copies of minus strand

RNA/µg tRNA.  The relative sensitivity of the two methods is consistent with our

detection frequency findings.


*Cytokine mRNA detection in the OB*

Expression of mRNAs for two pro-inflammatory cytokines [interleukin-1 beta (IL1β)

and tumor necrosis factor alpha (TNFα)] and two enzymes efficiently induced by type I

interferons (IFNs) [2',5' oligoadenylate synthetase 1a (OAS) and myxovirus resistance-1

GTPase (Mx1)] was evaluated.  These two enzymes are sensitive markers for type I IFN

expression (Samuel, 2001).  The results from three experiments are shown in Figure 2.2.

No cytokine or enzyme mRNAs were elevated at 4 h PI.  IL1β and TNFα mRNA levels

also were not elevated at 7 h PI, but both cytokine mRNAs were significantly up-

regulated at 15 h PI (Figure 2.2).  Significant up-regulation of both IFN-induced enzymes

occurred at both 7 h and 15 h PI (Figure 2.2).  We were unable to reliably detect the most

common type I IFNs themselves in the OB at 7 or 15 h PI using IFNα consensus primers

that detect all IFNα isoforms or with IFNβ primers (Traynor et al 2004) (data not shown).

*OB Immunohistochemistry at 15 h PI*

A large number of cells intensively stained for H1N1 were seen in the olfactory nerves and OB glomerular layer (GL) but rarely in the internal layers of the OB in mice inoculated with live PR8 (Figures 2.3C and 2.3D). In contrast, in mice inoculated IN with boiled PR8, very few influenza-H1N1-immunoreactive cells were evident 15 h PI (Figures 2.3A and 2.3B). A similar distribution of stain was seen when OBs were reacted with an anti N1 NP antibody in infected mice (Figures 2.3G and 2.3H) or control mice (Figures 2.3E and 2.3F).

**Discussion**

Our results confirm and extend the studies of Mori *et al.* (1995) using PR8 in mice, but we have examined the brain when objective illness is first detected at 13 to 15 hr (Figure 2.1), or well before detectable illness at 4 h or 7 h PI, rather than at 5 days when the infection is advanced.  We show by RT-PCR that virus is undergoing at least partial replication (expression of the NP plus strand) in brain and in the intact OB (Tables 2.1, 2.2), and that expression of the IFN-induced enzyme transcripts is up-regulated in the OB by 7 h and 15 h (Figure 2.2).  IL1β and TNFα mRNAs are both significantly up-regulated at 15 h PI in infected OBs (Figure 2.2) when symptoms are apparent.

The IHC analysis at 15 h PI indicates that an antibody to H1N1 (reported to bind to PR8, and presumably reactive with a viral surface protein) and an antibody to N1 NP stains cells mainly within the glomerular layer (GL) of the OB as well as cells within the olfactory nerves (Figure 2.3).  The stained cells morphologically resemble microglial cells.  Preliminary data also indicate that these microglia-like cells are IL1β-immunoreactive, and thus they may be the source of that cytokine in the OB that we demonstrated by qPCR at 15 h (Figure 2.2).

Our heat-inactivation studies also extend those of Bussfeld *et al.* (1998), who have demonstrated that virus heated at 56°C for 30 min (a commonly employed heat inactivation regimen) is capable of cytokine induction and that boiling the virus for 15 min eliminates cytokine induction.  Our nPCR data indicated that virus heated to 56°C for 30 min is still capable of at least partial replication in that plus strand was often detected in the lung.  Boiling the virus eliminated the plus strand expression in the lung

and gave consistently negative findings in the OB when examined by nPCR and qPCR, as well as negative cytokine mRNA induction and negative IHC findings.

It is noted that the intact OB is often not collected in studies of viral neurotropism (as was the case in our initial studies) and the rostral portion of the OB may not be included in whole brain analyses. To acquire the intact OB along with the bulk of the mouse brain the olfactory nerves must be carefully cut along the cribriform plate, which is not always done. From our results it would appear that the OB is an important brain region for analysis of IN infections, regardless of whether the agent is defined as neurotropic. Further, examination of the OB with extremely sensitive techniques such as nPCR earlier than is traditionally done (usually no earlier than 2-3 days PI) can be informative as abortive viruses may not be detectable after one replication cycle even though they may induce cytokines *in situ*.

Viremia has been reported early in mouse PR8 infections (Frankova and Rychterova, 1975; Ishida *et al.,* 1959; Mori *et al.,* 1995), and we sporadically saw viral NP RNA in whole blood and spleen by nPCR as early as 4 h PI (Table 2.2). Therefore, residual blood as a source of the PCR signals in the brain must be considered. Virus may enter the blood by penetrating the fenestrated endothelium of the olfactory mucosa following IN inoculation (Schlesinger *et al.,* 1998). From the blood the virus may potentially traverse the blood-brain barrier via extracellular channels (Banks, 2004). However, blood sources are unlikely in view of the focal distribution of the viral antigen in a very circumscribed area of the rostral OB as visualized by IHC (Figure2.3) and the very infrequent detection of viral RNA in the SCtx (Table 2.2), a brain region lacking direct neural projections from the OB. Instead, viral antigen localization within the olfactory nerves and at the site

of the first synapse of those nerves (the GL) strongly points to invasion of the OB by PR8 via the olfactory nerve.  Olfactory nerves have some unique anatomical features (Mori *et al.,* 2005), discussed below, that may promote viral transport.

Olfactory nerve transport of virus has been demonstrated by two established mechanisms: the axonal transport pathway and the olfactory epithelial pathway (Dahlin *et al.,* 2000).  Mouse hepatitis virus (Barnett and Perlman, 1993), vesicular stomatitis virus (Huneycutt *et al.,* 1994), herpes simplex (Johnson, 1964) and large protein complexes (Thorne *et al.,* 1995) can enter the OB via axonal transport through olfactory receptor neurons (Mori *et al.,* 2005).   Axonal transport would allow influenza virus to move a distance of about 8 mm in 4 h assuming the transport speed is similar to that of herpes virus (Maratou *et al.,* 1998); the length of the olfactory receptor axons in the mouse is likely to be less than 8 mm and thus this pathway may be relevant to our observations. Axonal transport would be consistent with the dense localization of viral antigen in the GL (Figure 2.3B) where the first synapse of olfactory neurons takes place.

The olfactory epithelial pathway is relevant when a virus enters into the nasal lamina propria (Dahlin *et al.,* 2000).  From this tissue layer the virus can enter the perineural space of nerves projecting to the subarachnoid space of the OB and other regions of the brain.  Herpes virus can use both axonal transport and perineural transport simultaneously (Johnson, 1964).

A newly discovered route to the GL, which has been demonstrated to permit passive transport of ultrafine carbon particulates in the size range (100 nm) of viruses (Oberdorster *et al.,* 2004), are the channels formed by a fibroblastic sheath surrounding the network of olfactory ensheathing cells that wrap olfactory nerve fascicles (Li *et al.,*

2005).  Transport of carbon particles to the OB via these channels was detected as early as day 1 post challenge (Oberdorster *et al.,* 2004).  Viruses have not yet been demonstrated to enter the brain via these channels.

The model that we have constructed based on these observations is as follows:  Virus enters the upper nasal cavity and binds to the olfactory receptor dendritic cilia that probably bear the sialic acid viral receptor (Allen and Akeson, 1985); the virus is then rapidly taken up by the olfactory receptor neurons by endocytosis and is transported by axonal flow to the glomeruli, where the axon terminates in a synapse. Virions and/or viral proteins detected by our mouse H1N1 and NP antibodies as well as viral RNA are released into the synaptic cleft, perhaps by exocytosis. After release into the extracellular environment, these viral products are taken up by resident microglia or other glial cells. The glial cells are thus activated to produce cytokines such as IL1$\beta$ in response to viral RNA, which in turn initiate the APR by acting upon the hypothalamus.  Investigations are ongoing to resolve all involved cell types, the cytokines induced in them, and the other suppositions underlying this model.

Our studies have not yet examined whether PR8 influenza virus can be detected within the trigeminal nerve, vagus nerve or other demonstrated routes of viral nerve transport from the respiratory system (Johnson and Mims, 1968; Park *et al.,* 2002).  Some neurotropic viruses are selective as to which nerves are employed for transport [for instance, CNS invasion by mouse hepatitis virus is restricted to the olfactory nerve (Barnett *et al.,* 1993)] where other viruses such as the herpes viruses may employ numerous nerves to invade the CNS (Barnett *et al.,* 1993).

While conclusive studies of the route of PR8 transport to the OB will require a more extensive time series and finer resolution of virus, our observations suggest that PR8 can be considered neurotropic but not progressively neurovirulent. This conclusion is compatible with the absence of neuropathology seen by Iwasaki *et al.* in PR8 infected mice (Iwasaki *et al.,* 2004) and the general lack of neurological symptoms observed in mice infected IN with PR8. Cytokines such as those that we detect by qPCR are likely to be induced by the viral RNA [single-stranded and/or double-stranded (Diebold *et al.,* 2004)] that we detect in the OB by both nPCR and qPCR. Neural pathways from the OB to the hypothalamus (Aronsson *et al.,* 2003) could conceivably result in OB-synthesized cytokines activating the hypothalamus to induce the viral APR in the absence of actual hypothalamic infection or the action of cytokines made in the respiratory tree.

Because we detect viral RNA in the OB by 4 hr, the signal we detect is likely to derive from input virus. Therefore, a functional Mx enzyme that inhibits influenza virus replication (Haller *et al.,* 1980) is not present in our mice (or most inbred strains) and is unlikely to affect OB invasion, although its absence may affect cytokine induction and viral replication. Similarly, the NS1 gene that blocks IFN induction (Garcia-Sastre *et al.,* 1998b), which is present in both viral strains that we have used, would not be expected to affect passive viral invasion although it also may affect cytokine induction and viral replication. Pre-existing antibody may also inhibit CNS invasion. The effects of these viral and host factors on viral invasion of the OB are under investigation.

Clearly the vast majority of human influenza infections or live virus vaccinations do not lead to clinical neuropathology, and if the virus routinely invades the human brain, the brain's defense mechanisms (and/or influenza's replication properties in neural

tissues) nearly always eliminates progressive infection. Perhaps the most intriguing possibility raised by these findings is that the severe systemic symptoms associated with pandemic influenzal infections may reflect direct cytokine induction in the brain resulting from viral invasion via nerves, rather than, or in addition to, localized cytokine induction in the respiratory tree. Closer scrutiny of the brain following IN infections with viruses not known to be neurotropic may help us to better understand systemic viral illness.

In summary, in these studies we have demonstrated influenza viral RNA in extrapulmonary tissues of mature mice, including the intact OB, as early as 4 hr PI following IN infection. Viral RNA expression in the OB was accompanied by cytokine induction as early as 7 hr PI. Detection of the NP plus strand in many samples suggested that virus is undergoing at least partial replication in the OB. IHC methods indicated that viral antigen is primarily localized to the olfactory nerve and glomerular layers of the OB, probably in glial cells, at 15 hr PI. Anesthesia was not required for viral invasion of the OB, and all boiled-virus controls were negative for viral RNA and cytokine mRNA expression. Our basic observations were confirmed using two viral strains, primers for two different viral genes, three distinct RT-PCR methodologies, and two distinct virus-specific monoclonal antibodies over the course of these studies. Thus, we have demonstrated rapid invasion of the brain by mouse-adapted influenza virus, apparently via olfactory nerves. Determining the relevance of these findings to human influenza encephalopathy/encephalitis or influenza symptoms will require examination of appropriate clinical specimens using virus-detection techniques of comparable sensitivity.

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**Table 2.1 Frequency of NP Detection by nPCR in Olfactory Bulbs Using Different Anesthesia Protocols**

| Hours Post-Infection of Tissue Harvest | Anesthesia Used | Boiled PR8 *Minus Strand* # Positive/ # Mice Tested | Boiled PR8 *Plus Strand* # Positive/ # Mice Tested | Live PR8 *Minus Strand* # Positive/ # Mice Tested | Live PR8 *Plus Strand* # Positive/ # Mice Tested |
|---|---|---|---|---|---|
| 4 | INH | 0/6 | 0/6 | 6/6 | 6/6 |
| 7 | INH | 0/6 | 0/6 | 6/6 | 5/6 |
| 15 | INH | 0/6 | 0/6 | 6/6 | 2/6 |
| 4 | IP | 0/6 | 0/6 | 3/6 | 0/6 |
| 7 | IP | 0/4 | 0/4 | 3/4* | 2/4* |
| 15 | IP | 0/6 | 0/6 | 6/6 | 3/6 |
| 7 | None | 0/6 | 0/6 | 5/6* | 4/6* |

Data show the frequency of detection of minus or plus strand NP RNA using Method 2 in olfactory bulbs harvested at 4, 7 or 15 h post-infection using either Metofane inhalation anesthesia (INH), intraperitoneal ketamine/xylazine anesthesia (IP) or no anesthesia (None).  Boiled virus columns represent control mice, while live PR8 columns represent infected mice.  Ratios marked with an asterisk (*) indicate that a single mouse was negative for both minus and plus strands.

**Table 2.2 Comparison of Frequency of PR8 NP Detection in Various Tissues Using**

**Method 1 or Method 2 nPCR**

| Hours Post-Infection of Tissue Harvest | Tissue Sampled | Method 1 *Minus Strand* | Method 1 *Plus Strand* | Method 2 *Minus Strand* | Method 2 *Plus Strand* |
|---|---|---|---|---|---|
| 4 h | Spleen | 1/6 | 0/6 | 0/6 | 0/6 |
| 7 h | Spleen | 0/4 | 0/4 | 0/4 | 0/4 |
| 15 h | Spleen | 2/6 | 4/6 | 1/6 | 0/6 |
| 4 h | Blood | 2/3* | 2/3* | 0/6 | 0/6 |
| 7 h | Blood | 3/6 | 3/6 | 0/6 | 0/6 |
| 15 h | Blood | 2/3* | 2/3* | 0/6 | 0/6 |
| 4 h | OB | 6/6 | 2/6 | 3/6 | 0/6 |
| 7 h | OB | 3/4 | 3/4 | 3/4 | 2/4 |
| 15 h | OB | 6/6 | 2/6 | 6/6 | 3/6 |
| 4 h | SCtx | ND† | ND | 1/6 | 0/6 |
| 7 h | SCtx | ND | ND | 0/4 | 0/4 |
| 15 h | SCtx | ND | ND | 0/6 | 0/6 |

Data compare the frequency of detection of minus or plus strand NP RNA using either

Method 1 or Method 2 nPCR on the same cDNA samples from various tissues [spleen,

blood, olfactory bulb (OB) or somatosensory cortex (SCtx)] harvested at 4, 7 or 15 h

post-infection using intraperitoneal ketamine/xylazine anesthesia . All mice were

challenged with live PR8. Ratios marked with an asterisk (*) indicate that inadequate

cDNA was available from 3 of the mice to repeat with Method 1. † ND--Not done

**Table 2.3 Primer Sequences for RT-PCR and nPCR Analysis**

| Gene | Product Size (bp) | Bases Spanned | Sequence 5'-3' | Refs. |
|---|---|---|---|---|
| HA Sense | 524 | 1-31 | ACGCATCAATGCATGAGTGTAACACG A AGTG | Lab§ |
| HA Anti-sense | | 1366-1400 | GATTCTTCACATTTGAGTCATGGA AAT CCAGAGTC | Lab§ |
| NP1 Sense* | 1097 | 301-320 | GGGAAAGATCCTAAGAAAAC | (Mori *et al.,* |
| NP2 Anti-sense* | | 1398-1379 | TGCACTTTCCATCATCCTTA | (Mori *et al.,* |
| NP3 Sense† | 450 | 649-668 | AATGATCGGAACTTCTGGAG | (Mori *et al.,* |
| NP4 Anti-sense† | | 1098-1079 | CTTCGTCCCTTTGATGAAGC | (Mori *et al.,* |
| NP5 Sense‡ | 98 | 669-688 | GGGTGAGAATGGACGAAAAA | Lab§ |
| NP6 Anti-sense‡ | | 764-745 | TCCATCATTGCTTTTTGTGC | Lab§ |
| NP 5' terminus sense | 1570 | 1-20 | CCAGCAAAAGCAGGGTAGATAA | Lab§ |
| NP 3' terminus anti-sense | | 1546-1565 | CCCAGTAGAAACAAGGGTATTTT | Lab§ |
| Cyclophilin A Sense | 430 | 90-113 | GTCTCCTTCGAGCTGTTTGCAGAC | Lab§ |
| Cyclophilin A Anti-sense | | 497-519 | GTCCACAGTCGGAGATGGTGATC | Lab§ |

*PR8 NP primers used for cDNA synthesis for RT-PCR and first step (external) nPCR

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†PR8 NP primers used for second step (internal) nPCR

‡PR8 NP primers used for qPCR

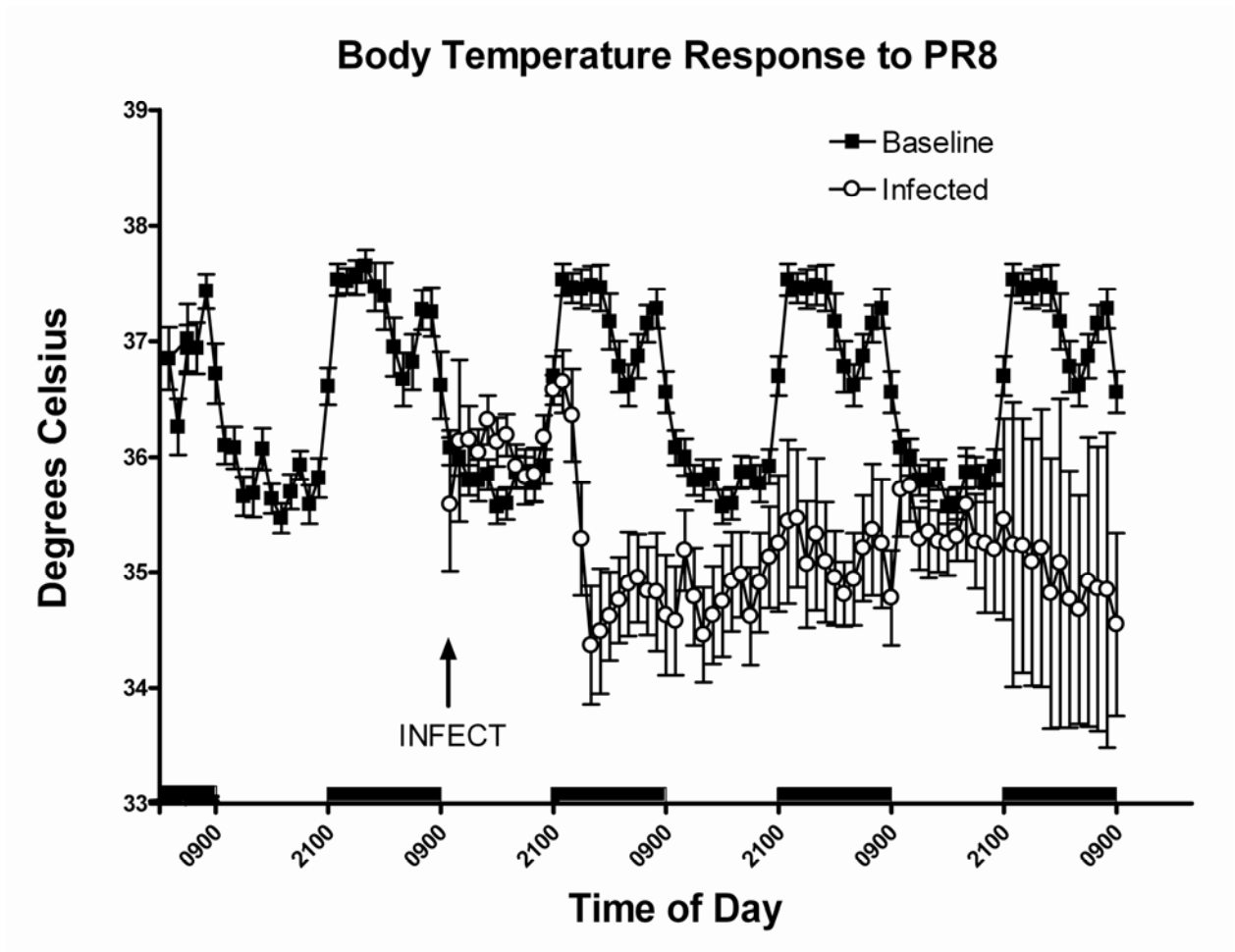§Primers developed in our laboratory using the criteria described in Materials and

Methods

**Figure 2.1** Body temperature curves of mice (n = 6) unexposed to virus (baseline) or infected IN with 2.5 x $10^6$ $TCID_{50}$ PR8 influenza virus under Metofane. Error bars denote SEM.

**Figure 2.2** Olfactory bulb cytokine and IFN-induced enzyme mRNA qPCR data from 3 experiments (corrected for boiled virus control values) at 4 hr (n = 6), 7 hr (n = 11) and 15 hr (n = 15). All mice were inoculated IN with $2.5 \times 10^6$ TCID$_{50}$ PR8 under Metofane anesthesia. The X axis shows the cytokine/enzyme evaluated and the time-point at which the mouse was killed PI. Error bars denote SEM. Asterisks denote statistically significant differences (* $p < 0.05$; ** $p < 0.001$).
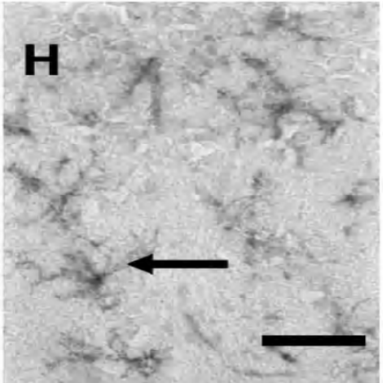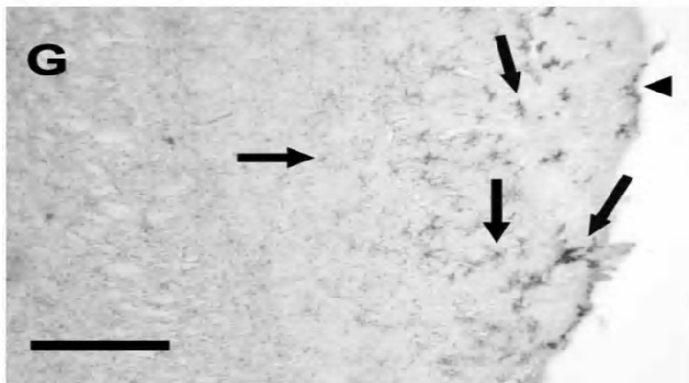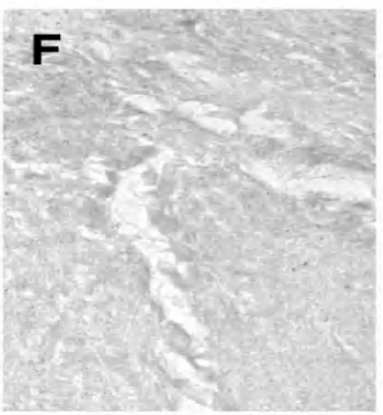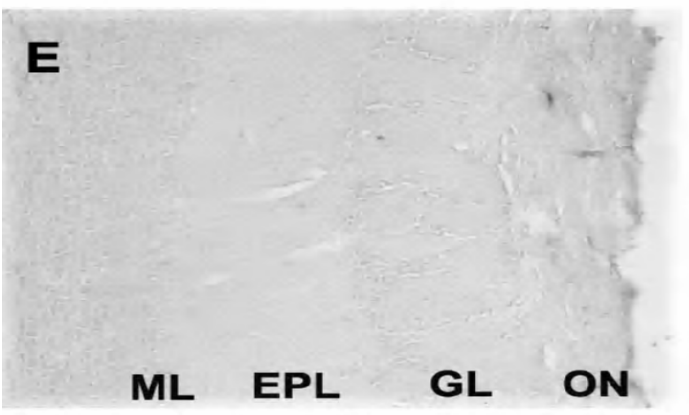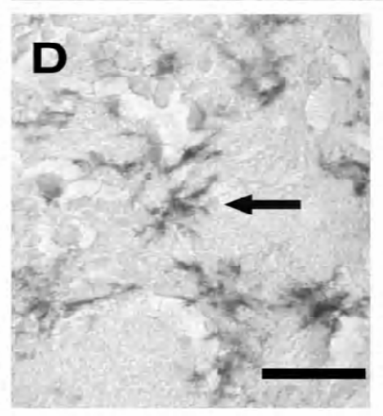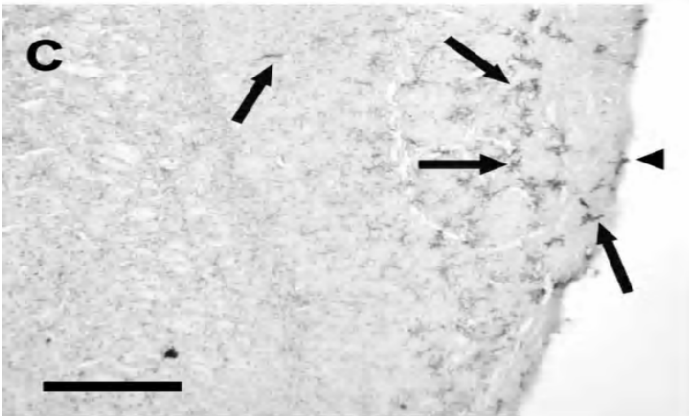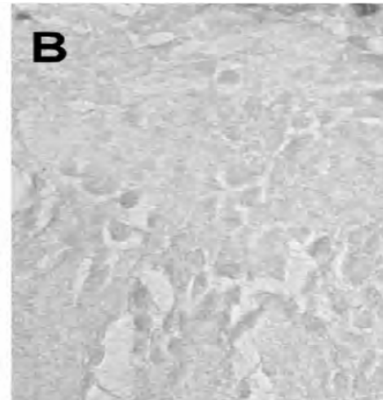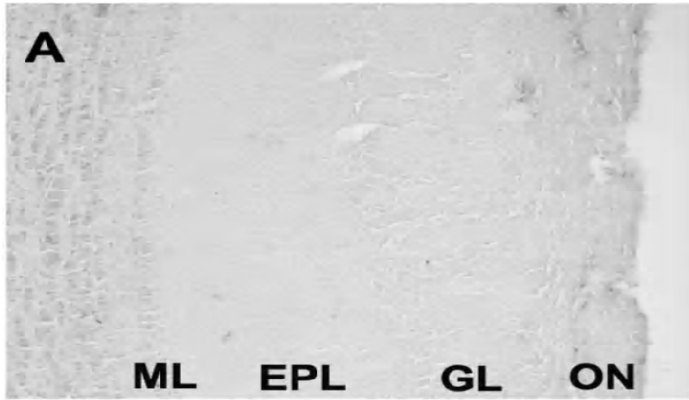
**Figure 2.3** Photomicrographs of OB coronal sections from mice killed 15 hr PI after IN challenge stained either for H1N1 influenza A (frames A-D) or for N1 NP (frames E-H). Mice receiving boiled virus are shown in frames A, B, E, and F.  Mice receiving live PR8 are shown in frames C, D, G and H.   Arrows in frames C, D, G and H indicate some of the stained cells in the GL while arrow heads point to some of the stained cells in the ON layer.  The scale bar for lower magnification pictures (frames A, C, E and G) is 0.2 mm; for higher magnification pictures (frames B, D, F and H) the scale bar is 0.025 mm. (Abbreviations: M, mitral cell layer; EPL, external plexiform layer; GL, glomerular layer; ON, olfactory nerve.)

# CHAPTER III

# INFLUENZA VIRUS AND CYTOKINE-IMMUNOREACTIVE CELLS IN THE MURINE OLFACTORY BULB AFTER INTRANASAL INOCULATION

Victor H. Leyva-Grado, Lynn Churchill, Melissa Wu, Jeannine A. Majde and James M. Krueger

Department of Veterinary and Comparative Anatomy, Pharmacology and Physiology, Washington State University, Pullman, WA 99164

**Abstract**

We previously demonstrated that viral RNA and cytokine mRNAs are elevated in the olfactory bulb (OB) by the time of illness onset (15 h post intranasal challenge) in mice infected with influenza virus. The human viral strain used in these studies is not neurovirulent or known to invade the brain. In this study, we mapped expression of cytokine and viral antigens in infected and boiled virus control mouse OBs obtained 15 h post inoculation using immunohistochemistry. Influenza viral antigen-immunoreactive (IR) cells were found in the olfactory nerve and glomerular layer (GL) and to a lesser extent in the external plexiform layer (EPL) of the OB. Double labeling demonstrated that viral antigen co-localized with a macrophage marker, F4/80, and an astrocyte marker, GFAP, but not with a neuronal nuclear protein, NeuN. In controls and virus-infected mice, cells expressing tumor necrosis factor alpha (TNF$\alpha$) or interleukin-1 beta (IL1$\beta$) immunoreactivity were detected in the GL, EPL and mitral cell layer (ML). TNF$\alpha$ immunoreactivity co-localized with NeuN-IR cells while IL1$\beta$ immunoreactivity co-localized with GFAP-IR and F4/80-IR as well as with NeuN-IR cells. The number of TNF$\alpha$-IR neuron-like cells increased in the EPL and the ML of infected mice, and the number of IL1$\beta$-IR neuron-like cells increased in the EPL of infected mice compared to controls that received boiled virus. These results demonstrate that influenza viral antigens are localized in glia within the OB and that TNF$\alpha$ and IL1$\beta$ are up-regulated in glia and/or neurons of live influenza virus-exposed mice at 15 h post intranasal infection.

# Introduction

The only mucosal surface where neurons are in direct contact with the environment is the olfactory epithelium lining the nasal cavities (Mori et al., 2005). These exposed olfactory neurons, protected only by a layer of mucus, provide a potential pathway for microorganisms to infect the brain. In fact, the olfactory nerve pathway is a route for central nervous system (CNS) invasion by various neurotropic viruses (Reiss et al., 1998; Iwasaki et al., 2004; Mori et al., 2005), including neurotropic strains of the avian influenza virus (Reinacher et al., 1983; Mori et al., 1995; Park et al., 2002). In contrast, non-neurotropic human strains of influenza virus are believed to be confined to the respiratory tract following intranasal (IN) infection (Ward, 1997).

The mouse-adapted human strain of influenza A (A/Puerto Rico/8/34, H1N1, abbreviated PR8) has no known neurotropic properties in adult mice as determined by immunofluorescence (Johnson and Mims, 1968; Schlesinger et al., 1998) and histological methods (Iwasaki et al., 2004), although virus can be demonstrated in the brain by sensitive molecular methods five days post IN infection (Mori et al., 1995). If PR8 is directly inoculated into the brain it is neurovirulent in neonatal mice but not in immunologically mature mice (Schlesinger et al., 1998). Nonetheless, the PR8-mouse model remains a useful model for studying influenza virus-induced, cytokine-induced acute phase response (Conn et al., 1995; Hennet et al., 1992). In recent studies (Majde et al., 2007), we demonstrated the presence of PR8 replication intermediates (both minus and plus viral RNA strands) in the olfactory bulb (OB) within 4 h after IN inoculation in adult mice. These unexpected results indicate that this mouse-adapted human strain of

influenza virus is able to rapidly reach the CNS and at least partially replicate within the OB. Moreover, after PR8 infection, transcripts for pro-inflammatory cytokines such as interleukin 1β (IL1β) and tumor necrosis factor-α (TNFα) were also significantly up-regulated in the OB within 15 h after IN inoculation with live virus, the time-frame for onset of hypothermia in these mice (Majde et al., 2007). In contrast, viral RNA was not detected in OBs collected from mice inoculated IN with boiled PR8. Collectively, these data indicate that after IN inoculation live PR8 influenza virus rapidly reached the OB and increased cytokine mRNA levels, although the cell types harboring the virus and producing the cytokines remain to be determined.

Cytokines are hypothesized to be responsible in part for the systemic symptoms comprising the acute phase response (APR), or 'flu' syndrome, observed in influenza infections (Conn et al., 1995). These symptoms include changes in body temperature, locomotor activity and sleep patterns (Conn et al., 1995; Fang et al., 1995; Toth et al., 1995; Opp, 2005). Cytokines such as TNFα and IL1β are directly involved in the systemic inflammatory response (Dinarello, 2000) and in associated constitutional symptoms (Majde and Krueger, 2005). Production of cytokines is also part of the mouse immune response against influenza virus infections. Studies using type I IL1 receptor-deficient mice infected with influenza virus demonstrate that an IL1-related ligand for this receptor is necessary to increase the survival rate in infected mice (Schmitz et al., 2005). Using IL1β- and IL6-deficient mice also demonstrates a role for these cytokines in the APR to PR8 influenza virus infection (Kozak et al., 1995; Kozak et al., 1997). In mice infected IN with PR8, levels of mRNA for IL1β and TNFα are upregulated in the lung and the hypothalamus (HT) 38 h after inoculation (Alt et al., 2007). Additionally,

an increase in the serum of IL1α and antiviral activity (perhaps type I IFN) occurs in mice infected IN with influenza (Kurokawa et al., 1996). IN infection results in viral replication in the upper and lower respiratory tract that is accompanied by an increase in cytokine production, including TNFα, IL1α, IL1β, IL6, and IFNγ (Hennet et al., 1992; Conn et al., 1995). However, it remains to be established whether the cytokines that act on the brain to induce the APR are produced in the brain or if they are made systemically and then reach the brain through the blood or other routes. Both mechanisms may be involved (Hopkins, 2007).

To better understand the pathophysiology of the APR, in this study we determined the type of cells involved using immunohistochemistry (IHC) of the OBs of immunologically mature C57BL/6 male mice taken 15 h after IN inoculation with live or boiled PR8 virus. Quantitative analyses of the number of cytokine-immunoreactive (IR) cells in different layers of the OB (GL, EPL and ML) were performed to determine if viral infection increased the number of IL1β- or TNFα-IR cells compared to boiled virus controls. Using double labeling with cell type markers and cell morphology we determined which cell types in the OB expressed viral or cytokine antigens.

**Material and Methods**

*Animals*

Twelve C57BL/6 male mice were purchased from Jackson Laboratories (Bar Harbor, ME) at 4-6 weeks of age and quarantined. They were used in the experiments when they were 8-12 weeks of age. After arrival, animals were housed in 48 x 25 x 16 cm polypropylene cages with filter tops to minimize intercurrent infections. Food and water were provided *ad libitum.* Mice were maintained on a 12:12 h light:dark cycle at an ambient temperature of 24° ± 1° C. Institutional guidelines for the care and use of research animals were followed and protocols were approved by the Washington State University Institutional Animal Care and Use Committee.

*Virus*

Influenza (A/Puerto Rico/8/34, H1N1) virus was supplied by Specific Pathogen-Free Avian Supply (SPAFAS, North Franklin, CT) where the virus was propagated in specific pathogen-free (SPF) chicken embryos and allantoic fluid was harvested using pyrogen-free materials. The virus was purified by sucrose-gradient sedimentation using pyrogen-free materials and the stock was tested for endotoxin and mycoplasma (negative), and titered in Madin Darby canine kidney cells as previously described (Chen et al., 2004).

*IN inoculation procedure*

Mice were inoculated at light onset. The mice (n=12) were inoculated IN by delivering 25 μl to each nostril using a 100 μl micropipette under light methoxyflurane

(Metofane, Schering-Plough Animal Health, Union, NJ) inhalation anesthesia. Infected mice (n=6) received 2.5 x $10^6$ TCID$_{50}$ purified PR8 diluted in Dulbecco's phosphate buffered saline (DPBS). Control mice (n=6) received the same diluted virus that was heat-inactivated prior to the inoculation by suspending the sample in boiling water for 25 min (boiled virus).

*Tissue collection*

Mice were returned to their home cages after virus inoculation. At 15 h post-inoculation under deep Metofane anesthesia the animals were perfused intracardially with warm saline (0.9% NaCl) containing 0.004% of heparin (Celsus laboratories, Cincinnati OH) followed by 35 ml of cold 4% paraformaldehyde in phosphate-buffered saline (PBS). Perfusion was performed using a Masterflex pump model 7014-20 (Cole-Parmer, USA) using a 21 G needle at a flow rate of 2.0 ml/min. Brains were carefully removed from the cribriform plate to maintain an intact OB. Brains were placed in ice-cold 4% phosphate-buffered formaldehyde to post-fix for 6 h, and then were sunk in 20% sucrose overnight. The OBs were separated from the rest of the brain, frozen in crushed dry ice, and stored at -80° C until sectioned.

*Immunohistochemistry (IHC)*

OBs were processed in pairs using OBs from a mouse inoculated with live virus and OBs from another mouse inoculated with boiled virus. Tissue sections of both sides of the OB were processed as previously reported (Churchill et al., 2005; Majde et al., 2007).

*Single labeling for light microscopy (DAB staining)*

Adjacent tissue sections were incubated with one of the following antibodies; anti-influenza A H1N1 virus monoclonal antibody produced in mice (Chemicon International, Temecula, CA, catalog # MAB8621, dilution 1:100), mouse anti-influenza nucleoprotein (NP) monoclonal antibody (Chemicon, catalog # MAB8257, dilution 1:100), rabbit anti-recombinant mouse IL1β (Chemicon, catalog # AB1413, dilution 1:100), goat anti-recombinant rat TNFα [17 kD secreted form, R&D, Minneapolis MN, catalog # AF-510, dilution 0.5 μg/ml], and rat anti-mouse F4/80 [a macrophage marker that also stains microglia in the OB (Mori et al., 2005), Serotec, catalog # MCA497GA, dilution 1:100]. The secondary antibodies were biotinylated horse anti-mouse, anti-rat or anti-goat IgG or biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA, dilution 1:500). Sections were stained using diaminobenzidine as a chromophore (DAB kit, Vector, catalog # SK4100).

*Antibody specificity*

The antibodies used in this experiment have been used in previous experiments and tested for specificity [McQuillin et al., 1985; Walls et al., 1986 (anti-viral antigens); Mori et al., 2005; Stichel and Luebbert, 2007 (F4/80 and TNF); Barbe et al., 2003 (IL1]. In addition, we confirmed IL1β and TNFα antibody specificity by several additional methods. (1) We omitted the primary antibody during the immunohistochemistry procedure. (2) Next, pre-absorption experiments were done by overnight incubation of the primary antibody with its target cytokine recombinant rat TNFα ( R&D systems catalog # RT-510 ) or recombinant mouse IL1β (R&D systems catalog # 401-ML/CF) at

a molar ratio of 1:45 . The primary antibodies were incubated with the appropriate recombinant protein for 24 h at 4°C. After the incubation the product was centrifuged at 10,000 $g$ for 15 min and the supernatant was used for incubation with sections (Eriksson et al., 1991). (3) In a third set of specificity experiments, we used OB tissues from non-infected TNFα knock out (KO) (Jackson Laboratories, stock #: 005540) or IL1β KO mice (a gift from Dr Kerry O'Banion, University of Rochester) according to the IHC described in section 2.5.1. (4) Western blot analyses for TNFα and IL1β were performed as previously described (Szentirmai et al., 2007) using mouse OBs to demonstrate antibody recognition of the two forms of TNFα and IL1β. Briefly, samples were homogenized, the homogenates were centrifuged at 10,000 $g$ for 10 min at 4°C, and the supernatants were collected. Sample protein concentration was determined by the DC Protein Assay Kit (Pierce, Rockford, IL). Twenty micrograms of total protein was separated by electrophoresis through a denaturing 4-15% SDS polyacrylamide gel, and the proteins were transferred to a nitrocellulose membrane. The membranes were blocked in 5% milk and then bathed in a 1:150 dilution of goat anti-rat TNFα (R&D systems) or 1:1000 dilution of mouse anti-IL1β antibody (Chemicon) in 5% nonfat dry milk/tween tris-buffered saline (TTBS) overnight at 4°C. After three washes in TTBS, the nitrocellulose membranes were incubated in either a 1:4,000 dilution of horse anti-goat HRP conjugated secondary antibody (TNFα) or a 1:4,000 dilution goat anti-rabbit horseradish peroxidase (HRP) conjugated secondary antibody (IL1β). Immunoreactive protein was detected by the enhanced chemiluminescence detection reagent (ECL kit) according to manufacturer's instructions (GE Healthcare Bio-Sciences Corp, Piscataway, NJ), and bands were visualized by exposure to autoradiographic film Cruz Marker (Santa

Cruz Biotechnology, Santa Cruz, CA). Molecular weight standards were used to identify the molecular weights of the bands. (5) Finally, in previous work using rats and a TNFα small interfering (si)RNA, we demonstrated that a TNFα siRNA reduced mRNA levels and TNFα-immunoreactivity in the brain using the same antibody we used for our experiments (Taishi et al., 2007).

To confirm the specificity of the TNFα antibody used, omission of the primary TNFα antibody eliminated the immunoreactivity in the OB sections (data not shown). Pre-absorption of the primary antibody with the recombinant TNFα protein, blocked the staining in both WT and KO mice (Figures 3.1C and 3.1D). TNFα immunoreactivity was observed in neuronal-like cells in the OB of TNFα wild-type mice (WT) but not in the OB of the TNFα-KO mice (Figures 3.1A and 3.1B). Western blot analysis showed a 17 kD band for the recombinant protein (Figure 3.2) and a 26 kD band in the OB protein extracts; the molecular weight of membrane-associated TNFα is 26 kD (Solomon et al., 1999).

To confirm the specificity of the IL1β antibody used, OB sections were processed without the primary IL1β antibody; such sections showed no immunoreactivity. After pre-absorption with the recombinant protein, the immunoreactivity was greatly reduced but not completely blocked (Figure 3.3C). IHC for IL1β showed intensely stained IL-1β-IR cells in the EPL and GL (Figure 3.3A). These IL1β-IR cells were not observed in the EPL or GL of the OB from the IL1β KO mice (Figure 3.3B). Western blot analyses of OB protein extracts demonstrated the presence of a 17 kD band for the recombinant protein and a 37 kD band, which is the molecular weight of pro-IL1 (Deak et al., 2005)

(Figure 3.4). Pre-absorption treatment with the respective recombinant protein prior to Western blot completely eliminated the presence of these bands (Figures 3.2 and 3.4).

 *Double labeling for confocal microscopy*

After immersion in 3% blocking serum [a combination of normal chicken serum (NCS) and normal donkey serum (NDS)] for 1 h, adjacent sections were incubated with a mixture of the anti-influenza A H1N1 (Chemicon, 1:100) and F4/80 (Serotec, 1:100) or rabbit anti-mouse GFAP (an astrocyte marker; Chemicon, catalog # MAB360, dilution 1:1000) antibodies prepared in 2% serum (NDS and NCS) at 4°C for 3 days. For double labeling with the anti-mouse NeuN nuclear protein-neuronal marker (Chemicon, catalog # MAB377, dilution 1:1000) we used a polyclonal goat anti-H1N1 antibody (Fitzgerald Industries International, Inc., Concord, MA, catalog # 20IG23, dilution 1:100). Also, adjacent sections were incubated in rabbit anti-mouse IL1β (Chemicon, dilution 1:100) in combination with mouse anti-rat F4/80 (Serotec, dilution 1:100; Serotec), mouse anti-NeuN (Chemicon, dilution 1:1000) or rabbit anti-mouse GFAP (Chemicon, dilution 1:1000) antibodies. Finally, some OB sections were incubated with goat anti-rat TNFα (R&D systems, dilution 0.5 μg/ml) and anti-mouse NeuN (Chemicon, dilution 1:1000) antibodies. After incubation, the samples were washed with PBS and then were incubated in the dark for 2 h at room temperature with secondary antibodies using a combination of Alexa Fluor 488 chicken anti-rat (Invitrogen, Carlsbad CA, catalog # A21470, dilution 1:500), anti-rabbit (catalog # A21441) or anti-goat (catalog # A11055) plus Alexa Fluor 555 donkey anti-mouse (A31570) for the sections with virus antibodies, and a combination of Alexa Fluor 488 chicken anti-rat, anti-rabbit or anti-mouse (catalog

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# A21200) plus Alexa Fluor 568 goat anti-rabbit [catalog # A11011 (for the sections with the IL1β antibodies)] or donkey anti-goat [catalog # 11057 (for the sections with TNFα antibodies)]. After incubation the sections were washed and then mounted on gelatin-coated slides, dried, and cover slipped with fluorescent hard set mounting medium (Vecta shield, Vector, catalog # H1400).

*Image preparation*

For DAB sections, images were captured with a Spot camera and software (Diagnostic Instruments Inc., Sterling Heights MI), in a Leica DMLB microscope, before being transferred into Adobe Photoshop CS2 (Adobe Systems Inc., San Jose, CA). Adobe Photoshop was used only for brightness and contrast adjustments to produce the photographs used in the quantitative analysis. For the fluorescence IHC, double-stained sections were analyzed using a confocal laser scanning microscope (Zeiss LSM-510, Oberkochen, Germany) equipped with an AxioCam HR digital camera (Zeiss). Confocal microscope pictures were taken using the Argon 488 nm laser for Alexa fluor 488 and the HeNe 543 nm laser for Alexa fluor 568. Single plane with full resolution was used for each fluorescent channel.

*Quantitative analyses*

The number of IR-cells was determined using a transparent template with a rectangular box that measured 0.25 mm by 0.5 mm (TNFα) for photographs taken at 20X or 0.20 mm by 0.22 mm (IL1 and F4/80) for photographs taken at 40X in 6 different fields from several sections of the rostral OB. For mitral cell layer (ML) quantification,

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the rectangle was 0.1 mm by 0.5 mm for the 20X photographs and 0.04 mm by 0.20 mm for the 40X photographs. The analyzed areas were selected based on preliminary studies that showed the presence of viral and F4/80-IR cells in the olfactory nerve (ON) and the GL. Consequently, viral proteins (H1N1 and NP) and F4/80-immunoreactivity were evaluated in both ON and the GL by photographing every third field of view within these two layers. Also, preliminary data for the cytokines indicated that cytokine-immunoreactivity was mainly observed in the GL and the EPL; therefore, 6 fields were selected from these regions in the OB. The templates were placed over digital images prepared by photography using the 20X (TNF) or the 40X (IL1) objectives of a Leica DMLB microscope. An individual blinded to the experiment treatment completed the quantification. The total number of IR cells was averaged within the 6 different fields for each mouse and then was statistically analyzed using a paired Student's t-test. The data were expressed as mean ± the standard error of the mean. A p-value of 0.05 or less was considered statistically significant. Additionally, IR-cells were divided according to their morphology and size into two groups: glial cells [ramified cells between 5-15 microns in size that showed the immunoreactivity both in the cell body (< 5 microns) and in the ramifications] or neurons (non-ramified cells or cells with one or two branches between 10-30 microns in size that mainly showed immunoreactivity in the cell body) and then quantified as above.

**Results**

*Morphology of influenza virus-immunoreactive (IR) cells within the olfactory nerve and the OB*

In mice inoculated with live virus, the viral antigen-immunoreactivities both for H1N1 and NP antigens were present mainly in the olfactory nerve (ON), in the ON layer of the OB, and in the glomerular layer (GL) of the OB at 15 h post infection (Figure 3.5A). Viral antigen immunoreactivity was rarely detected in mice inoculated with heat-inactivated PR8 (Majde et al., 2007). The topographical distribution of the viral antigen-IR cells was characterized by a ventrolateral localization (Figure 3.5A) close to the entry of the ON into the OB from the cribriform plate.

The viral protein-IR cells in the ON, the GL and, to a lesser extent, the external plexiform layer (EPL) resembled F4/80-IR cells with numerous processes and microglia-like morphology (Figure 3.6). We also found that viral antigen immunoreactivity was present in a group of densely stained fusiform-like cells resembling olfactory ensheathing cells (OEC) in the ON. These cells were located along the ON parallel to the nerve fibers (Figure 3.7). No viral protein immunoreactivity was observed in neuron-like cells (see below).

*F4/80- immunoreactive cells in DAB-stained sections*

F4/80 antibody binds to an antigen common to all macrophage–related cells, including microglia (Perry et al., 1985) and dendritic cells (Fischer and Reichmann, 2001) within the brain. In the OB, F4/80 immunoreactivity was evident in numerous

ramified cells with microglia-like morphology; these cells had a topographic distribution similar to viral antigen-IR cells (Figure 3.5C).  F4/80-IR cells were most densely distributed in the GL with occasional F4/80-stained cells also seen in the ON layer and the EPL (Figure 3.5C).  The morphology of the F4/80-IR cells in the GL was altered by the live PR8 infection; the processes were shorter, thicker and more densely stained with F4/80 antibody (Figures 3.8B and 3.8D) in comparison with F4/80-stained cells in the GL from the mice inoculated with boiled virus (Figures 3.8A and 3.8C).  Further, the cell bodies of the F4/80-IR cells from the mice inoculated with live virus (Figure 3.8B) appeared to be more densely stained relative to cell bodies in boiled-virus control cells. The F4/80-IR cells in the infected mouse OB were morphologically similar to activated microglia (Perry, 1994).  The number of F4/80-IR cells in the GL was not statistically different at 15 h post inoculation between mice inoculated with live virus ($17.4 \pm 1.7$) and those inoculated with boiled virus ($17.9 \pm 2.0$, $p = 0.72$).  These data suggest that the microglia-like cells were resident and not migrating into the OB in response to infection.

*Co-localization of viral antigen with F4/80 staining using confocal microscopy*

H1N1 viral antigen frequently co-localized with the macrophage marker F4/80 within microglia-like cells in the GL (Figure 3.9A).  F4/80 positive cells were also observed in the ON and EPL layers.  However, co-localization of F4/80 antigen with the H1N1 viral antigen in the ON and EPL layers was not as frequent as within the GL.

*Co-localization of viral antigen with GFAP staining using confocal microscopy*

GFAP-immunoreactivity was observed in multi-branched cells of about 15-20 microns in size. These cells were observed in the ON, GL and EPL, with the GL showing a more abundant population of the GFAP-IR cells. However, in double-labeled OB sections the GFAP-IR cells that co-localized with the anti-H1N1 antibodies were observed only in the GL (e.g., Figure 3.9B). There were only a few double labeled GFAP-H1N1-IR cells in the OB. This is in contrast with the relatively abundant double-labeled cells detected with the F4/80 and H1N1 antibodies in the same layer (Figure 3.9A).

*Co-localization of viral antigen with NeuN staining using confocal microscopy*

NeuN-IR cells were found in both the GL and the EPL; however, not all cells with neuronal morphology in the analyzed regions showed NeuN-immunoreactivity. None of the NeuN-IR cells showed co-localization with the viral H1N1 antigen in any of the analyzed layers (Figure 3.9C).

*Quantification of TNF$\alpha$-IR cells in OBs from infected versus control mice*

A large number of TNF$\alpha$-IR cells were observed in the GL, EPL and ML of the DAB-stained sections. The morphology of the TNF$\alpha$-IR cells resembled neurons, i.e., the cells were between 10 to 30 μm in size with a lightly stained rounded nucleus surrounded by more darkly stained cytoplasm (Figures 3.10A and 3.10B). An apical dendrite was usually evident in the TNF$\alpha$-IR cells in the EPL but not in the GL. TNF$\alpha$-immunoreactivity was present in cell bodies as well as in fiber-like processes in the EPL

(Figures 3.10C and 3.10D). The morphologies of the TNFα−IR cells were similar in infected and control mice. There was, however, a significant increase in the number of TNFα-IR cells in the EPL from live virus-inoculated mice (19.7 ± 1.1) compared with the same layer in the EPL from boiled virus-inoculated mice (15.7 ± 1.0, p = 0.005) (Figure 3.11A). In both groups, the TNFα-IR cells were mainly observed in the superficial one-third of the EPL, adjacent to the GL. TNFα-IR cells in the ML also increased significantly in mice inoculated with live virus (16.0 ± 1.3) in comparison with control mice (12.8 ± 1.3, p=0.003) (Figure 3.11A). However, there was no significant difference in the number of TNFα-IR cells in the GL of mice inoculated with live virus (34.7 ± 2.4) compared with mice inoculated with boiled virus (33.7 ± 2.2, p=0.23) (Figure 3.11A). Double labeling of the OB with a combination of anti-TNFα and anti-NeuN antibodies confirmed that TNRα-immunoreactivity was localized in the cytoplasm of neurons labeled with NeuN in the nucleus (Figure 3.12A).

*Quantification of IL1β-IR cells in OBs from infected versus control mice*

IL1β-IR cells were detected in the GL and the EPL. Some IL1β-IR cells morphologically resembled neurons (as described above for TNFα-IR cells) and glia [i.e., ramified cells between 5-15 microns in size that showed several darkly-stained projections with a small dark nucleus (Figure 3.13)]. There was a significant increase in the number of IL1β-IR neuron-like cells in the EPL of mice inoculated with live virus (20.2 ± 1.8) in comparison with mice inoculated with boiled virus (15.8 ± 2.3, p = 0.006) (Figure 3.11B). Similar to TNFα-immunoreactivity, IL1β-IR neuron-like cells in the

EPL were mainly observed in the superficial two-thirds of the EPL. The number of

IL1β-IR neuron-like cells did not significantly change in either the GL (live $42.8 \pm 2.5$;

boiled $38 \pm 4.0$; $p = 0.072$) nor in the ML (live $13.7 \pm 1.2$; boiled $12.3 \pm 0.7$; $p = 0.09$)

(Figure 3.11B). The number of IL1β-IR glial-like cells within the various OB layers was

similar in live virus and control groups and consequently significant differences between

the two groups were not found (data not shown). Adjacent sections processed for double

labeling with the cytokine antibody and cell markers confirmed that IL1β-

immunoreactivity co-localized with NeuN (Figure 3.12B) in the GL and EPL and with

GFAP (Figure 3.12C) or F4/80 (data not shown) in the GL.

**Discussion**

The primary result described herein is that in the GL and the EPL glia-like cells, positive for F4/80-immunoreactivity and GFAP-immunoreactivity, harbor the viral antigen at 15 h after IN infection with PR8 influenza virus. The majority of these cells appeared morphologically similar to microglia. Microglia originate from a myeloid lineage (CD45+ bone marrow precursor) that enters the brain during embryonic development (Santambrogio et al., 2001). This cell population comprises about 20 % of all cells in the brain parenchyma and is usually found in a resting state (Hauwel et al., 2005; Banati, 2003). Microglial cells are considered to be immune-effector cells in the brain (Guillemin and Brew, 2004) with the capability to clear viruses and virus-infected cells (Hauwel et al., 2005). Additionally, we found that viral protein-immunoreactivity also co-localized with the astrocyte marker GFAP, suggesting that these cells also take up the virus after its entrance into the GL. Similarly, infection of astrocytes has been demonstrated during West Nile (Diniz et al., 2006) and herpes viral infections (Aravalli et al., 2006).

F4/80-IR OB cells in mice receiving live virus were morphologically distinct from those F4/80-IR cells of mice inoculated with boiled virus. The thicker and shorter processes, as well as the darkly stained cell bodies, suggest that the cells are likely activated. After viral or bacterial infections microglia are rapidly activated as indicated by a change in the morphology of the cells as well as the expression of phagocytosis markers (Kumaraswamy et al., 2006; Lehnardt et al., 2006; Ghoshal et al., 2007; Lemstra et al., 2007). Morphological changes include an augmentation in the size of cell bodies

with thicker processes and darker immuno-staining (Deng et al., 2006) similar to that reported here. These morphological changes observed during viral infections may be induced by the accumulation of viral double-stranded (ds)RNA (Guillot et al., 2005) in the form of replication intermediates and its binding to Toll-like receptor (TLR)3 in microglia (Town et al., 2006), as discussed below. The number of F4/80-IR cells did not significantly change after infection with live virus, thereby suggesting that the F4/80-IR cells were resident microglia and not dendritic cells or macrophages migrating from the blood (Perry et al., 1985; Deshpande et al., 2007). Microglia-like cells that stain for both F4/80 and CD11c, a dendritic cell marker, are resident in the brain (Fischer and Reichmann, 2001; Santambrogio et al., 2001, Bulloch et al., 2008) and are activated during the process of neuroinflammation (Deshpande et al., 2007) and aging (Stichel and Luebbert, 2006). Furthermore, the topographical distribution of the viral antigen and F4/80 immunoreactivity in the region of the OB nearest to the entry site of the ON resembles the topographical localization of these dendritic cells (Bulloch et al., 2008). Consequently it is possible that some of the virus-IR cells may in fact be resident dendritic cells.

The pathway used by PR8 to reach the OB after IN inoculation remains unknown, but as with other viruses and proteins, may include the axonal transport pathway or the perineural space pathway (Dahlin et al., 2000). The endings of the olfactory receptor neurons are located in the nasal olfactory epithelium. These neurons project unbranched axons to the OB where they synapse with mitral and tufted neurons in specialized glomeruli located in the GL (Walz et al., 2006). The olfactory receptor neurons express surface glycoproteins that include D-galactosyl and sialic acid components (Allen and

Akeson, 1985).  These substances are likely recognition sites for the influenza HA protein and are necessary to initiate cell infection (Smith, 1951; Steinhauer and Skehel, 2002), suggesting that the olfactory receptor neurons may take up the virus and translocate it to the OB via axonal transport.  Studies with neurotropic viruses such as mouse hepatitis virus suggest that the ON is necessary for the virus to reach the brain after IN inoculation (Barnett and Perlman, 1993).  However, with our approach we were unable to observe viral protein-IR in the ON nerve fibers.

On the other hand, we did observe that the viral proteins were present in large ON fusiform cells resembling olfactory ensheathing cells.  These cells wrap the axons of the olfactory receptor neurons and with the ON fibroblasts form channels that cross the cribriform plate and terminate in the glomeruli (Li et al., 2005; Vincent et al., 2005). Recent studies using ultra fine carbon (Oberdorster et al., 2004) or ultra fine manganese oxide particles (Elder et al., 2006) demonstrated that nanoparticles less than 100 nm in diameter translocate from the nasal epithelium to the OB using these olfactory ensheathing cell channels.  Further, these nanoparticles induced a local increase in the OB of TNFα mRNA and protein (Elder et al., 2006).  Olfactory ensheathing cells phagocytize degenerating axons (Li et al., 2005) and are activated by the presence of bacterial lipopolysaccharide and the synthetic dsRNA, poly I:C, to activate nuclear factor kappa B (Vincent et al., 2007).  Even though there is no direct evidence that viruses employ olfactory endothelial cell channels after IN inoculation, their size range is similar to that of the nanoparticles described above.  Further, our results showing the presence of viral protein-immunoreactivity in the putative olfactory ensheathing cells suggest that this perineural pathway may be involved in the transport of the influenza virus to the OB.

Much of our knowledge regarding the expression and distribution of cytokines in the CNS has been generated with IHC studies (Sweitzer et al., 2001; Mausset-Bonnefont et al., 2003). However one of the limitations of this technique is antibody specificity and reproducibility of the staining (Sweitzer et al., 2001). In the present study, we confirmed the specificity of our antibodies by different tests including the omission of the primary antibody, preabsorption with specific recombinant cytokines, the use of OB tissue from TNFα KO and IL1β KO mice, and Western blot analyses. Furthermore, we previously conducted studies that relied on the nucleic acid sequence of TNFα mRNA to reduce TNFα protein levels; this procedure also reduced the specific TNFα immunoreactivity in brain sections using the same antibody that we used for our studies (Taishi et al., 2007). To address the reproductibility issue of the technique, we paired OBs from mice that were challenged with boiled or live virus using the same DAB solutions and times of incubation for each pair. This allowed us to evaluate the number of cells in a semi-quantitative manner.

The second major finding described herein was the presence of enhanced cytokine–IR cell numbers after viral challenge. Double labeling showed that cytokines co-localized with GFAP (IL1β), F4/80 (IL1β) and NeuN (TNFα and IL1β)-IR cells in the OB. Pro-inflammatory cytokines such as TNFα and IL1β are expressed in microglia and astrocytes under physiological and pathological conditions (Konsman et al., 2002; Owens et al., 2005). Consequently, we acknowledge the possibility that cytokines released by glial cells may be taken up into nearby neurons. However, neurons also produce pro-inflammatory cytokines in response to pathological conditions and even activate the microglia to become antigen-presenting cells (Liu et al., 1994; Ohtori et al., 2004; Yang

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et al., 2004; Figiel and Dzwonek, 2007).  Furthermore, our findings of TNFα- and IL1β-immunoreactivity in neurons confirm prior results demonstrating neuronal expression of these cytokines (Bandtlow et al., 1990; Breder et al., 1993; Breder et al., 1994; Ignatowski et al., 1997; Lim and Brunjes, 1999; Gao et al., 2000; Acarin et al., 2000; Ji et al., 2005; Figiel and Dzwonek, 2006; Mao et al., 2006; Kwon et al., 2008)

The increase in the number of cells expressing TNFα and IL1β proteins in the OB in response to live virus extends our previous observations of an increase in TNFα and IL1β mRNAs in the OB (Majde et al., 2007).  The differences in distribution of cytokine-IR cells in the GL, EPL and the ML may represent sequential events within the OB.  Previously, we reported that viral RNA, both minus and plus strand, is strongly expressed in the OB as early as 4 h post-inoculation, suggesting the virus enters the OB sometime before 4 h.  At 15 h post-inoculation the virus has had time to complete its first replication cycle (7-8 h) and to complete or nearly complete its second replication cycle within the OB.  The differences in cytokine expression within the OB layers may reflect the dynamics of virus distribution or that of its dsRNA replication intermediates that we previously demonstrated (Majde et al., 1998).  Additionally, it is likely that the activated microglia-like cells in the GL release cytokines, which may in turn activate the production of cytokine proteins in the mitral and tufted cells within the inner layers of the OB.

Cytokine induction by influenza virus occurs in response to both viral genomic single-stranded (ss) RNA recognized by TLR7 (Diebold et al., 2004) and viral dsRNA recognized by TLR3 (Schulz et al., 2005).  Viral dsRNA is spontaneously released from dying influenza virus-infected cells (Majde et al., 1998).  Since both glial cells and

neurons bear TLR3 (Bsibsi et al., 2002; Jackson et al., 2006), it is possible that the dsRNA released by dying microglia is activating other glia and neurons to produce cytokines that, among other functions, activate neighboring cells or cells in distant tissues to initiate the immune response (Watkins and Maier, 2005). Similarly, in the event that some influenza particles are degraded in the cytosol by cellular proteases, the viral ssRNA may interact with the TLR7 to induce cellular activation (Diebold et al., 2004).

Cytokines such as TNF$\alpha$ and IL1$\beta$ are associated with the APR, including the induction of sleep and variations in body temperature and motor activity (Kent et al., 1992; Krueger and Majde, 2003). It has not been established as to whether the APR is induced by cytokines produced in the brain and/or by cytokines produced in the periphery. The OB has direct connections with several structures in the brain including the anterior olfactory nucleus, the piriform cortex, the olfactory tubercle, the taenia tecta, the amygdala, and the bed nucleus of the stria terminalis (BNST) (Shipley et al., 2004; Wang and Swann, 2006). Moreover, there are indirect connections between the OB and hypothalamus through the olfactory tubercle, the taenia tecta, the amygdala and the BSNT (McGregor et al., 2004; Gelez and Fabre-Nys, 2006). We found that both TNF$\alpha$- and IL1$\beta$-IR cells in the EPL were located primarily in the superficial two-thirds of the EPL. This region of the EPL is mainly populated with middle tufted cells. These cells are morphologically and functionally distinct from mitral cells and project their axons primarily to the anteromedial regions of the olfactory cortex (Schoenfeld et al., 1985; Nagayama et al., 2004). The presence of these neuronal connections suggests a route by which OB cytokines can communicate with the hypothalamus to induce the APR. In fact, preliminary results showed a significant increase in the number of TNF$\alpha$-IR and IL1$\beta$-IR

cells in specific regions of the primary olfactory cortex (piriform cortex and olfactory tubercle) and central amygdala, and in the number of IL1β-IR cells in the hypothalamus (arcuate nucleus) of mice 15 h after IN inoculation with live virus (Leyva-Grado et al., see Chapter IV). Additional preliminary data showed that transection of the olfactory nerve delays the onset of IN influenza virus-induced hypothermia by 13 hours (Leyva-Grado et al., see Chapter IV). These results strengthen the idea that the cytokines produced by the OB may have an effect on the behavioral and immunological changes observed in rodents after being challenged IN with an infectious agent.

Previously we showed that mRNA for the interferon-induced enzyme 2'-5'-oligoadenylate synthetase (OAS) is upregulated in the OB of PR8-IN challenged mice (Majde et al., 2007). Preliminary IHC studies using brain tissues obtained from the mice used in this study indicate the presence of OAS-IR cells in the GL and EPL. Further, there was a significant increase ($p < 0.01$) in OAS-IR cells in the GL and EPL in cells resembling glia but not in cells resembling neurons ($p > 0.1$). However, the quality of the OAS-IHC was insufficient to allow firm conclusions as to the identity of OAS-IR cell type and changes in OAS-IR cell numbers.

Human influenza strains are not recognized as neurotropic, though influenza-associated encephalopathies are seen with increasing frequency (Majde et al., 2007). PR8 infection of mouse embryo brain cell cultures transiently increases hemagglutinin (HA) and neuraminidase (NA) proteins in both astrocytes and neurons *in vitro* (Bradshaw et al., 1989). We were not able to demonstrate the co-localization of viral proteins within neurons using the neuronal marker NeuN by double labeling. However, recent studies (Kumar and Buckmaster, 2007; Parrish-Aungst et al., 2007)

indicated that the neuronal marker NeuN did not label all OB neurons, leaving open the possibility that *in vivo* neurons could take up viral protein and/or virus. Most studies of H1N1 influenza virus in the brain have used neurovirulent strains, such as WSN, derived by serial passages in the brain via intracerebral inoculation (Schlesinger et al., 1998). Although it is generally thought that intracerebral-inoculated PR8 does not replicate in the brain, all of those studies were conducted using large doses of virus; such doses promote the formation of incomplete virus ending in an abortive infection (Cairns, 1951). When lower doses of PR8 are employed, the virus undergoes complete replication in the brain for one or two cycles (Cairns, 1951). However, there is no evidence indicating that this strain of influenza is able to replicate in neurons even at low doses. Together, our results suggest that 15 h after infectious challenge the virus does not localize to neurons at levels detectable by our methods, although the virus is found in glial cells. These cells may be the source of the PR8 replication intermediates in the OB reported previously (Majde et al., 2007). The majority of virus-IR cells were observed in the ON and GL suggesting that the virus was taken up by glial cells before it could reach the deeper layers of the OB by 15 h post IN infection.

In conclusion, the results presented here indicate that PR8 influenza virus localizes to glia and induces the production of pro-inflammatory cytokines in the GL, the EPL and the ML by neurons and/or glial cells. This response may play a significant role in the activation of the APR observed during influenza virus infection.

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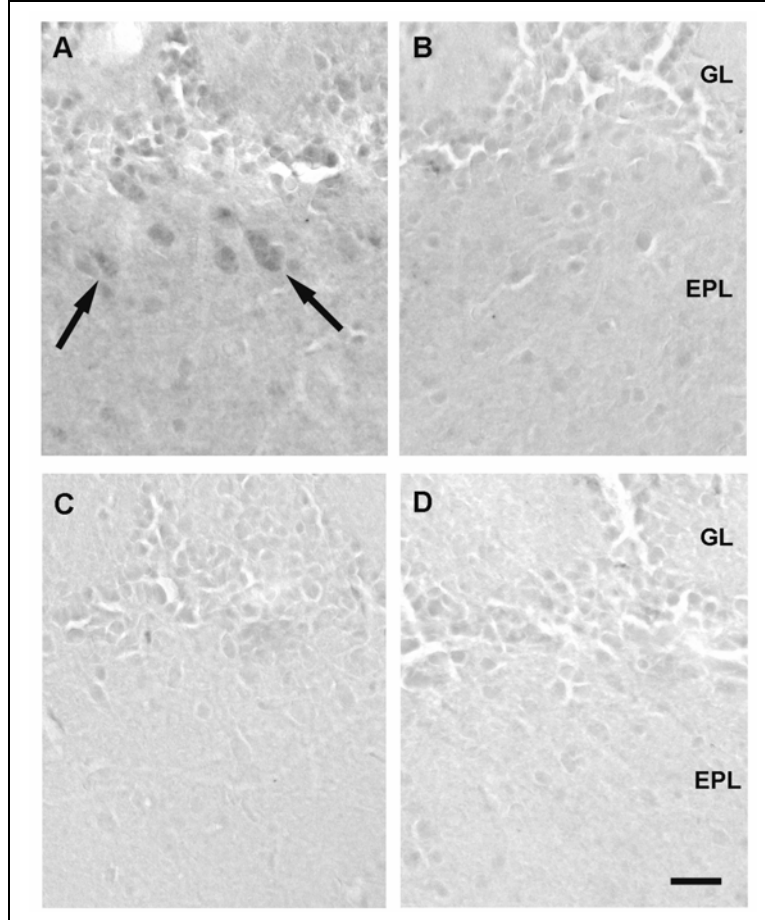
**Figure 3.1** Tumor necrosis factor alpha (TNFα)-IR cells in the OB of non-infected wild-type (WT) (A & C) and TNFα knockout (KO) mice (B & D). Immunoreactivity was observed in neuron-like cells in the glomerular layer and in the external plexiform layer in the WT mice [(A) arrows] but not in the KO mice (B). Preabsorption of the TNFα antibody with the recombinant TNFα for 24 h blocked the immunostaining in both the wild type (C) and the KO mice (D). Scale bar = 0.025 mm.
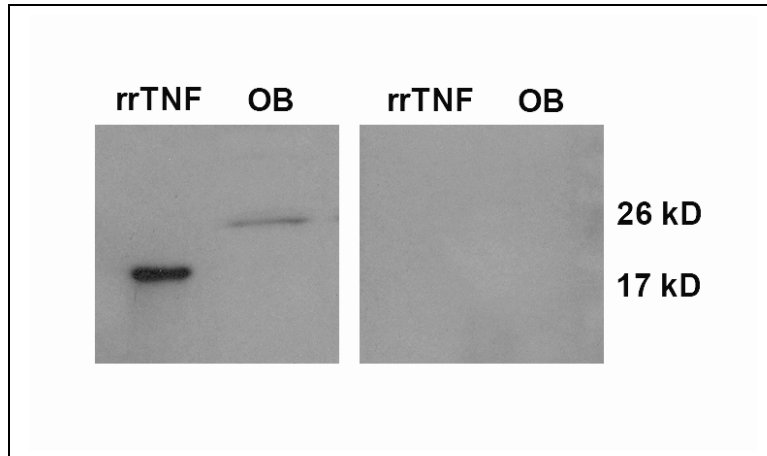
**Figure 3.2** Western blot analysis of TNFα antibody alone and combined with
olfactory bulb protein extracts. Recombinant rat TNFα (rrTNF) is evident as a 17 kD
band (left panel, left side), while the TNFα band combined with the OB extracts is a 26
kD band (left panel, right side). Pre-absorption treatment with rrTNF prior to running the
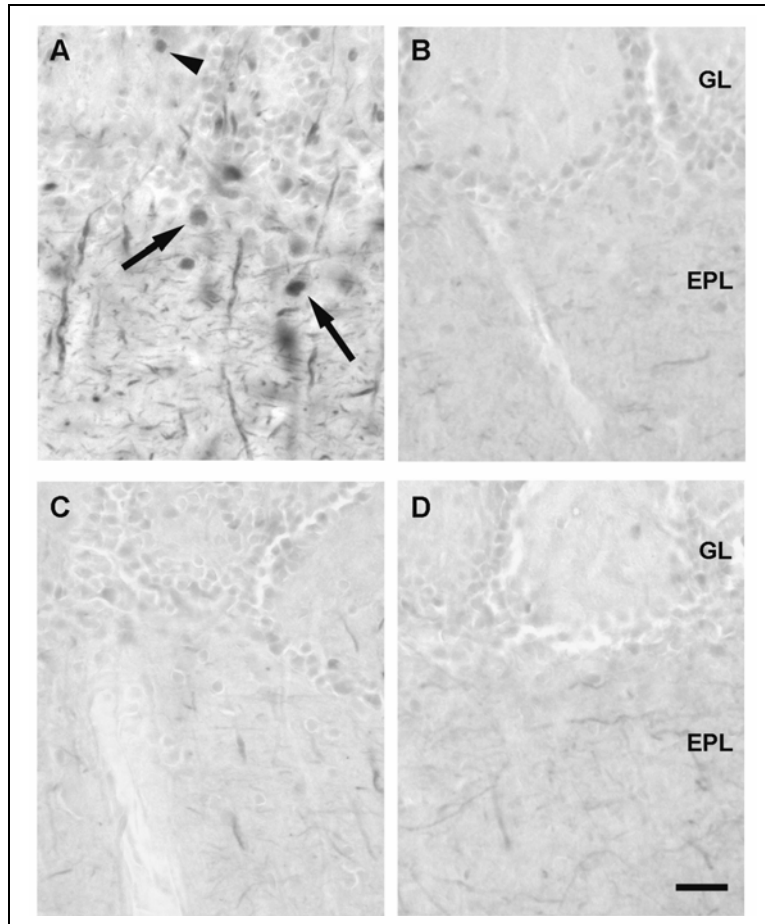Western blots eliminated the presence of both bands (right panel).

**Figure 3.3** Interleukin-1 beta (IL1β)-IR cells in the OB of non-infected IL1β WT (A & C) and KO mice (B & D). Immunoreactivity was observed in neuron-like cells in the glomerular layer (GL) and the external plexiform layer (EPL) in the WT mice (A) but not in the KO mice (B). Preabsorption of the IL1β antibody with the recombinant IL1β for 24 h substantially reduced the immunostaining in the WT (C) and KO (D) mice. Scale bar = 0.025 mm.
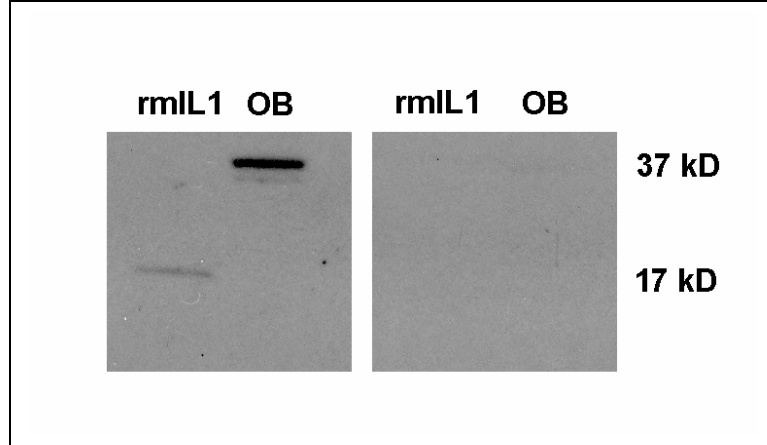
**Figure 3.4** Western blot analysis of IL1β antibody alone and combined with olfactory bulb protein extracts. Recombinant mouse IL1β (rmIL1) is evident as a 17 kD band (left panel, left side), while the IL1β band combined with OB extracts occurs at 37 kD (left panel, right side). Pre-absorption treatment with rmIL1 prior to performing the Western blot eliminated the presence of both bands (right panel).
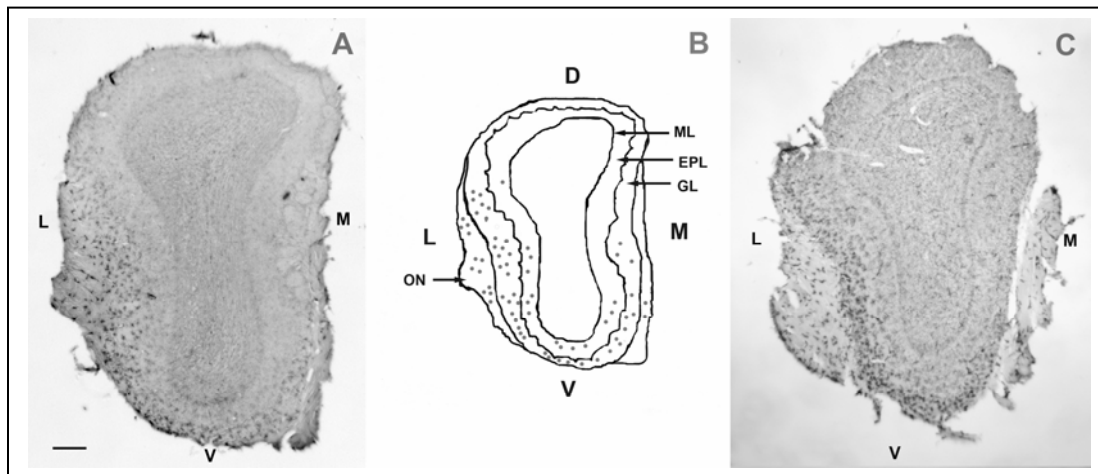
**Figure 3.5** Distribution of viral protein and F4/80 immunoreactivity within cross-sections of the whole olfactory bulb (OB). Viral H1N1 immunoreactivity is mainly present in the olfactory nerve (ON) and glomerular layer (GL) with less detectable immunoreactivity in deeper layers such as the external plexiform layer (EPL). The H1N1 immunoreactivity is largely concentrated in the ventro-(V) lateral (L) area of the OB (A) A schematic representation of the OB section in (A). Each dot represents 2 H1N1-IR cells (B). F4/80 immunoreactivity in the olfactory bulb showed a similar pattern of distribution to the one observed for the viral antigen (C) Scale bar= 0.2mm. Mitral layer (ML), medial surface (M), and dorsal surface (D).
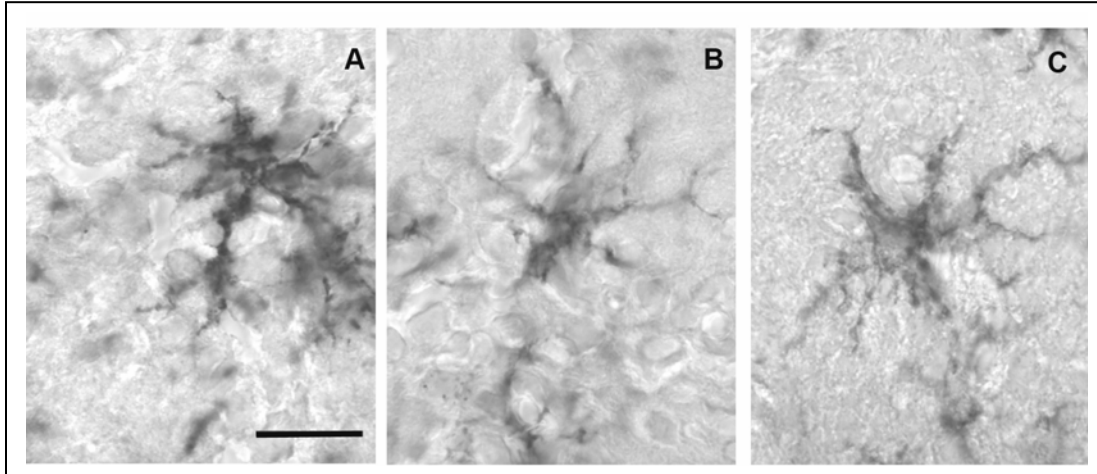
**Figure 3.6** Morphological comparison of immunoreactivity in OB sections of mice inoculated with live PR8 using F4/80 antibody (A), viral H1N1 antibody (B) and viral nucleoprotein (NP) antibody (C) respectively. Viral protein-IR cells (B and C) showed similar shape and size to the F4/80-IR cells (A), including several darkly stained processes. Scale bar = 0.01mm.
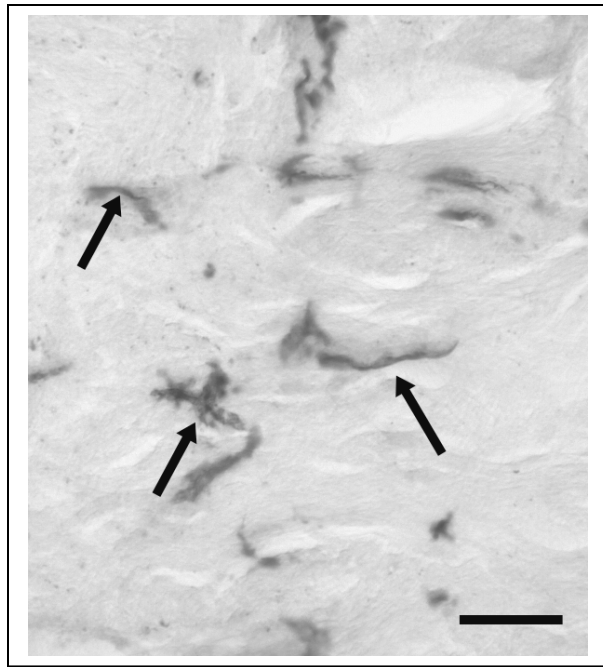
**Figure 3.7** Photomicrograph of H1N1-immunoreactivity in the olfactory nerve of a mouse inoculated with live PR8. Arrows designate fusiform cells running along the nerve fibers. These H1N1-IR cells resemble olfactory ensheathing cells (Ubink et al., 1994). An arrow head illustrates H1N1-IR in a glia-like cell. Scale bar = 0.025mm.
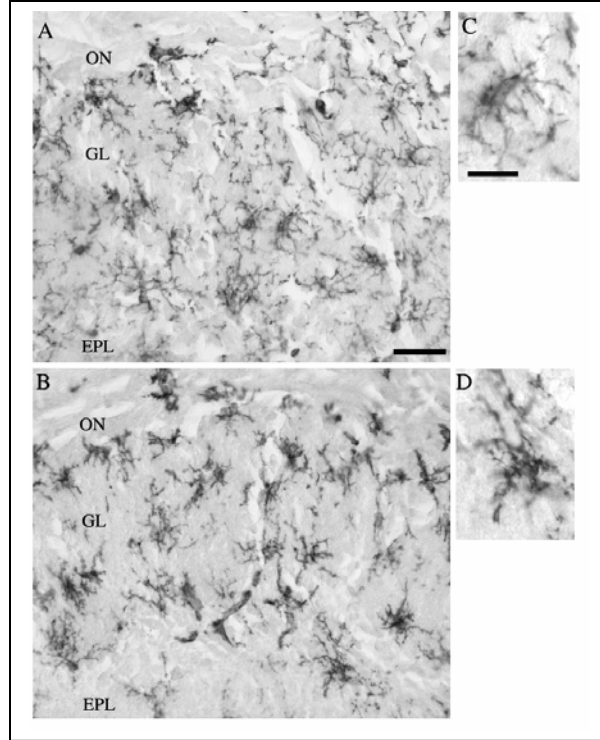
**Figure 3.8** Photomicrographs of the OB glomerular layer showing coronal sections from boiled and live virus-inoculated mice at 15 h post IN inoculation stained for F4/80. At the lower magnification numerous intensely-stained ramified microglia-like cells (arrows) were seen in the OB of mice challenged with boiled (A) or live (B) PR8. Higher magnifications of the microglia-like cells are also shown for the boiled (C) and live (D) PR8-infected mice. Cells in the OB of mice inoculated with live virus were more darkly stained and showed thicker process than the cells in the OB from mice inoculated with boiled virus. Scale bar = 0.025mm (A & B) or 0.01 mm (C & D).
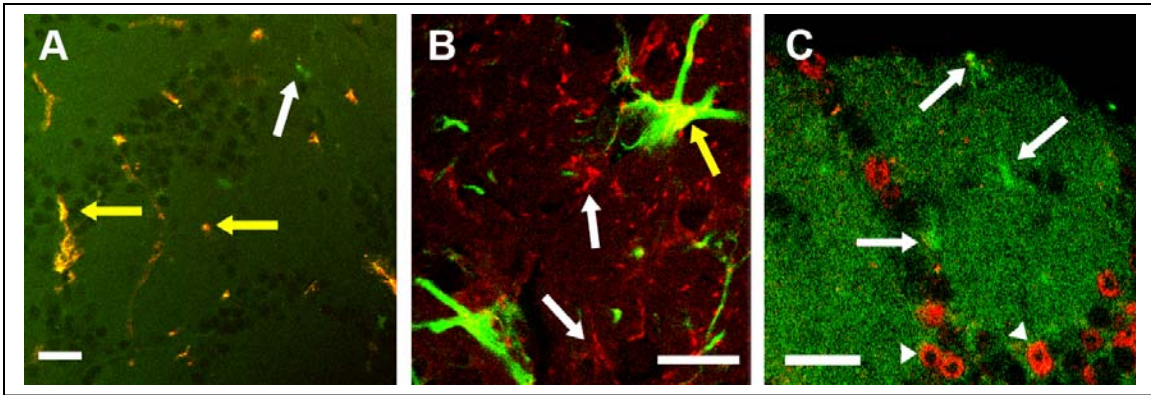
**Figure 3.9** Confocal photomicrographs of H1N1-IR cells and cellular markers in the OB of mice inoculated with live PR8 at 15 h post inoculation. Yellow arrows indicate co-localization, while white arrows indicate single-labeled cells. Viral H1N1-immunoreactivity (red) co-localized with F4/80-immunoreactivity, suggesting that microglia-like cells (green) in the GL take up virus (A). Viral H1N1-immunoreactivity (red) co-localized with some cells expressing the astrocyte marker GFAP (green) in the GL, suggesting that astrocytes in the GL also take up virus (B). Double-labeling with anti-viral H1N1 antibodies (green) and the neuronal marker NeuN (red) in adjacent sections of the OB did not show co-localization of the virus within neurons in the GL (C). Scale bar = 0.02mm (A & C) or 0.01mm (B).

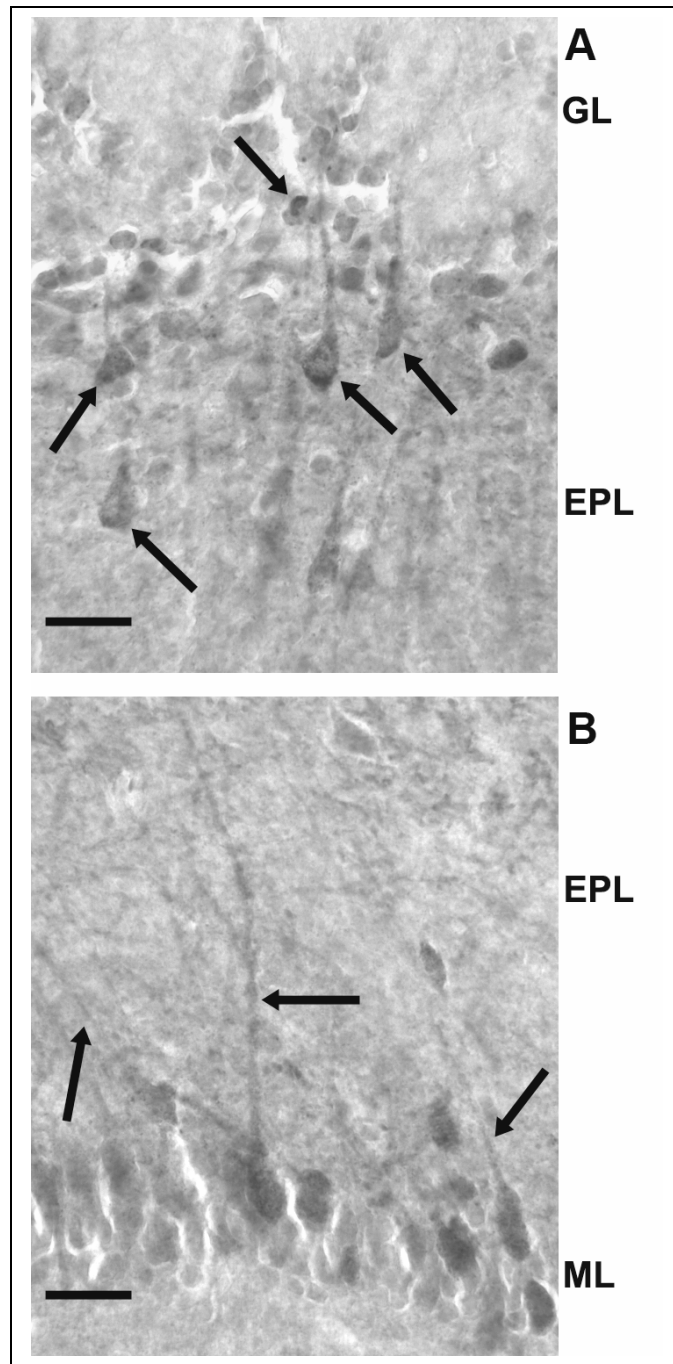**Figure 3.10** Tumor necrosis factor alpha (TNFα)-IR cells in the OB of mice inoculated with live PR8. Immunoreactivity was observed in neuron-like cells in the external plexiform layer (EPL) (A).. TNFα-IR was also observed in the long neuronal projections of mitral cells in the EPL (B). Scale bar = 0.025 mm.
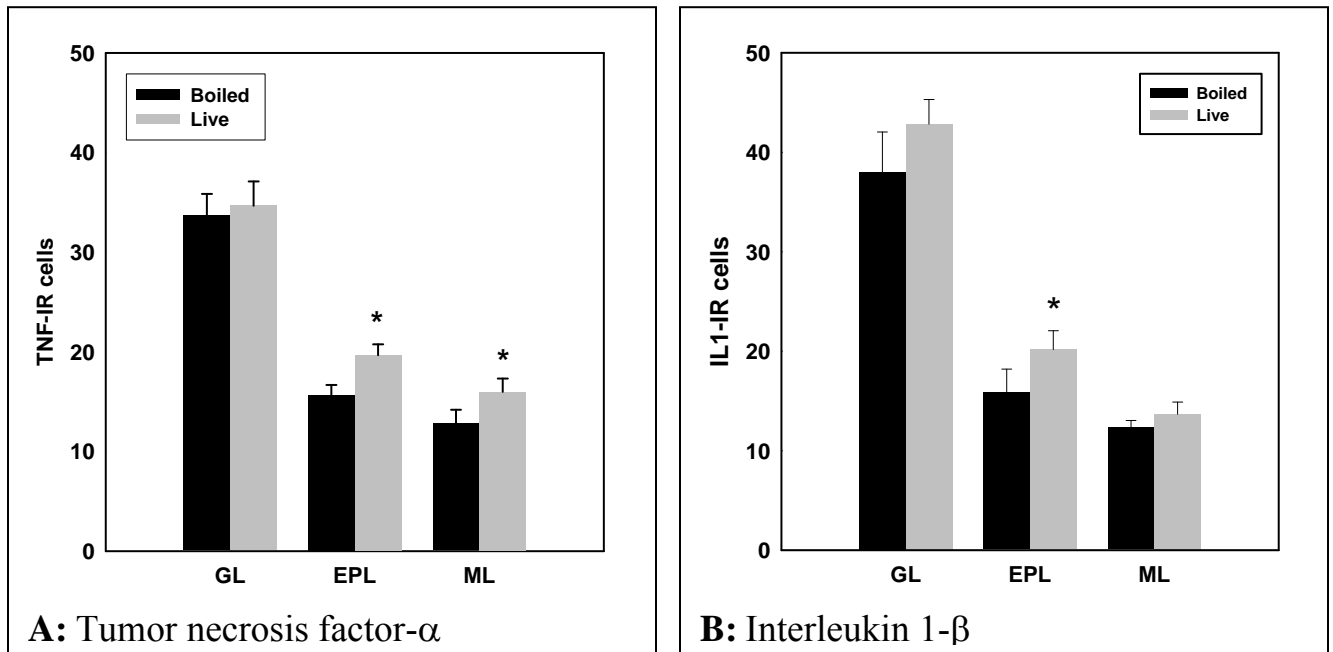
**Figure 3.11** (A) The number of TNFα-IR cells increased in the EPL and the ML of the OB in mice inoculated with live virus in comparison with mice inoculated with boiled virus.  No significant changes were observed in the GL. An asterisk (*) indicates a significant difference ($p < 0.05$) using the paired Student's t test. The area used for TNFα quantification in the GL and EPL was $0.125$ mm$^2$ and for the ML was $0.05$mm$^2$.  (B) The number of neuron-like IL1β-immunoreactive cells was significantly different between mice inoculated with live or boiled PR8 only in the EPL. An (*) indicates significant differences ($p < 0.05$). The area used for quantification in the GL and EPL was $0.144$ mm$^2$ and for the ML was $0.008$ mm$^2$.
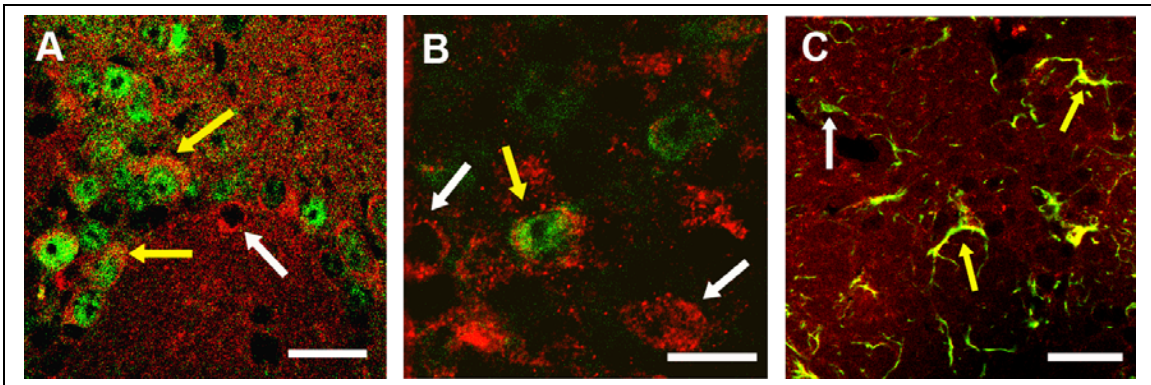
**Figure 3.12** Confocal photomicrographs of cytokines and cellular markers in the OB of PR8-infected mice. Yellow arrows indicate co-localization, while white arrows indicate single-labeled cells. Two cells showing double-labeling (yellow arrows) with TNFα (red) and NeuN (green) indicate the presence of TNFα in the cytoplasm of some juxtaglomerular neurons in the external plexiform layer. Some cells only showed TNFα-immunoreactivity (white arrow) (A). IL1β-immunoreactivity (red) co-localized with one cell also labeled with NeuN (green) in the external plexiform layer, demonstrating that IL1β was present in neurons (yellow arrow) (B). Some cells in the glomerular layer labeled with IL1β (red) also expressed GFAP (green) indicating the presence of IL1β in astrocytes of the OB (C). Scale bar = 0.02 mm (A & C) or 0.01mm (B).
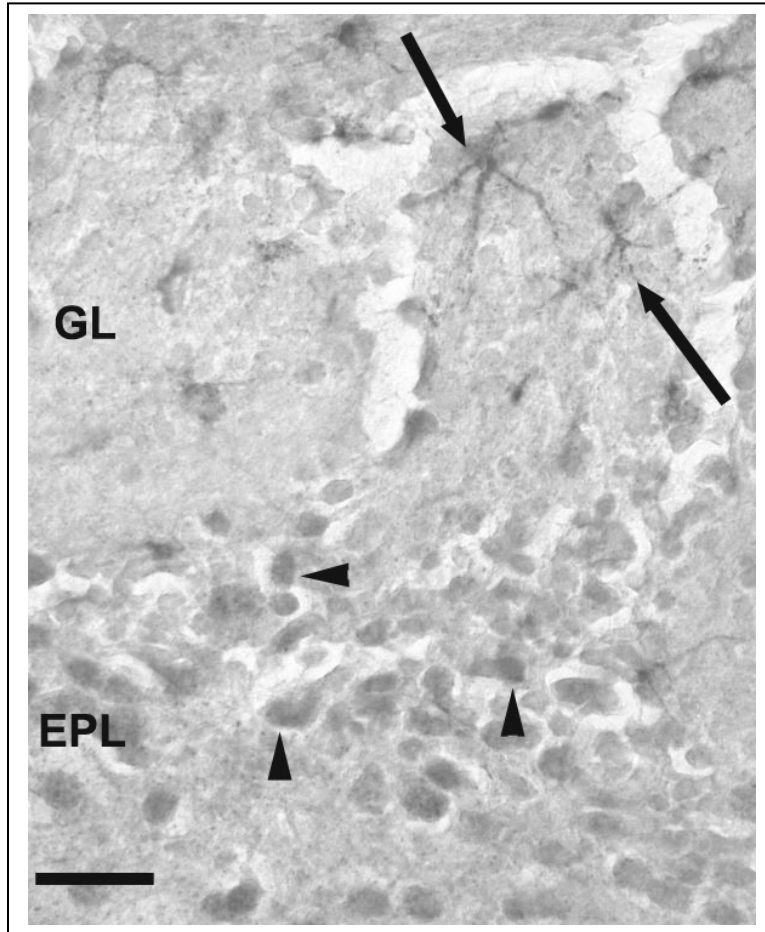
**Figure 3.13** IL1β-immunoreactivity in the OB of mice inoculated with live PR8 virus. The IL1β-immunoreactivity was observed in the GL and the EPL. The morphology of IL1β–IR cells suggests that both neuron-like cells in the EPL (arrow heads) and glia-like cells in the GL (arrows) are expressing this cytokine. Scale bar = 0.025 mm.

# CHAPTER IV


# THE OLFACTORY NERVE PATHWAY HAS A ROLE IN THE ACUTE

# PHASE RESPONSE TO INTRANASAL INOCULATION WITH INFLUENZA

# VIRUS

Victor H. Leyva-Grado, Lynn Churchill, Timothy J. Williams, Jeannine A. Majde,

Joseph Harding and James M. Krueger


Department of Veterinary and Comparative Anatomy, Pharmacology and Physiology,

Washington State University, Pullman, WA 99164

**Abstract**

Mouse-adapted human influenza virus is detectable in the olfactory bulbs of mice within hours after intranasal challenge and is associated with enhanced local cytokine mRNA and protein levels. To determine whether signals from the olfactory nerve influence the unfolding of the acute phase response (APR), we surgically transected the olfactory nerve in mice prior to influenza infection. We then compared the responses of olfactory nerve-transected (ONT) mice to those recorded in sham-operated control mice. ONT did not change baseline body temperature (Tb); however, the onset of virus-induced hypothermia was delayed for about 13 h in the ONT mice. Locomotor activity, food intake and body weights of the two groups were similar. At 15 h post-challenge fewer viral antigen-immunoreactive (IR) cells were observed in the olfactory bulb (OB) of ONT mice compared to sham controls. The number of tumor necrosis factor alpha (TNFα)- and interleukin 1 beta (IL1β)-IR cells in ONT mice was also reduced in the OBs compared to sham controls. In separate experiments, we examined brain regions connected to the OB. Mice were inoculated with live or boiled (control) virus and sacrificed 10 or 15 h later. The number of TNFα- and IL-1β-IR cells increased in the piriform cortex (Pir), olfactory tubercle (Tu) and central amygdala (CeA) at 15 h but not in mice sacrificed at 10 h after viral challenge. In the hypothalamic arcuate nucleus (Arc), the number of IL-1β-IR, but not TNFα-IR, cells also increased at 15 h but not at 10 h. No significant differences were observed in the basolateral amygdala (BLA) for TNFα or IL1β at either 10 h or 15 h. No viral antigen immunoreactivity was observed in the Pir, Tu, CeA, BLA or Arc. These results suggest that the olfactory nerve pathway is important for the initial pathogenesis of the influenza-induced APR.

**Introduction**

Cytokines such as tumor necrosis factor α (TNFα) and interleukin 1β (IL1β) are prominent mediators of the acute phase response (APR) (Bluthe et al., 2000). In influenza virus-infected mice, the APR includes enhanced sleep accompanied by reduced body temperature, locomotor activity and food intake (Kent et al., 1992; Fang et al., 1995; Toth et al., 1995; Swiergiel et al., 1997; Schmitz et al., 2005; Szretter et al., 2007). Some facets of the APR manifest within 15 h post-inoculation (PI) (Fang et al., 1995). Previously, we showed that TNFα and IL1β olfactory bulb (OB) transcripts are up-regulated within 15 h after intranasal (IN) challenge with influenza virus A (H1N1 PR8/34, abbreviated PR8 (Majde et al., 2007). Additionally, at 15 h PI the number of OB TNFα and IL1β immunoreactive (IR) cells is enhanced (Leyva-Grado et al., see chapter III). The APR is posited to be regulated, in part, by hypothalamic cytokines (Kent et al., 1992, Opp, 2005; Alt et al., 2007). In fact, by 38 h post-influenza viral inoculation, hypothalamic IL1β and TNFα mRNAs are up-regulated (Alt et al., 2007). However, our previous studies of influenza-infected mice suggest that OB cytokines could also potentially play a role in the APR.

The amygdala influences the APR via the hypothalamic-pituitary-adrenal axis (Xu et al., 1999; Lin et al., 2004). Specifically, activation of the central amygdala (CeA), as evidenced by an increase of c-Fos or of cytokines such as TNFα and IL1β, occurs after immunological challenges in rodents, including herpes virus infection (Ben-Hur, 1996), lipopolysaccharide (LPS) (Frenois et al., 2007) and enterotoxins (Rossi-George et al., 2005). Similarly, increases in c-Fos-IR cells are observed in CeA following systemic

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IL1β injection (Xu et al., 1999). After systemic or local injections with LPS or IL1β, c-Fos-IR cells increase in the hypothalamic arcuate nucleus (Arc) (Reyes and Sawchenko, 2002; Scarlett et al., 2007). The Arc plays a role in sleep regulation, food intake, energy balance and body weight (Obal and Krueger, 2003; Bouret et al., 2004) and may also have a role in the APR (Reyes and Sawchenko, 2002). Such data indicate that the amygdala and hypothalamus (HT) are involved in the APR.

Projections from the OB also reach the amygdala at the anterior cortical nucleus and the posterolateral cortical amygdala (Price, 2003; Ubeda-Bañon et al., 2007). In addition, the basolateral amygdala (BLA) and CeA receive indirect connections from the OB through the olfactory tubercle (Tu) and piriform cortex (Pir) and through the anterior and posterolateral amygdala (Johnson et al., 2000; LeDoux, 2007). The Tu and Pir are also indirectly connected to the Arc through the lateral hypothalamus (Lin et al., 2004; Price et al., 1991) and medial preoptic area (Chiba and Murata, 1985). This anatomical relationship of the olfactory pathway with centers involved in the APR and the early increased expression of TNFα and IL1β in the OB after IN influenza infection led us to hypothesize that the olfactory pathway is important in early APR ontogenesis.

In the present study, therefore, the olfactory nerve was transected at the level of the cribriform plate prior to viral infection. We then evaluated body temperature (Tb), locomotor activity and food intake responses and quantified the number of TNFα and IL1β-IR cells in the OB of olfactory-nerve transected (ONT) mice. We also evaluated TNFα and IL1β immunoreactivity in the Pir, Tu, amygdala and Arc at 10 h (before APR onset) and at 15 h [after onset of hypothermia (see Chapter II)] after IN inoculation with

influenza PR8. We demonstrate a role for the ON in the APR and that the number of

TNF$\alpha$- or IL1$\beta$-IR cells was time-dependently enhanced in the olfactory pathway.

## Material and Methods

*Animals*

C57BL/6 male mice were purchased from Jackson Laboratories (Bar Harbor, ME) at 4-6 weeks of age. After arrival, animals were housed in 48 x 25 x 16 cm polypropylene cages with filter tops to minimize intercurrent infections. Food (rodent chow Purina 5001) and water were provided *ad libitum.* Mice were maintained on a 12:12 h light:dark cycle at an ambient temperature of 24° ± 1°C. They were used in the experiments when they were 8-12 weeks of age and their body weights were between 26-30 grams.

*Experiment I. ONT*

A total of 52 mice were used and divided into groups as follows; 14 mice received the ONT and live virus challenge; 12 mice received the sham surgery and live virus challenge; and 8 mice received sham surgery and boiled virus challenge (n=8). For food intake and body weight analyses, subsets (n=5 per group) of these same mice were used. For immunohistochemistry (IHC) studies of ONT mice, two groups of separate mice were inoculated with live virus, one with ONT (n=6) and the other with sham surgery (n=6). These mice were also used in a task to test the latency to find buried food. An additional group of mice were used for the histological analysis to test the effectiveness of ONT (n=4) and compared with sham mice (n=2).

*Experiment II. IHC analysis in the olfactory pathway*

Two groups of mice (total 24) were used in this experiment. One group was sacrificed at 10 h PI after receiving boiled (n=6) or live (n=6) virus and another group was sacrificed at 15 h PI after receiving boiled (n=6) or live (n=6) virus. Institutional guidelines for the care and use of research animals were followed and protocols were approved by the Washington State University Institutional Animal Care and Use Committee.

*Virus*

Allantoic fluid containing PR8 influenza virus prepared under pyrogen-free conditions was purchased from Specific Pathogen-Free Avian Supply (SPAFAS, North Franklin, CT). The virus was purified by sucrose-gradient sedimentation using pyrogen-free materials and the stock was tested for endotoxin and mycoplasma (both were negative), and titered in Madin-Darby canine kidney cells as previously described (Chen et al., 2004).

*IN inoculation procedure*

Mice were inoculated IN at light onset by delivering 25 μl to each nostril using a 100 μl micropipette under light methoxyflurane (Metofane, Schering-Plough Animal Health, Union, NJ) inhalation anesthesia. Mice received either live virus: $2.5 \times 10^6$ TCID$_{50}$ purified PR8 suspended in Dulbecco's phosphate buffered saline (DPBS) or boiled virus (same amount of virus after being heat inactivated in boiling water for 25 min).

*Surgical procedure for ONT*

The surgical procedure was adapted from Yee and Constanzo (1995). Briefly, mice were anesthetized using intraperitoneal (IP) ketamine (87 mg/kg) and xylazine (13 mg/kg) prepared in pyrogen-free saline (0.1 ml/10 g body weight each). A skin incision was made at the midline over the anterior skull and nasal bones. A small section of the frontal bones was removed with a dental drill to expose the dorsal surface of the OBs. A microdissecting knife (blade 3.0 mm; Roboz surgical instruments, Gaithersburg, MD) was inserted between the OB and the cribriform plate to cut all the olfactory axons projecting from the nasal cavity. Sham animals received identical surgical exposure of the OB as the experimental mice except the blade was not used to cut the nerve. Following surgery, mice were allowed to recover for 7 days, and then challenged with PR8 virus on day 10 post-surgery.

*Effectiveness of the ONT*

In a separate experiment, we tested the mice for their latency in finding buried food to determine whether mice could detect odorants after the ONT. Briefly, mice (n=12) were trained for three consecutive days to find a sugar cube randomly buried under approximately 1 inch of pine shavings. The mouse was removed from the test cage after eating the food or after 5 min. Mice were tested on days 5 and 7 after surgery. The average time to find the sugar cube was significantly higher in the mice with ONT (n=6) compared to the sham group (n=6) (sham 79.21 ± 7.9 sec; ONT 166.83 ± 30.55 sec, p=0.02). Results indicate the effectiveness for the ONT surgery was approximately 85% [1 out of 6 mice fell outside 2 standard deviations (Glantz, 2002)].

The brains from six additional mice (ONT=4; sham=2) were examined histologically

for ON connections between the olfactory epithelium and the forebrain using light

microscopy on hematoxylin-eosin stained sections. Mice were killed 3 days after surgery;

they were not infected. Primary fibers connecting the olfactory epithelium with the

forebrain were not evident in the four ONT mice but were in the sham mice (Figure 4.1).

*Body temperature and motor activity analyses*

During the sham or ONT operations, radio transmitters (XM-FH, Minimitter, Bend,

OR) were implanted into the peritoneum of mice. Then individual cages (1 mouse/cage)

were placed on telemetry receivers (TR-3000, Minimitter) and Tb and locomotor activity

were recorded using VitalView data acquisition software (Minimitter). After recovery

from surgery, baseline data were collected for 2 days and then the mice were challenged

with live (12 sham, 14 ONT) or boiled (8 sham) PR8 virus. Temperature and activity

values were collected every 6 min and averaged over 60 min for each animal (Traynor et

al., 2004) for 6 days after viral challenge. Activity values on the baseline day were

averaged across the entire 24-h recording period for each mouse separately to obtain a

reference value.  Hourly activity data on each experimental day were expressed as a

percentage of this reference value (Kapas et al., in review).

*Food intake and body weight*

Food intake and body weights (5 sham, 5 ONT) were determined daily for six days

starting on the day of inoculation.  Food consumption was determined by weighing the

food pellets one hour before dark onset. The weight of each mouse was recorded at the

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same time. Body weight values are expressed as percentage of the initial body weight (Szretter et al., 2007).

*Brain collection*

Under deep isoflurane anesthesia the animals were perfused with warm saline (0.9% NaCl) containing 0.004% of heparin (Celsus laboratories, Cincinnati OH) followed by 35 ml of cold paraformaldehyde in phosphate-buffered saline (PBS). Brains were carefully removed from the skull and postfixed for 6 h at $4^{\circ}$C and then sunk in 20% sucrose overnight. The brains were frozen in crushed dry ice, and stored at -80° C until sectioned. For the ONT-IHC studies, mice were killed at 15 h PI. For the IHC studies of the olfactory pathway mice were killed at 10 h (prior to hypothermia onset) or at 15 h PI (after the time of hypothermia onset).

*Immunohistochemistry (IHC)*

IHC was performed as previously described (Churchill et al., 2005; Majde et al., 2007). Brain sections were processed in pairs and adjacent coronal tissue sections were cut at 30 μm thickness and floated onto PBS.

*Antibody and labeling specificity testing*

The anti-TNFα and anti-IL1β antibodies used in this experiment were previously tested for specificity (Leyva-Grado et al., see chapter III). For this experiment, we

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incubated sections without the primary antibody or with pre-absorbed antibody (using specific recombinant cytokines as done previously); both procedures blocked the immunoreactivity. We also examined midbrain sections from TNFα and IL1β knockout mice; these mice lacked TNFα or IL1β immunoreactivity using the procedures described herein.

*DAB staining for IHC single labeling*

Adjacent tissue sections were incubated with one of the following antibodies; mouse anti-influenza A H1N1 virus monoclonal antibody (Chemicon International/Millipore, Temecula, CA, dilution 1:100), rabbit anti- recombinant mouse IL1β (Millipore, dilution 1:100) and goat anti-recombinant rat TNFα (R&D, Minneapolis MN, dilution 0.5 μg/ml). The sections were developed in 3, 3'- diaminobenzidine tetrahydrochloride (DAB kit, Vector) as a chromophore.

*Double labeling*

Preliminary results indicated that TNFα and IL1β immunoreactivities were present in cells that resemble neurons. To determine whether cytokines localized in neurons, double labeling for TNFα or IL1β plus NeuN was performed. After immersion in 3% blocking serum [a combination of normal chicken serum (NCS) and normal donkey serum (NDS)] for 1 h, adjacent sections were incubated with a mixture of goat anti-rat TNFα (R&D systems, dilution 1:100) or rabbit anti-mouse IL1β (Chemicon, dilution 1:100), in combination with mouse anti- nuclear protein NeuN (Chemicon, dilution 1:1000). After incubation, the samples were washed and then incubated in the dark for 2 h at room

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temperature with secondary antibodies (dilution 1:500) using a combination of Alexa

Fluor 488 chicken anti-mouse (Invitrogen, Carlsbad CA,) plus donkey anti-goat (for the

sections with TNFα antibodies) or Alexa Fluor 488 chicken anti-rabbit (for the sections

with the IL1β antibodies) plus Alexa Fluor 555 donkey anti-mouse.  After incubation, the

sections were washed and then mounted on gelatin-coated slides, dried, and cover slipped

with fluorescent hard set mounting medium (VectaShield, Vector Laboratories, Inc,

Burlingame, CA).


*Image preparation and quantitative analyses*

Images were captured with a Spot camera and software (Diagnostic Instruments Inc.,

Sterling Heights MI), in a Leica DMLB microscope. The number of IR-cells in the OB of

mice that received ONT in Experiment I was determined as previously described (Leyva-

Grado et al., see Chapter III). For Experiment II, the number of IR-cells was determined

using a rectangular box that measured 0.15 mm by 0.35 mm (Pir and Tu); 0.15 mm by

0.075 mm (Arc) or a circle with a radius of 0.100mm (CeA). Three different sections for

each area in each mouse were analyzed. An individual blinded to the experiment

treatment completed the quantification.


*Statistical analyses*

To compare body temperature and activity, three way ANOVA was used; surgery

was the between factor and days and hours within the day, the repeated factors.

 For the food intake and body weight among the ONT, sham and boiled groups two-way

ANOVA was used (between factor: surgery, repeated factor: day). When ANOVA

showed significance, post hoc comparisons were performed using the Student-Newman-Keuls test or Student's *t*-test. For IHC, the total number of IR cells was statistically analyzed using a paired Student's t-test to compare experimental and control samples for each brain. The data were expressed as mean ± the standard error of the mean. A p-value equal or lower than 0.05 was considered significant.

## Results

*Experiment I.*

*Body temperature and locomotor activity*

There were no significant differences in baseline Tbs between the ONT and the sham operated mice over the 12:12 h light-dark cycle [Figure 2 (hours -24 to 0); $F_{(1, 24)} = 1.2$, p= 0.32]. Mice in both groups had higher Tbs during the night and lower Tbs during their inactive day period (Figure 4.2). After viral challenge, both groups of mice became hypothermic compared to their respective baseline values [Figure 4.2; $F_{(1, 36)} = 2.15$, p<0.01]. However, on day one PI, Tbs in ONT mice were significantly higher when compared to Tbs in the sham mice between 15 and 27 h PI (p< 0.05). After 28 h PI, Tbs were not significantly different (p> 0.05) between the two groups.

The baseline locomotor activity responses were not significantly different between the ONT and the sham mice over the 12:12 h light-dark cycle [Figure 4.3 (hours -24 to 0); $F_{(1, 20)} = 0.86$, p= 0.36]. Activity was higher during the night and lower during the day (Figure 4.3). After influenza challenge, there was a transient increase in activity of about 4 h in both groups (Figure 4.3). After the initial increase in activity, locomotor activity fell in both ONT and sham groups compared to their baseline values [$F_{(1, 20)} = 32.16$, p< 0.01]. This reduced activity continued until the end of recording. No significant differences in activity were observed between the ONT and sham groups after viral infection [Figure 4.3; $F_{(1, 20)} = 0.46$, p= 0.50].

*Food intake and body weight*

There was a significant treatment effect on both food intake [Figure 4.4A; $F_{(2, 12)}$ = 7.25, p<0.01] and body weight [Figure 4.4B; $F_{(2, 12)}$ = 22.03, p<0.01] among the three treatment groups. The Student-Newman-Keuls posthoc comparison test revealed that both food intake and body weights of the mice that received boiled virus did not change over time and values were significantly higher than those measured in the live virus-treated groups on day 2 post-challenge. There were no significant differences in food intake or body weights between the infected ONT and sham groups that received the live virus.

*Mortality rate*

There were no differences in the percentage of deaths between the two groups of mice inoculated with live virus (ONT or sham). Mice started dying on day 5 after infection with more than 70% of the deaths occurring between day 5 and day 8 PI [ONT 71% (10 out of 14 mice) and sham 75% (9 out of 12 mice)]. None of the mice inoculated with boiled virus died.

*Immunohistochemistry of OBs from ONT and sham mice.*

*Viral antigen immunoreactivity*. H1N1 immunoreactivity was mainly present in the olfactory nerve (ON) and in the glomerular layer (GL) of the OB, and was observed in both the ONT mice (Figures 4.5A and 4.5B) and the sham mice (Figures 4.5C and 4.5D). The number of H1N1-IR cells was significantly reduced in the GL of the ONT group (8.4± 1.47) when compared with the sham group [18.7 ± 3.69; p=0.02 (Figure 4.6)].

*TNFα and IL1β immunoreactivities.* TNFα- and IL1β-IR cells were evident 15 h PI in

the GL and external plexiform layer (EPL) of the OB from infected sham mice,

confirming our earlier report from studies of unoperated mice (Leyva-Grado et al., see

Chapter III). In infected ONT mice, the number of TNFα- (Figure 4.7A) and IL1β-IR

cells (Figure 4.7B) was significantly reduced in the EPL when compared with the sham

group [TNFα (ONT 14 ± 1.15; sham 20 ± 2.4; p=0.05); IL1β (ONT 12 ± 2.4; sham 15.3

± 1.3; p=0.05)]. In ONT mice, the number of TNFα-IR cells in the GL was less than the

corresponding number in sham mice (27± 6.1 versus 45 ± 5.5) but these differences only

showed a trend toward significance [p=0.08 (Figure 4.7A)]. The number of IL1β-IR cells

did not significantly change in the GL of the ONT group (31± 3.71) when compared with

the sham group [31 ± 3.51; p=0.73 (Figure 4.7B)].


*Experiment II*

*Immunohistochemistry of midbrains from intact mice sacrificed at 10 or 15 h PI.*

*Viral antigen immunoreactivity.* No H1N1 or NP viral antigen immunoreactivity was

found in the Pir, Tu, the amygdala or Arc either at 10 h or at 15 h PI.


*TNFα immunoreactivity.* In the Pir at 10 h PI, the number of TNFα-IR cells in mice

treated with live virus (15 ± 2.22) was not significantly different from the number found

in mice treated with boiled virus (12 ± 1.87, p= 0.2) (Figure 4.8). By 15 h PI there was a

significant increase in the number of TNFα-IR cells in the Pir of live virus-inoculated

mice (40 ± 6.86) compared with the same region from boiled virus-inoculated mice (31 ±

4.76, p = 0.04) (Figure 4.8). In the Pir, the majority of the TNFα-IR cells were located in layer II (Figure 4.9). Similarly, in the Tu at 10 h PI, there was no significant difference in the number of TNFα-IR cells between the groups receiving live virus (24 ± 4.99) and boiled virus (25.5 ± 7.32, p= 0.57) (Figure 4.8). In contrast, at 15 h PI the number of TNFα-IR cells increased in the mice inoculated with live virus (43± 2.48) compared to control mice (31 ± 3.71, p= 0.02) (Figure 4.8). TNFα-immunoreactivity was mainly located in the dense cell layer (DCL) of the Tu (Figure 4.9).     In the BLA at 10 h PI, there was also no significant difference in the number of TNFα-IR cells between mice that received boiled virus (5 ± 1.52) and mice that received live virus (6 ± 2.01, p=0.28) (Figure 4.8). Similarly, at 15 h PI, there were no significant differences between the mice inoculated with live virus (19 ± 2.6) compared with mice inoculated with boiled virus (16 ± 2.7, p=0.18) (Figure 4.8). In the CeA at 10 h PI, live virus inoculation did not alter the number of TNFα-IR cells [boiled (11 ± 1.18) and live (13 ± 1.6, p=0.6)]. In contrast, by 15 h PI an increase in the number of TNFα-IR cells was observed in the CeA (live virus: 34 ± 2.12; boiled virus: 30 ± 1.42, p=0.05) (Figure 4.8). Finally, in the Arc, no significant differences between the boiled virus- and the live virus-inoculated groups were observed in the number of TNFα-IR cells either at 10 h PI (p= 0.57) or at 15 h PI (p= 0.23) (Figure 4.8).

*IL1β immunoreactivity.* In the Pir at 10 h PI, there was no significant difference in the number of IL1β-IR cells between live virus- inoculated mice (44 ± 6.91) and mice that received boiled virus (45 ± 7.61, p= 0.46) (Figure 4.10). By 15 h PI, however, the number

of IL1β-IR cells in the Pir significantly increased ($58 \pm 3.27$ versus $49 \pm 3.12$, p = 0.02) (Figure 4.10). IL1β immunoreactivity was mainly observed in layer II of the Pir (Figure 9). Similarly, in the Tu at 10 h PI, there was no significant difference in the number of IL1β-IR cells between the live virus-inoculated mice ($29 \pm 5.93$) and the boiled virus-inoculated mice ($34 \pm 10.9$, p= 0.65). However, at 15 h PI the number of IL1β-IR cells increased in mice inoculated with live virus ($36 \pm 2.68$) when compared with the same region in control mice ($28 \pm 2.71$, p= 0.04) (Figure 4.10). IL1β immunoreactivity was observed in cells located in the DCL of the Tu (Figure 4.9). In the BLA at 10 h PI, there was no significant difference in the number of IL1β-IR cells between mice that received boiled virus ($20 \pm 3.13$) and mice that received live virus ($20 \pm 2.3$, p=0.39) (Figure 4.10). By 15 h PI, however, the number of IL1β-IR cells in mice inoculated with live virus ($25 \pm 2.1$) was higher compared with the corresponding number in boiled virus-inoculated mice ($21 \pm 1.4$) but these differences only showed a trend toward significance (p=0.06) (Figure 4.10). In the CeA at 10 h PI, no significant differences were observed between the mice that received boiled virus ($23 \pm 4.73$) and the mice that received live virus ($24 \pm 5.21$, p=0.7) (Figure 4.10). However, by 15 h PI the number of IL1β-IR cells in the CeA was significantly higher in live virus-inoculated mice ($38 \pm 1.64$) compared to the boiled virus-inoculated mice ($33 \pm 1.40$, p= 0.03) (Figure 4.10). Finally, in the Arc at 10 h PI, there was no significant increase in the number of IL1β-IR cells in the live virus-inoculated mice ($34 \pm 3.8$) compared to the boiled virus-inoculated mice ($32 \pm 7.44$, p= 0.43). In contrast, by 15 h PI, a significant increase in the number of IL1β-IR cells was observed (live virus $56 \pm 6.29$ versus boiled virus $44 \pm 2.67$, p= 0.03) (Figure 4.10).

*Double labeling.* Both TNFα and IL1β immunoreactivity co-localized with NeuN in the

examined areas. Figure 4.11 shows an example of co-localization in the CeA for TNFα

and NeuN (Figure 4.11 A) and IL-1β and NeuN (Figure 4.11 B).

**Discussion**

The transection of the olfactory nerve at the level of the cribriform plate 10 days prior to intranasal inoculation with influenza PR8 affected the APR by delaying the onset of hypothermia for about 13 h. Furthermore, this surgical procedure also reduced the number of OB TNF$\alpha$- and IL1$\beta$-IR cells at 15 h PI after influenza viral challenge, suggesting that the ON serves as a cytokine-activation pathway.

Hypothermia, rather than fever, is usually observed in mice in response to challenge with influenza virus, regardless of the virus dose (Conn et al., 1995; Toth et al., 1995; Traynor et al., 2007). In rodents with systemic inflammation, hypothermia functions as an adaptive mechanism that correlates with enhanced protection and survival (Leon, 2004). Influenza virus-induced hypothermia is a regulated response in the sense that infected mice choose cool ambient temperatures even during the advance stage of the disease when hypothermia is more evident (Klein et al., 1992). Furthermore, influenza-infected mice show hypothermia even in warm environments (30 $^o$C) (Jhaveri et al., 2007). Current results showing the delay of the hypothermic response for about 13 h in mice that received the ONT are consistent with the idea that the ON is involved very early in the onset of hypothermia. However, this delay in the onset of hypothermia did not affect the survival rate after the infection. Hypothermia, like fever, is mediated by cytokines such as TNF$\alpha$, IL-1$\beta$ and interferons (Leon, 2004; Traynor et al., 2007).

TNFα and IL1β are associated with the initiation of the APR after influenza infection, acting in the brain regions responsible for regulation of temperature, sleep, food intake and sickness behavior (Hennet et al., 1992; Fang et al., 1995; Van Reeth, 2000; Schmitz et al., 2005). We found that the number of TNFα and IL1β–IR cells was reduced in the EPL of the OB in the mice that received the ONT in comparison with the sham group. Primary olfactory neuronal axons synapse in the EPL with dendrites of second-order neurons such as the tufted cells (Astic and Saucier, 2001). Tufted cells, along with the mitral cells in turn, extend axons to different regions of the brain. Although, the mechanisms involved in the reduction of the number of cells expressing TNFα and IL1β are not clear, current results clearly indicate that some signal from the ON hastens the early expression of TNFα and IL1β in the OB.

The reduced locomotor activity observed in mice after influenza challenge (Conn et al., 1995; Toth et al., 1995) was confirmed in the current studies after inoculation with live PR8. However, we did not find significant differences between the ONT and Sham groups, suggesting that the ON is not involved in the locomotor activity response after the virus challenge. Consistent with our findings, no significant changes in locomotor activity are observed as an effect of the ONT in uninfected animals (Harding and Wright, 1979; Yee and Constanzo, 1995; Astic and Saucier, 2001).

The reduced body weight and food intake after virus challenge was also consistent with prior findings (Conn et al., 1995; Swiergiel et al., 1997). The fact that we found no significant differences between the ONT and the Sham groups suggests that the ON is not essential to induce changes in food intake and body weight responses to influenza challenge. Previous studies in uninfected rodents also show that the ONT does not affect

normal food intake and body weight parameters (Yee and Constanzo, 1995; Yee and Rawson, 2000). Since Tb, locomotor activity, food intake and body weight during the APR are all thought to be regulated in part via enhanced cytokine production, current results of only Tb being affected by the ONT indicate that the importance of the ON and OB cytokines induced after influenza challenge is limited to hypothermia. The reason why this would be the case is not known. Perhaps the hypothermic-responsive brain regions are more sensitive to the olfactory input than the brain regions regulating locomotor activity or food intake.

The olfactory system has the capacity to undergo neurogenesis and continuously replace its primary olfactory neurons (Harding and Wright, 1979; Yee and Constanzo, 1995; Yee and Rawson, 2000). The olfactory receptor neurons (ORNs) are replaced when cells undergo normal aging, nerve injury or following toxic chemical exposure. After the ONT, an extended neuronal degeneration occurs within the first 2 days post-surgery (Holcomb et al., 1995; Astic and Saucier, 2001). Immediately after the injury, a proliferative response starts to replace the lost ORN (Constanzo et al., 2006). Restoration of the olfactory mediated behavior is achieved within 20 to 30 days after surgery (Harding and Wright, 1979; Yee and Constanzo, 1995). In our experiments we infected the mice 10 days after the ONT. We use this time frame because we wanted the mice to recover from the surgery before the infection. Furthermore, the expression and transport within the ORN of carnosine and the olfactory marker protein (both markers of mature ORN) do not start until between 15 and 45 days after surgery (Wright and Harding, 1982; Constanzo et al., 2006). It is possible, that by the time of infection and in the days following some cells have already reestablished connections with the OB. Accordingly,

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the conclusions drawn for our transaction experiment regarding the role of the primary olfactory tract and the secondary OB projections likely underestimate the importance of this pathway.

The second major finding described herein was the presence of enhanced cytokine–IR cell numbers after viral challenge in brain regions such as Pir, Tu, CeA and Arc. Studies using bilateral lesions of the CeA demonstrate that herpes simplex virus-1 induced fever and increased locomotor activity are attenuated in rats with a lesioned CeA compared with a sham group (Weidenfeld et al., 2005). Furthermore, after systemic injections with LPS (Konsman et al., 1999) or IL1β (Day et al., 1999) CeA c-fos mRNA and protein expression increase in the CeA indicating activation in response to antigen-induced stimulation (Sagar et al., 1995) and suggesting a role for the CeA in the regulation of the APR (Weidenfeld et al., 2005). Our results are consistent with this idea because we found that the number of TNFα and IL1β-IR cells increased in the CeA in response to viral challenge.

The number of IL1β-IR cells also increased in the Arc after viral challenge. Proinflammatory stimuli such as IL1β or LPS given systemically enhance the expression of the neuronal activity marker c-Fos in the Arc (Bluthe et al., 2000; Reyes and Sawchenko, 2002; Scarlett et al., 2007), thereby showing the activation of cells in this area in response to the immune challenge. Our results are consistent with those findings to the extent that after viral challenge the cells in the Arc are activated as evidenced by an increase in the production of cytokines such as IL1β. After IP injection with Staphylococcus enterotoxin, there is an increase in c-Fos-IR cells in the Arc that is not observed in TNFα KO mice or when an anti-TNFα antibody is used (Rossi-George et al.,

2005). However, we only saw limited TNFα immunoreactivity in the Arc and the number of TNFα-IR cells was not different between the mice inoculated with boiled virus and the mice inoculated with live virus.

The Arc is close to the median eminence, a circumventricular organ (CVO). It is thus possible that IL1β response in the Arc may be to signals of blood origin. CVOs are highly vascularized structures that lie outside the blood brain barrier (Rivest et al., 2000) and have cells that express cytokine receptors as well as toll-like receptors (Hopkins, 2007). Peritoneal injection of bacterial LPS increases IL1β (Konsman et al., 1999) and TNFα (Breder et al., 1994) in CVOs. After influenza PR8 challenge, virus is sporadically detected in blood (Mori et al., 1995; Majde et al., 2007) suggesting the possibility that this source of virus stimulates Arc production of IL1β. Another possibility is that cytokines of peripheral origin (e.g. lungs) signal the brain through afferent nerves such as the vagal nerves that project to the nucleus tractus solitarius (NTS) in the caudal brainstem and from the NTS through the afferent fibers that project to the Arc (Hansen et al., 1998; Goehler et al., 2000; Kubota et al., 2001; Matsuda et al., 2004; Wieczorek et al., 2005). For example, vagotomy blocks intraperitoneal IL1β-induced up regulation of hypothalamic IL1β transcripts (Hansen et al., 1998).

Influenza PR8 is considered a neurotropic, but not neurovirulent, strain of virus (Majde et al., 2007), and consequently the spread of this virus in the brain is unexpected. In this study we were not able to observe viral antigen immunoreactivity in any of the midbrain regions. This is consistent with previous studies by Iwasaki et al. (2004) who did not observe viral antigen immunoreactivity in the central nervous system of PR8 infected mice. Other studies using a different non-neuroadapted strain of influenza and

inoculation directly into the brain parenchyma also showed that the virus fails to spread beyond the injection site (Stevenson et al., 1997; Stevenson et al., 2002). These results suggest that the increase in cytokines we observed in the midbrain is not directly induced by viral antigens. Furthermore, after intranasal inoculation with a neurovirulent strain of influenza, the virus replicates in the olfactory epithelium and spreads to the OB with no further replication or spread of the virus to other regions of the brain. However, in immunodeficient mice lacking the recombination activating gene 1 (necessary for maturation of B and T lymphocytes) the virus persists in the OB for as long as 65 days PI and spreads to different regions of the brain such as the primary olfactory cortex, raphe nucleus and hypothalamus (Aronsson, et al., 2003). Results suggest that replication and spread of the virus beyond the OB is controlled in part by innate immune factors in the OB including microglia activation as we reported previously (Leyva-Grado et al., see Chapter III).

TNFα and IL1β induce each others and their own production (Vitkovic et al., 2000; Silverman et al., 2005). A possible mechanism involved in neuronal expression of TNFα and IL1β along the olfactory pathway may involve cell to cell communication through the release of nucleotides (Inoue et al., 2007). In response to the presence of the virus there is likely an increase in neuronal activity (perhaps the ORNs in the olfactory epithelium) that, in turn, is associated with release of adenosine triphosphate (ATP). ATP binds to the P2 receptors on glial cells and stimulates the release of cytokines from these cells (Domercq et al., 2006; Stock et al., 2006). Glial-released cytokines such as TNFα and IL1β, as well as ATP, are considered gliotransmitters that likely stimulate nearby neuronal TNFα and IL1β production.

In conclusion, our findings indicate that the surgical transection of the ON delays the onset of hypothermia for 13 h and decreases the number of TNF$\alpha$- and IL1$\beta$-IR cells in the OB 15 h after PR8 inoculation, thereby strongly suggesting a role for the ON pathway in the initial pathogenesis of influenza infection. Further, the enhancement of the number of TNF$\alpha$- and IL1$\beta$-IR cells in different regions of the olfactory pathway; i.e., Pir, Tu (part of the olfactory cortex), CeA and Arc at 15 h but not at 10 h PI in intact mice suggest that the activation of the different regions in this pathway is time dependant. Finally, we found that the cytokine-producing cells in these regions are mainly neurons suggesting that these cells are actively involved in the response to brain disturbances caused by the virus challenge. Together, these data suggest a role for the olfactory pathway in the activation of the APR after influenza infection that is mediated by the increase in cytokines production.

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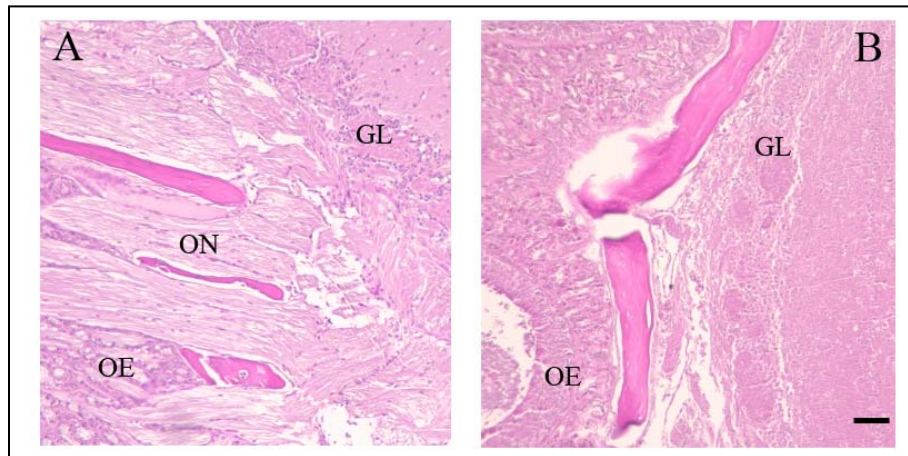
**Figure 4.1** Photomicrographs of sagital sections of the olfactory epithelium showing their connectivity with the olfactory bulb. (A) A sagital section showing the axons of the olfactory receptor neurons connecting with the glomerular layer of the olfactory bulb in a mouse that received a sham surgery. (B) A sagital section showing that the connectivity between the axons and the olfactory bulb is lost after the olfactory nerve transaction surgery. The scale bar is 0.02 mm. OE, olfactory epithelium; ON, olfactory nerve; GL, glomerular layer.
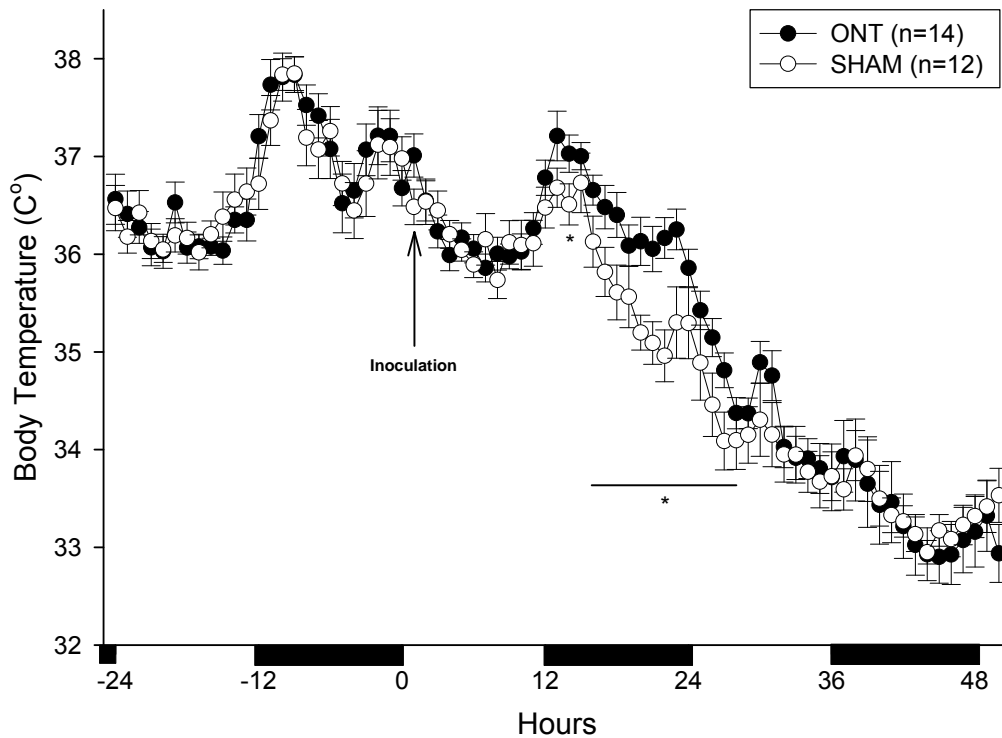
**Figure 4.2** Time course of body temperature changes in ONT or sham mice following intranasal inoculation with influenza virus. The mice with a transected olfactory nerve (ONT) delay their hypothermia response to influenza virus by 13 h. Data points represent the means +/- SEM at 1 hour intervals. * represents significant differences, p<0.05 between the ONT and the sham groups. The solid horizontal bars along the abscissa mark the 12 h dark periods.
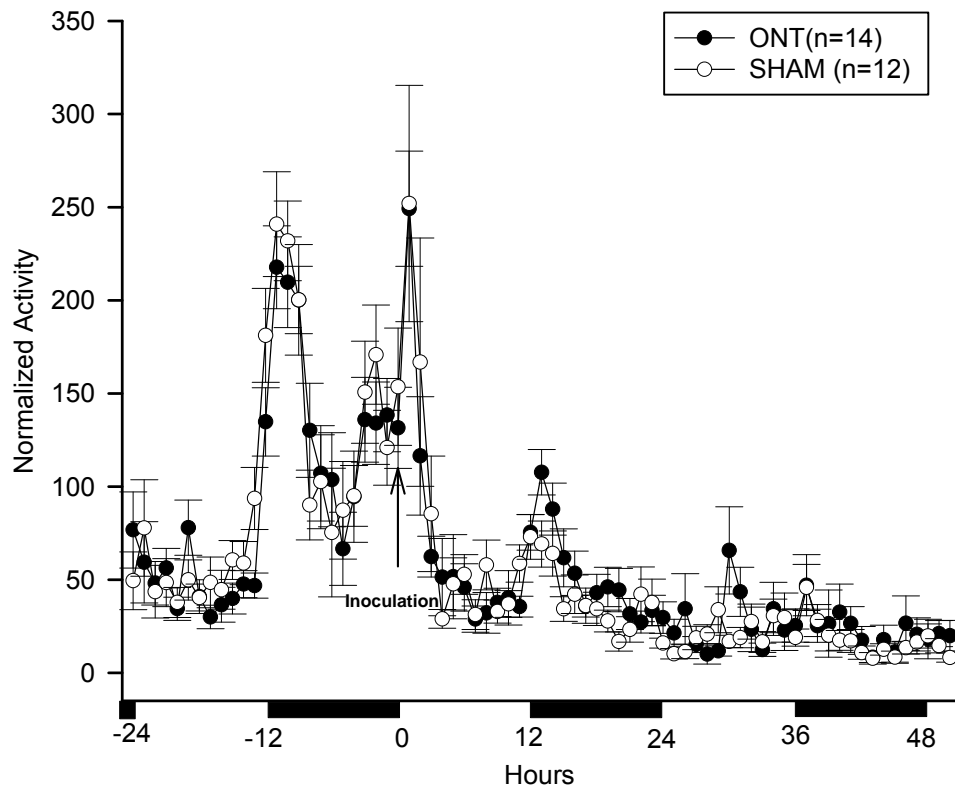
**Figure 4.3** Locomotor activity responses to PR8 challenge in ONT or sham mice.

Data points represent the means +/- SEM at 1 hour intervals. No significant differences

were observed between the olfactory nerve transected (ONT) and the Sham groups. After

live viral challenge, activity increased for about 4 h compared with the same time of the

baseline day. It then decreased below baseline values in both groups. The second day

after viral challenge, activity remained below baseline levels. The solid horizontal bars

along the abscissa mark the 12 h dark periods.
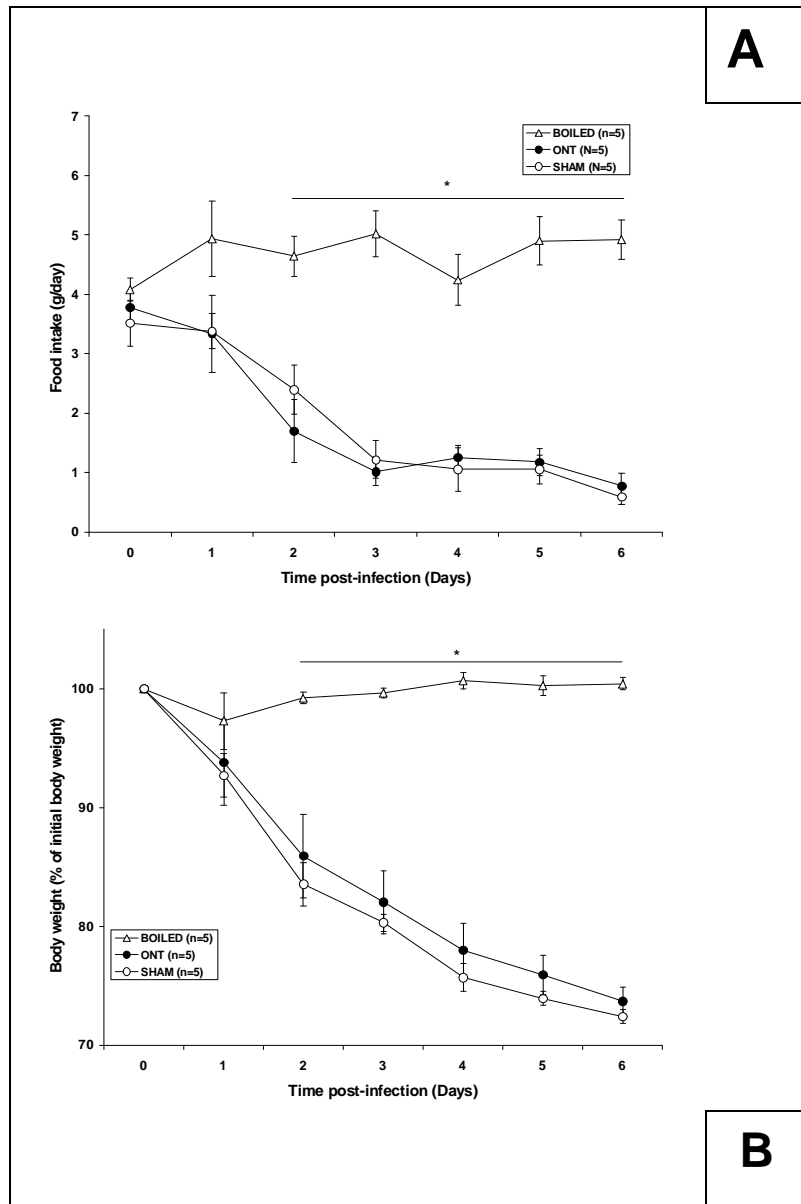
**Figure 4.4** Food intake and body weight following intranasal inoculation with influenza virus in ONT or sham mice. Food intake (A) and weight (B) were determined for 6 days. (A) Data points represent the food consumption for the 24 h previous to the measurement. A decrease in food intake was observed in the ONT and sham groups that received live virus compared to the sham mice that received the boiled virus. (B) Data points represent the percentage of body weight in relationship to the initial body weight

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(day of virus challenge or day 0). Mice inoculated with live virus (ONT and sham) lost

weight when compared with the sham mice that received boiled virus. (*) represents

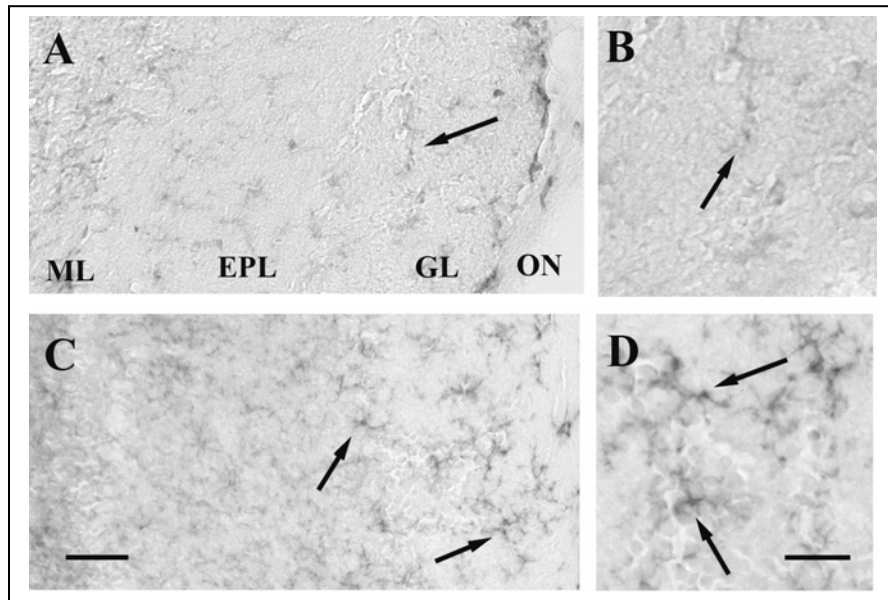significant differences, $p<0.05$ between the ONT and the sham groups compared to the

boiled group.

**Figure 4.5** Photomicrographs of olfactory bulb coronal sections from ONT or sham mice killed 15 h post intranasal challenge stained for influenza H1N1. (A) Coronal section from an ONT mouse that received live PR8, no H1N1 immunoreactivity is evident. (B) A higher magnification of the GL on (A). (C) Coronal sections from a sham mouse showing the H1N1 immunoreactivity in the ON and the GL. (D) A higher magnification of the GL in (C). Arrows point to some stained cells. The scale bar for lower magnification pictures (A and C) is 0.02 mm; for higher magnification pictures (B and D) the scale bar is 0.025 mm. ML, mitral cell layer; EPL, external plexiform layer; GL, glomerular layer; ON, olfactory nerve.
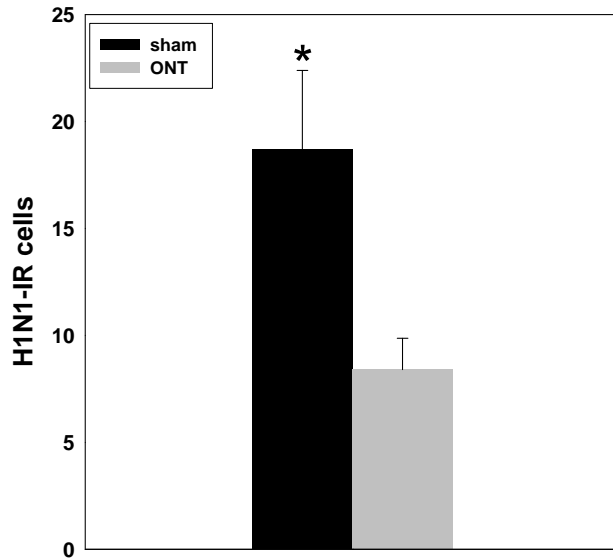
**Figure 4.6** Quantitative analyses of the number of the viral antigen H1N1-immunoreactive (IR) cells in the OB of ONT or sham mice inoculated with live virus. One group of mice had their olfactory nerves transected (ONT) prior to the challenge (n=6), the other group received a sham surgery (n=6). The number of H1N1-IR cells was significantly different between the two groups. Mice were inoculated with virus 10 days subsequent to olfactory nerve transaction. (*) indicates $p < 0.05$.
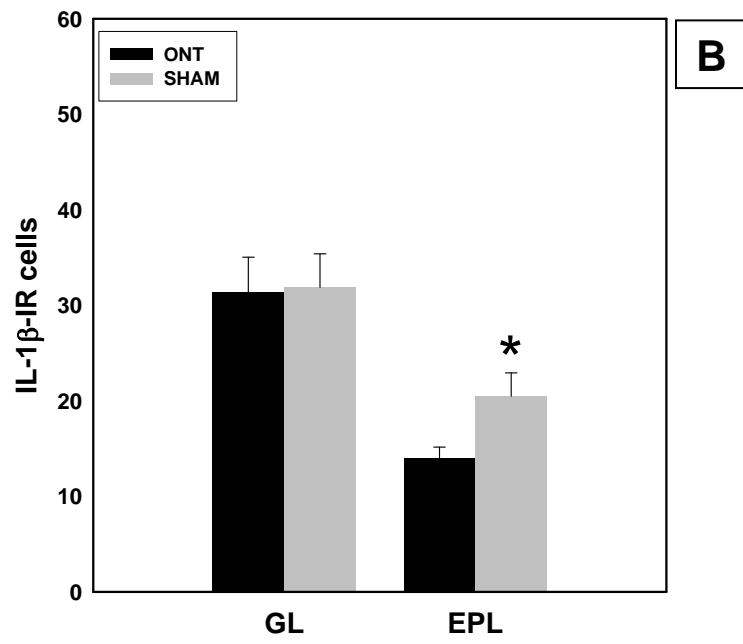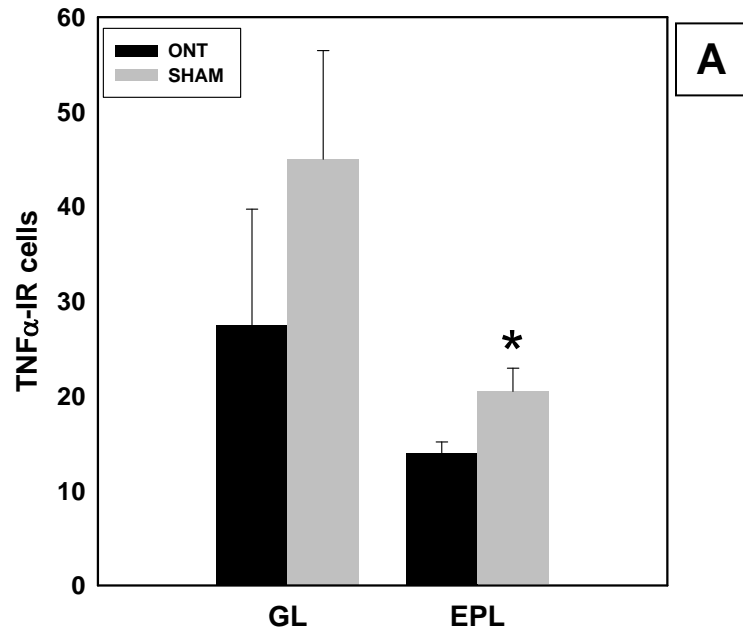
**Figure 4.7** Quantitative analyses of the number of TNFα- and IL1β-immunoreactive (IR) cells in the OB of ONT or sham mice inoculated with live virus. One group of mice had their olfactory nerves transected (ONT) prior to the challenge (n=6), the other group received a sham surgery (n=6). The number of TNFα-IR (A) and IL1β-IR cells (B) cells was significantly different between the two groups in the external plexiform layer (EPL). No significant differences were observed in the glomerular layer (GL). Mice were inoculated with virus 10 days subsequent to olfactory nerve transaction.(*) indicates $p < 0.05$.
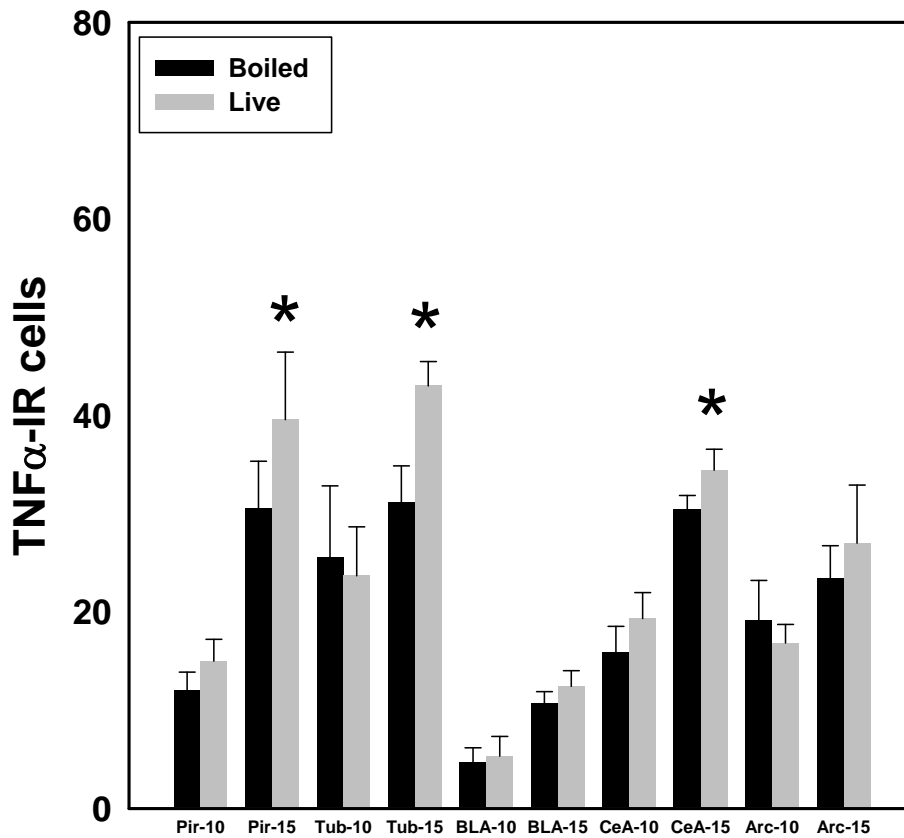
**Figure 4.8** The number of TNFα- immunoreactive (IR) cells in the piriform cortex (Pir), the olfactory tubercle (Tu), the basolateral amygdala (BLA), the central amygdala (CeA) and the hypothalamic arcuate nucleus (Arc) at 10 h and 15 h after intranasal inoculation with influenza virus. At 10 h post inoculation no significant differences between the two groups were observed in any of the regions. At 15 h, the number of TNFα-IR cells increased in Pir, Tu and CeA in mice inoculated with live virus compared to the mice that received boiled virus. No significant changes were observed in the BLA or Arc. (*) indicates a significant difference ($p < 0.05$). The area used for TNFα quantification in the Pir and Tu was 0.053 mm$^2$, the BLA and the CeA was 0.012 mm$^2$ and the Arc was 0.03 mm$^2$.

TNFα    IL-1β

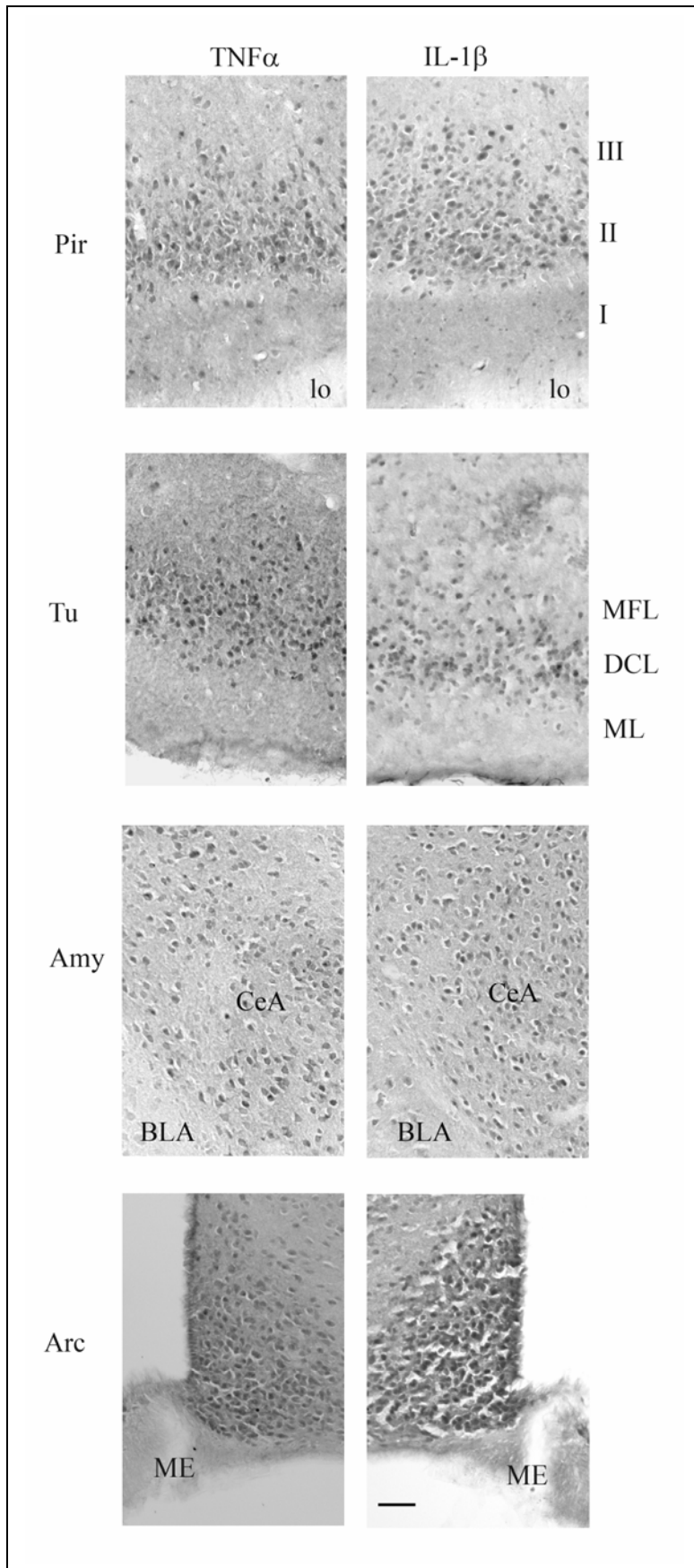Pir — III, II, I, lo

Tu — MFL, DCL, ML

Amy — CeA, BLA

Arc — ME

**Figure 4.9** Distribution of the TNFα- and IL1β- immunoreactive (IR) cells in the piriform cortex (Pir), olfactory tubercle (Tu) amygdala (Amy) and the hypothalamic arcuate nucleus (Arc) at 15 h after intranasal inoculation with live PR8 influenza virus. In the Pir, IR cells for both cytokines were observed in the layer II; in the Tu, TNFα and IL-1β-IR cells were located in the dense cell layer (DCL). Both the central amygdala (CeA) and basolateral amygdala (BLA) also showed immunoreactivity for TNFα and IL1β. Scale bar= 0.05 mm. Abbreviations: lo (lateral olfactory tract), ML (molecular layer), MFL (multiform layer), ME (median eminence).
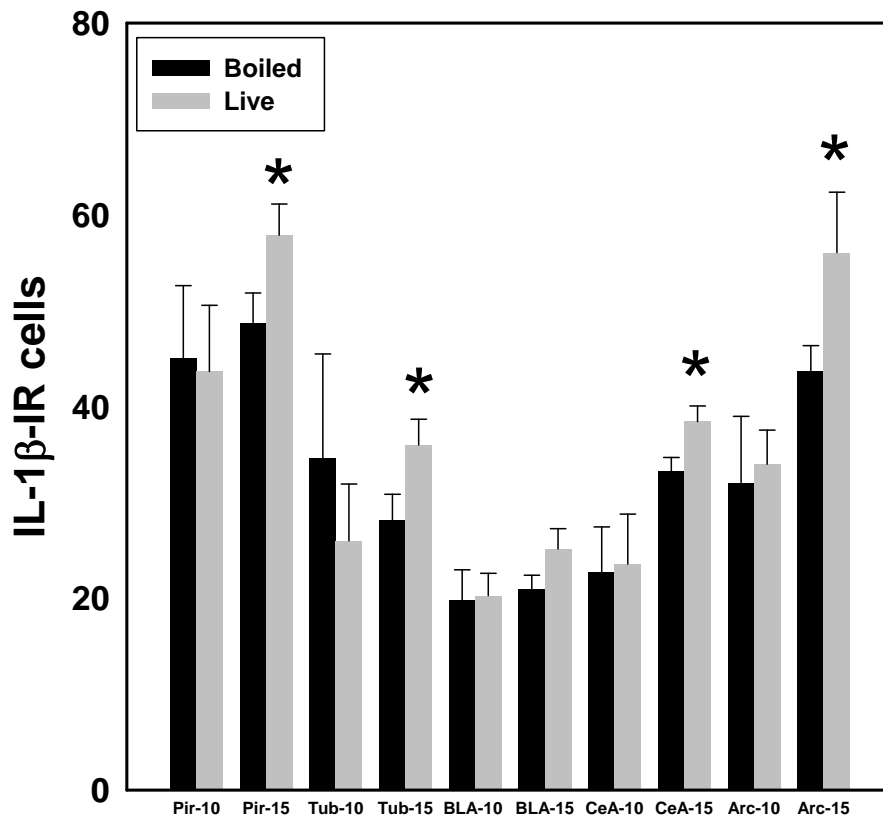
**Figure 4.10** Quantitative analyses of the number of IL-1β- immunoreactive (IR) cells in the piriform cortex (Pir), the olfactory tubercle (Tu), the basolateral amygdala (BLA), the central amygdala (CeA) and the hypothalamic arcuate nucleus (Arc) at 10 h and 15 h after intranasal inoculation with influenza virus. At 10 h post inoculation no significant differences between the two groups were observed in any of the analyzed regions. At 15 h, the number of IL-1β-IR cells increased in Pir, Tu, CeA and Arc in mice inoculated with live virus compared to those that received boiled virus. No significant changes were observed in BLA. (*) indicates a significant difference (p< 0.05). The area used for IL-1β quantification in the Pir and Tu was 0.053 mm$^2$, the BLA and the CeA was 0.012 mm$^2$ and the Arc was 0.03 mm$^2$.
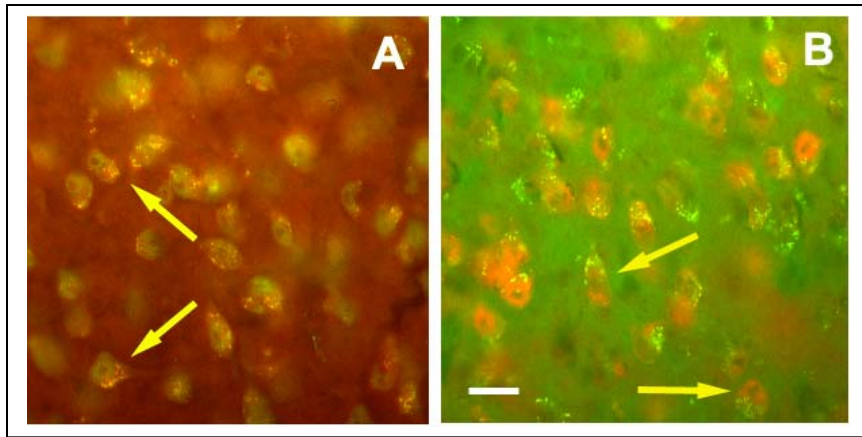
**Figure 4.11** Double labeling immunofluorescence photomicrographs of TNFα or IL1β and the neuronal marker (NeuN) in the central amygdala of PR8-infected mice. Yellow arrows indicate cells exhibiting co-localization of one of the cytokines with NeuN. (A) TNFα (red) and NeuN (green) indicate the presence of TNFα in the cytoplasm of some neurons in the central amygdala.  (B) IL1β-immunoreactivity (green) co-localized with cells also labeled with NeuN (red) in the central amygdala. (B). Scale bar = 0.025 mm.

# CHAPTER V

## GENERAL DISCUSSION

The experiments detailed in this dissertation describe one of the potential mechanisms involved in the genesis of the influenza virus-induced acute phase response (APR). In this chapter, the major findings of this thesis work will be briefly summarized and discussed in the broader context of the pathogenesis of the APR.

Data presented in chapter II demonstrate that after intranasal inoculation with influenza virus, the virus localizes and partially replicates in the olfactory bulb (OB) of the brain. This is remarkable because most human mouse-adapted strains of influenza are thought to replicate only in the respiratory tract (Hennet et al., 1992; Ward, 1997). Although Mori et al. (1995), did show the presence of the virus in the brain at day 5 post infection (PI), when the clinical signs of disease are advanced, we found genomic RNA as well as replication intermediates in the brain as early as 4 h PI. One of the hallmarks of influenza infection in mice is the presence of hypothermia (Hennet et al., 1992; Conn et al., 1995). The hypothermia peak is between 13 and 15 h PI (chapters II and IV; Toth et al., 1995; Fang et al., 1995). This physiological response at 13-15 h PI may be caused by the presence of the virus in the OB at 4 h after inoculation.

Within hours of exposure, the viral antigen was found in the olfactory nerve (ON), the glomerular layer (GL), and, to a lesser extent, in the external plexiform layer (EPL) of the OB. It was localized in microglia and astrocytes, but not in neurons (Chapter III). Microglial cells are the resident macrophages of the CNS that function as part of the

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mechanisms of immune defense against microorganisms and injury (Hanisch and Kettenmann, 2007). During the immune response to an insult, astrocytes participate by promoting neuroinflammation through NF-κB-dependant pathways and by restoring brain homeostasis (Farina et al., 2007). In agreement with my results, previous studies show that PR8 localization in the CNS is restricted to glial cells (Bradshaw et al., 1989; Wang et al., 2008). Results also suggested that PR8 goes at least through a partial replication in the OB as previously demonstrated in mouse brain cell cultures (Bradshaw et al., 1989).

One of the main effects observed in response to the presence of the virus in the OB was the production of cytokines as evidenced by an increase in TNFα- and IL1β- mRNA (chapter II) and TNFα- and IL1β- IR cells (chapter III). An increase in cytokines, particularly pro-inflammatory cytokines, is a hallmark of the initial immune response to the presence of microorganisms (Dantzer et al., 2008). In the CNS, microglia, astrocytes, oligodendrocytes and neurons may express and produce cytokines in response to the presence of a microorganism. Also neuroimmunomodulatory signals, such as cytokines or gliotranmitters (ATP or glutamate), may be produced by microglia and astrocytes, which first recognize the presence of the attack (Konsman et al., 2002; Liu et al., 1994; Ohtori et al., 2004; Owens et al., 2005). Our studies confirm that microglia, astrocytes and neurons in the OB are able to respond to the presence of the virus by increasing the synthesis and expression of both TNFα and IL1β.

The increased expression of cytokines also extended to other regions of the brain (chapter IV). We found an increase in the number of TNFα and IL1β-immunoreactive cells in different regions that receive direct or indirect input from the OB, including the

CeA and the Arc. The amygdala is involved in the APR; in particular the CeA mediates the neuroendocrine, febrile and behavioral responses to HSV-1 infection (Weidenfeld et al., 2005). The Arc is involved in different physiological activities, including energy homeostasis and food intake. Anorexia is considered part of the sickness behavior response (Konsman and Dantzer, 2001) and it is observed during infection (Reyes and Sawchenko, 2002). The mechanism of how the OB cytokines communicate with the different regions in the brain to induce cytokine expression has not been completely elucidated but may include signaling through adenosine or ATP (Hasko et al., 2005; Inoue et al., 2007).

The ON pathway plays an important role in the expression of hypothermia following an influenza virus challenge. Mice that received an olfactory nerve transection (ONT) show a 13 h delay in the onset of hypothermia compared to sham-operated mice (chapter IV). The ON pathway is used by several viruses to reach the brain after intranasal inoculation (Barnett and Perlman, 1993; Becker, 1995; Park et al., 2002). The ON transport of virus may be accomplished by axonal transport or by an olfactory epithelial pathway (Dahlin et al., 2000). Furthermore, the presence of channels formed by olfactory ensheathing cells and ON fibroblasts that surround the ON (Li et al., 2005) may be another pathway used by the virus to reach the OB. Our data suggests that the virus enters through the olfactory ensheathing cells, but we have not completed the electron microscopic analyses to prove which particular pathway is used by the PR8 strain in our model. However, my results clearly indicate that transection of the ON pathway delays the onset of hypothermia.

In summary, our results indicate that the PR8 strain of influenza virus that was considered a non-neurotropic strain is indeed a neurotropic strain without neurovirulent characteristics. The virus is able to reach the OB very early after intranasal inoculation and colocalizes within microglia and astrocytes. The presence of the virus induces the production of cytokines such as TNF$\alpha$ and IL1$\beta$ in the OB as well as in specific regions of the brain that receive direct or indirect connection from the OB. We also conclude that the ON plays a very important role in the manifestation of the APR following the challenge with influenza virus. Overall, the results presented in this thesis elucidate in part the possible mechanism involved in the pathogenesis of the APR in influenza infected mice.

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