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**Effects of Isolation or Isolation and LPS During Postnatal Days 6, 12 & 21
on Rat Brain: Blood Brain Barrier and IgG Expression**

A thesis submitted to
The Department of Psychology
Carleton University

In Partial Fulfilment
Of The Requirements For The Degree Of
Master of Science Psychology

by
James N. McIntyre
August 1998

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Effects of Isolation or Isolation and LPS During Postnatal Days 6, 12 & 21
on Rat Brain: Blood Brain Barrier and IgG Expression

submitted by

James N. McIntyre

in Partial fulfillment of the requirements for
the degree of Master of Science

Chair

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September, 1998

Abstract

Early life events influence the degree to which subsequent stressors promote activation of the HPA axis. Likewise, immunological challenges, including administration of endotoxins and cytokines, also result in the activation of the HPA axis and may proactively influence HPA responses to subsequently encountered stressors. The mechanisms by which endotoxins and early life stressors have their sustained effects on HPA functioning has not been fully elucidated. While variations of HPA activity could account for these effects, it is possible that other factors play a role as well. For instance, it is possible that stressors, such as maternal separation and endotoxin challenge, compromise the blood brain barrier, thus permitting entry to the brain of neuroactive and/or neurotoxic-like substances and proteins, including cytokines and even corticoids. The degree to which any of these phenomena occur may have distinct genetic underpinnings. The present research was aimed at assessing what role, if any, early maternal separation and endotoxin challenge had on blood brain barrier (BBB) permeability, as measured by the large blood-borne protein, immunoglobulin G (IgG).

In the initial experiment, the effects of acute isolation (3 hr) on BBB permeability was assessed in rats of different developmental ages (6, 12 and 21 days of age), and genetic strains (Fast and Slow kindling strains of rat).

In the second experiment, we assessed the effects of LPS on subsequent IgG expression in the brain of Slow rats. Slow rats of three different ages (6, 12, and 21 days) experienced a combination of LPS and isolation from the dam. The isolation was necessary, since illness induced by LPS could influence the dam's treatment of her pups.

In both experiments, it was found that IgGs were normally present in several, specific brain areas at 6 days of age, but diminished by 12 days, and largely disappeared by

21 days of age. In experiment 1, acute isolation of Slow and Fast rats did not promote any changes in normal IgG expression seen in neurons. However, on day 6 and 12, Slow rats expressed more significantly more IgGs than their Fast counterparts. On the other hand, by 21 days of age IgG expression in the two strains was absent.

Since isolation itself did not cause differences in IgG expression, it would appear that the IgGs alone are not responsible for known long term changes associated with acute separation. Conversely, differences in IgG expression between the strains might reflect some of the distinct behavioural differences observed elsewhere in the adults.

In experiment 2, saline injections did not promote significant changes in the normal pattern of IgG expression in Slow rats from all 3 age groups. Conversely, exposure to LPS caused large increases in IgG expression at days 12 and 21, but not day 6, compared to control values, suggesting that the younger rats are somehow 'resistant' to the effects of the endotoxin. Thus, although the acute stress of isolation alone did not alter IgG expression, perhaps in combination with an endotoxic challenge, it might have contributed to the dramatic age-specific increase in expression that was observed.

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Glossary

Antibodies	amino acid produced by B lymphoid cells in response to stimulation with an immunogen. Antibody molecules are immunoglobulins found in the blood and body fluids. Thus, all antibodies are immunoglobulins formed in response to immunogens.
Antigen	a substance that reacts with the products of an immune response stimulated by a specific immunogen, including both antibodies and/or T lymphocyte receptors. Considered to be one of many kinds of substances with which an antibody molecule or T cell receptor may bind (e.g., sugars, hormones, proteins).
Cytokines	coordinate antibody and T cell immune system interactions and amplify immune reactivity. Include monokines (e.g., IL-1, TNF- α and β interferons) synthesised by macrophages, microglia and lymphokines (e.g., interleukins, γ interferon, colony stimulating factor) produced by activated T lymphocytes and NK cells.
CRH	or corticotropin releasing hormone is secreted by neurons in the hypothalamic region and is responsible for the release of corticotropin.
Cytotoxicity	the fatal injury of target cells by either specific antibody and complement or specifically sensitised cytotoxic T cells, activated macrophages, or NK cells.

Immunoglobulin	a mature B cell product synthesised in response to stimulation by an antigen. Antibody molecules are immunoglobulins of defined specificity produced by plasma cells. The five immunoglobulin classes are termed isotypes based on the heavy chain specificity of each immunoglobulin class.
Immunoglobulin G	is the most abundant of the circulating antibodies. It readily crosses the walls of blood vessels and enters tissue fluids. IgG also crosses the placenta and confers passive immunity from the mother to the fetus. IgG protects against bacteria, viruses, and toxins circulating in the blood and lymph, and triggers action of the complement system.
Interleukins	group of cytokines synthesised by lymphocytes, monocytes, and selected other cells that promote growth of T cells and B cells.
Interleukin 1	synthesised by activated mononuclear phagocytes that have been stimulated by ribopolysaccharide or by interaction with CD4 ⁺ T lymphocytes. Comprised of two principal polypeptides (IL-1 α , IL-1 β). A mediator of inflammation, IL-1 stimulates multiple cells to act as immune or inflammatory response effector cells.
Lymphocyte	a round cell, divided into two principle groups termed B and T lymphocytes, which are distinguished by the expression of distinctive surface molecules that have precise roles in immune reaction.
Macrophages	mononuclear phagocytic cells derived from monocytes in the blood that were produced from stem cells in the bone marrow.

Microglia	mononuclear glial cells which act in a variety of ways similar to macrophages. Partly responsible for immune reaction in the CNS.
Monocytes	mononuclear phagocytic cells in the blood that are derived from promocytes in the blood. They quickly migrate to tissues and are transformed into macrophages.
OVL	is the short form for organum vasculosum laminae terminalis. This region of the brain includes many hypothalamic nuclei and is known to have a thin blood brain barrier.

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Introduction

It has been suggested that early life experiences may profoundly influence the response to later stressor experiences. Whereas mild stimulation, such as handling or brief maternal separation, appears to limit the response to stressors encountered during adulthood, maternal separation for extended periods of time (e.g., 3 hr per day) has the opposite effect (Anisman, Zaharia & Merali, in press; Dinan, 1994; Issa, Rowe, Gauthier, & Meaney, 1990; Meaney, Tannenbaum, Francis, Bhatnagar, Shanks, Viau, O'Donnel & Plotski, 1994). The functional consequences of these stressors appear to be mediated through the hypothalamic-pituitary-adrenal (HPA) axis (De Kloet & Reul, 1987; Dinan, 1994). The analysis of stressor effects on behavioural and physiological processes is complicated by the fact that diverse stressors may yield different types of outcomes. For instance, Herman and Cullinan (1997) have characterised two types of stressors which activate the HPA axis: processive stressors, which involve higher order sensory processing (such as exposure to predators); and systemic stressors, which provoke swift physiological reactions that may be required to deal with intrinsic changes (e.g., the response to bacterial insults). Moreover, the effects of systemic stressors, like those of maternal separation, may have long-lasting repercussions. For instance, lipopolysaccharide (LPS), a glycoprotein sheath surrounding gram negative bacteria, administered to rat pups induces variations of HPA activity that persist into adulthood (Shanks, Larocque & Meaney, 1995). Ordinarily, endotoxins give rise to the release of cytokines (IL-1, IL-6 and TNF- α) from immune cells (macrophages). Inasmuch as cytokines, the regulatory proteins of the immune system, profoundly influence HPA activity (Besedovsky, Del Rey, Klusman, Furukawa, Monge, & Kabiersch, 1991;

Bodurka, Caputa & Bodurka, 1997; Sarlis, Chowdrey, Stephanou & Lightman, 1992; Vilcek & Le, 1994), it is not surprising that endotoxin challenge might have long term consequences on HPA activity.

In addition to acting as mediators of HPA activity, cytokines such as IL-1, IL-6 and TNF- α also possess potent inflammatory actions in both the peripheral and central nervous systems (Rothwell, 1991; Hopkins & Rothwell 1995; Rothwell & Strijbos, 1995; Vallières & Rivest, 1997). One of the key components of inflammation is vasodilation which, in the brain, results in the blood brain barrier (BBB) being compromised (Claudio, Martiney & Brosnan, 1994; Quagliarello, Wispelwey, Long & Sheld, 1991; Sternberg, 1997; Shöbitz, de Kloet & Holsboer, 1994; Terada, Willingham & Guidot, 1992). Once interrupted, the BBB allows passage of blood products such as macrophages, T-cells and immunoglobulins to the CNS (Roitt, 1994; Upender, Dunn, Wilson & Naegele, 1997; Wright & Merchant, 1994). While some research exists concerning the impact of blood products on the development of the CNS (Habgood, Sedwick, Dziegielewska & Saunders, 1992; Upender et al., 1997), it is curious that limited information is available concerning the contribution of altered BBB permeability early in life to the adult response to stressors. For that matter, it remains to be established whether early life stressors (e.g., early life maternal separation, and endotoxin challenges) influence brain development, including HPA reactivity, owing to variations of BBB permeability.

The purpose of the present investigation is to evaluate whether early maternal separation and administration of an endotoxin compromise BBB permeability as reflected by the increase of immunoglobulin G (characteristic blood product) in brain.

As well, we will establish whether the results of these manipulations vary as a function of the age of the animals during which the insults were applied.

Overview of localisation and function of IL-1 β , IL-6 and TNF- α

It has been established that various cytokines initiate activation of the HPA axis, and may play a pivotal role in modulation of brain injury associated with various types of insults. Furthermore, as will be discussed later, it appears that cytokine administration may have enduring effects on HPA activity. Given that endotoxin challenge (which stimulates cytokine release) and maternal deprivation have similar long-term effects, a role for interleukins in such effects is implicated. Before discussing this proposition, it is only prudent to first provide a brief overview of structure and action of the proinflammatory cytokines, IL-1 β , IL-6 and TNF- α .

It has been said that cytokines are simply small regulatory proteins (molecular weights ≤ 30 kDa) with pleiotropic activities (Vilcek & Le, 1994). However, this view is likely too simplistic. A cytokine may have various destinations or may act to increase and/or decrease the production of either another cytokine, or the expression of its receptors. We will first examine IL-1 β before progressing to TNF- α and IL-6.

In the periphery, interleukin-1 β is primarily secreted by monocytes and macrophages (Rothwell, 1991). Central synthesis occurs primarily in microglia, while lesser amounts can be attributed to astrocytes as well as neurons within the hypothalamus, hippocampus, choroid plexus, olfactory bulb and cerebellum (Cunningham & de Souza, 1993; Rothwell & Hopkins, 1995; Rothwell & Strijbos, 1995; Szaflarski, Burtrum, Faye & Silverstein, 1995; Shöbitz et al., 1994). Dinarello (1994), describes IL-1 primarily as an inflammatory cytokine that is closely related to

TNF- α in its reactivity. There are several forms of IL-1 that include IL-1 α , IL-1 β and the endogenous IL-1 antagonist, IL-1ra (Rothwell & Hopkins, 1995; Rothwell & Stijbos, 1995; Shöbitz et al., 1994). There also exist two types of IL-1 receptor: IL-R_I and IL-R_{II}. The IL-R_I has a high affinity for both IL-1 α and IL-1 β , while IL-R_{II} binds preferentially to IL-1 β . In the periphery, IL-R_I is found primarily on T-cells and fibroblasts (Deyerle, Sims, Dower & Bothwell, 1992). On the other hand, the IL-R_{II} receptor is found on B-cells, hematopoietic cells, T-cells and endothelial cells (Simms & Giri, 1994). This type II receptor has been suggested to be a “decoy” receptor by binding IL-1 β and thus limiting its exposure to the primary receptor. Hence, its ultimate function is essentially similar to that of IL-1ra in that it serves as a negative regulator of the action of IL-1 (Simms & Giri, 1994). Although it is premature to exclude a role for other cytokines, the majority of changes in form and function that occur following systemic immunological challenges such as LPS, ischemia and excitotoxic injection have been ascribed to the actions of IL-1 β (Rothwell & Hopkins, 1995; Rothwell & Stijbos, 1995; Shöbitz et al., 1994).

The main sources of TNF- α are much the same as those of IL-1. Further similarities include the fact that it is primarily an inflammatory cytokine that mediates both local and systemic features of inflammation, such as leukocyte release, thermogenesis, activation of T-cell toxicity and HPA activation (Shöbitz et al., 1994). It has been found that TNF- α is maximally secreted from astrocytes following the administration of a bacterial endotoxin such as LPS (Tracey, 1994). A unique feature of this cytokine is that it has been found to be most effective when only 5% of its receptors are activated (Shöbitz et al., 1994). Like IL-1 β , TNF- α has two types of receptors.

The type-I receptor mediates cell toxicity, while the type-II receptor responds by engendering a proliferative signal for cytotoxic T cells (Tartalia, Weber, Figari, Reynolds, Palladino, & Goeddel, 1991; Shöbitz et al., 1994).

Interleukin-6 is secreted by many of the same cells as IL-1 β and TNF- α (Shöbitz et al., 1994). In addition to performing functions similar to IL-1 β & TNF- α , this cytokine also aids in differentiating B- and cytotoxic T-cells (Lyson & McCann, 1992). Apart from these apparently shared actions of TNF- α , Hirano (1994) suggests that a unique function of IL-6 is that it can act as an inhibitor or promoter of malignant cell lines. While IL-6 has been receiving increasingly more attention, the remainder of this document will emphasize the roles of IL-1 β and TNF- α in response to immune challenge.

Expression of cytokines after injury

While there is evidence to suggest that the frontal cortex, hypothalamus, hippocampus and cerebellum secrete low levels of cytokines under normal conditions, exact baseline values have not been agreed upon (Bandtlow, Meyer, Lindholm, Spranger, Heunemann & Theonen, 1990; Rothwell, 1991; Rothwell & Strijbos, 1995; Shöbitz et al., 1994). Notwithstanding, a variety of physical stressors including systemic LPS administration (Ban, Haour & Lenstra, 1992; Buttini & Boddeke, 1995; Gabellec, Griffais, Fillion & Haour, 1995; Layé, Parnet, Goujon & Dantzer, 1994; Liu, Kita, Tanaka & Kinoshita, 1996), central LPS injection (Alheim, Chai, Fantuzzi, Hasanvan, Malinowsky, Di Santo, Ghezzi, Dinarello & Bartfai, 1997; Bodurka et al., 1997; Buttini, Sauter & Bodekke, 1994; Hillhouse & Mosley, 1993; Layé, Goujon, Combe, VanHoy, Kelley, Parnet & Dantzer, 1996; Quan, Whiteside & Herkenham, 1998; Tasaki,

Reutzler, Ohtsuke, Martin, Nawashiro & Hallenbeck, 1997; Vallières & Rivest, 1997; Yamasu, Onoe, Soma, Oshima & Mizuno, 1989), brain injury, cerebral ischemia and seizure (Buttini et al., 1994; de Bock, Dorand & Rondouin 1996; Hopkins & Rothwell, 1995; Iadecola, 1997; Minami, Kuraishi, Yamaguchi, Nakai, Hirai & Satoh, 1990; Rothwell & Hopkins, 1995; Sairanen, Lindsberg, Brenner & Sirén, 1997; Szaflarski et al., 1995; Yabuuchi, Minami, Katsumata & Satoh, 1993) have been found to induce IL-1 β , IL-6 and TNF- α mRNA expression in the olfactory bulb, lateral septum, hypothalamus, hippocampus, piriform cortex, amygdala, and entorhinal and cerebral cortices. As well, there have been several reports indicating that stressors increase IL-1 mRNA and protein levels in the brain (Nguyen, Deak, Owens, Kohno, Fleshner, Watkins & Maier, 1998). Thus, it seems possible that cytokines play a role in modulating brain function after physiological stress and may be a catalyst for long-term change.

Early life experiences cause changes in the adult animal

Early life experiences alter HPA activity, and these effects persist into adulthood (Meaney et al., 1994). For instance, Issa et al. (1990) demonstrated that handled animals (defined as brief separation from the dam) show delays in the appearance of spatial learning deficits and hippocampal cell loss that are normally associated with ageing. It has been suggested (De Kloet & Reul, 1987; Dinan, 1994) that these adult phenomena are attributable to a modified forebrain amine system, whose roots can be found in altered HPA axis functioning. To fully understand the implications of these arguments, it is only prudent to include a brief overview of the role that both the endocrine and immune systems play in HPA axis functioning.

HPA axis links endocrine with immune system and responds to cytokines

Circulating glucocorticoids released from the adrenal glands may affect hippocampal functioning by interacting with glucocorticoid receptors (Reul & de Kloet, 1985), resulting in the inhibition of further CRH release (Jacobson & Sapolsky, 1991; for a comprehensive review see Haas & Schauenstein, 1997). It has been proposed that sustained glucocorticoid release may be critical in provoking age-related behavioural disturbances, by inducing hippocampal cell loss (Sapolsky, 1992; Sapolsky, Krey & McEwen, 1986). Early life-stimulation limits the release of CRH and AVP from the parvocellular portion of the periventricular nucleus (PVN), thus modulating the adult response to stressors, and consequently limiting glucocorticoid stimulation of hippocampal receptors. As a result, the feedback process remains unimpaired, permitting effective release of corticoids in response to further stressor experiences (Meaney et al., 1994). Conversely, protracted stimulation from the dam may increase HPA responses to subsequently encountered stressors, hence promoting excessive hippocampal stimulation and the resultant cell loss.

Like exogenous stressors, systemic and central administration of cytokines promote HPA activation (Sarlis et al., 1992). Administration of IL-1 β stimulates the release of corticotrophin releasing hormone (CRH) and arginine vasopressin (AVP) from PVN neurons of the hypothalamus in much the same way as footshock has such an effect (Tilders, Schmidt & de Goeij, 1993; Watanobe & Takebe, 1993). These peptides stimulate the release of ACTH from the pituitary, which in turn causes glucocorticoid secretion from the adrenal glands (Plotsky, 1991). Interestingly, both IL-1 and early life stress have been shown to increase the coexpression of AVP within PVN CRH neurons terminating in the external zone of the median eminence (Schmidt, Janszen, Wouterlood,

long lasting) consequence of the stimulation (Tilders et al., 1993). Inasmuch as these secretagogues act synergistically to promote ACTH secretion, this coexpression may be responsible for the sustained increases in ACTH and corticosterone reactivity associated with stressful events encountered during early life.

It has been demonstrated that intravenous administration of IL-1 β provokes a dose-dependant increase in *c-fos* expression in CRH neurons of the PVN, supporting the idea that IL-1 β may act at the level of the hypothalamus to elicit the release of ACTH (Ericsson, Kovacs & Sawchenko, 1994). Aside from IL-1 β , other cytokines, including IL-6 and TNF- α , may influence HPA activity (Bernardini, Kamilaris, Calogero, Johnson, Gomez, Gold & Chrousos, 1990; Blalock, 1994; Rothwell & Hopkins, 1995, Schöbitz et al., 1994), although their effects are not as pronounced as those of IL-1 β (Bernardini et al., 1990; Del Rey & Besedovsky, 1992). It remains to be established whether early life events, including endotoxin challenges, permanently influence HPA reactivity owing to the effects of cytokines.

Isolation and maternal separation also activate the HPA axis.

Short and long term separation of pups from their mother result in different behavioural and neuroendocrine effects. As indicated earlier, chronic handling during the first 21 days of life results in blunted ACTH and corticotrophin responses to various stressors that are subsequently encountered (Meaney et al., 1994; Meaney, Diorio, Francis, Widdowson, LaPlante, Caldji, Sharma, Seckl & Plotsky, 1996). This appears to be linked with a more efficient negative feedback cycle due to an increased number of glucocorticoid type II receptors (Meaney et al., 1996; Meaney et al., 1994; Meaney, Aitken, Sharma, Viau, & Sarriau, 1989). It has been suggested that following brief

periods of separation, the dam engages in more frequent bouts of grooming/licking and arched-back nursing of pups, which in turn is responsible for the altered hippocampal receptor sensitivity (Liu et al., 1998).

There is less information available concerning the impact of protracted maternal separation, and the mechanisms responsible for the behavioural effects of such treatments. As already indicated, protracted separation (considered to be 3 hrs or more) has been shown to increase stress reactivity in pups. This could be due to an increase in CRH receptor density in the frontal cortex, amygdala, hypothalamus, hippocampus and/or cerebellum (Nemeroff, Owens, Plott & Levine, 1993). Plotsky & Meaney (1993) demonstrated an exaggeration of the normal stress response in adults that have undergone 3 hours of daily separation during the first 2 weeks of life. Similarly, an LPS injection on postnatal days 3 & 5 results in a heightened HPA response when presented with stressors as an adult (Shanks, Laroque & Meaney, 1988). As will be seen later, it is possible that these results are mediated by cytokines.

Role of cytokines in central processes and stress response

While the present review focuses only on IL-1 β , IL-6 and TNF- α , other cytokines, including IL-2, IL-4, IL-8 and IL-10, may contribute to stressor-induced changes in adult animals. However, the data concerning these latter cytokines are still too sparse to warrant inclusion in this review. It has been suggested that the brain interprets immune activation as if it were a stressor (Anisman, Zalcman & Zacharko, 1993; Dunn, 1990). Several lines of evidence support this notion confirming that IL-1 β , and to a lesser extent TNF- α and IL-6, can engender plasma endocrine and central neurochemical alterations analogous to those seen after stressor exposure (Dunn, 1992,

Lyson & McCann, 1992). Conversely, when animals undergoing immobilisation stress were pre-treated with the endogenous receptor antagonist, IL-1ra, expected hypothalamic norepinephrine alterations and plasma ACTH did not appear (Shintani, Nakake, Kanba, Sato, Yagi, Shiozawa, Aiso, Kato & Asai, 1995). Furthermore, the data support the notion that the modulating effects of cytokines are limited to the period immediately before stressor onset, and once applied, subsequent administration is without effect (Shintani et al., 1995). These data suggest that cytokines play a consistent role in the initiation of the neuroendocrine response to stressors, but may be difficult to control once established.

Cytokines and other blood products are associated with BBB semipermeability

Cytokines mediate a variety of activities in the central nervous system following immune challenge, including inflammation, glial cell activation and subsequent nitric oxide production. The remainder of this document will focus primarily on these properties of cytokines.

Cytokine effects on endothelial cells and BBB permeability

Astrocytes form a tight junction (resistance $\sim 1000 \Omega/\text{cm}^2$) around cerebral blood vessels that restricts access of blood products to the brain. Another defining feature of the BBB is that brain capillary endothelial cells are not capable of transporting molecules through their cell bodies (Rowland, Fink & Rubin, 1991). However, there are several brain areas that lack an effective BBB, such as the posterior pituitary and the circumventricular organs (Dinarello, 1988; Quan et al., 1998). Another critical role of the BBB is that it protects the brain from both direct and secondary effects of

inflammation. Although maintaining the integrity of the BBB is vital in the protection of the CNS, cytokines and/or other blood products may influence its structure and overall continuity. For example, meningitis (Tuomanen, 1993), seizure (de Bock et al., 1996; Yabuuchi et al., 1993), ischemia (Buttini et al., 1994) and endotoxin challenge (Habgood et al., 1992; Shöbitz et al., 1994; Upender et al., 1997) are associated with increased BBB permeability, which results in the passage of large molecules, like cytokines, into the brain.

Using LPS as a case study, i.p. administration of the endotoxin initiates inflammatory cytokines in the periphery. These cytokines appear to be able to pass through areas with thin capillary walls, such as the OVLT (Dinarello, 1988; Quan et al., 1998). This triggers *de novo* synthesis of cytokines, which in turn provokes astroglia to produce cell adhesion molecules, such as ICAM-1 and VCAM-1 (Neumann & Wekerle, 1998). Furthermore, these molecules allow for the binding of T-cells to the inner surface of the BBB endothelium. Enzymes (and cytokines) released from the bound T-cells cause a loosening of the BBB structures, which subsequently signals surrounding astrocytes to secrete more inflammatory cytokines (Springer, 1994; as cited in Neumann & Wekerle, 1998). These cytokines, along with other inflammatory mediators, also function as chemoattractors, drawing additional activated T-cells to the area, thus accelerating the BBB disruption (McGeer & McGeer, 1995). Finally, cytokines, along with the presence of T-cells, activate neighbouring microglia to become antigen presenting cells (APC). The activated (or ramified) microglia are then attracted to degenerating brain areas, which no longer express MHC molecules (essential for the recognition “self” cells) and phagocytosis occurs (Newnam & Wekerle, 1998).

It has been suggested that both IL-1 β and TNF- α likely influence BBB permeability owing to the astrocytic connection with the endothelial cells (Libby, Ordorvas, Auger, Robbins, Birinyi, & Dinarello, 1986). Although contrary evidence has been reported by Saija et al. (1995), who found no such effects following systemic administration of IL-1 β , systemic administration of IL-2 & IL-6 increased leakiness in several brain areas, including the hypothalamus, hippocampus, occipital cortex, frontal cortex and temporoparietal cortex. Supporting Libby's work, Claudio et al. (1994) found that intraperitoneal administration of both IL-1 β and TNF- α resulted in a peak infiltration of polymorphonuclear cells and monocytes between 6 to 24 hours post injection. Last, Wright & Merchant (1994) found that TNF- α injection enhanced IgG passage into the brain, suggesting that the BBB was compromised since IgG's are approximately 150 KDa in size, thus far too large to pass through an intact endothelial barrier.

Glial cell types galvanised after CNS injury.

There are two major subtypes of glial cells: microglia and macroglia (astrocytes, oligodendrocytes and ependymal cells). This presentation will concentrate on astrocytes and microglia and how cytokines, particularly IL-1 β , TNF- α and IL-6, modulate their activities in response to immune challenge.

Astrocytes, which outnumber neurons (10:1), play an essential role in regulating the ionic composition around neurons, as well as in maintaining the integrity of the BBB. The presence of cytokines in CNS tissues during injury disrupts these functions (Banati & Graeber, 1994). For example, there is an excessive, and potentially lethal, influx of calcium through neuronal NMDA channels in mixed cultures prepared with IL-1 β and

TNF- α . Presumably, this stems from reactive astrocytes being incapable of properly balancing extracellular ionic concentrations (Mattson, 1997). In addition, these astrocytes no longer form a tight junction with the endothelium and allow passage of large proteins into brain. While astrocytes are more important for homeostatic functioning, it is the microglia that are critical during injury or disease. Microglia may either be fully phagocytic (activated, or ramified) or non-phagocytic (stellate). Stellate microglia are activated by MHC presenting cells, antigens or by cytokines (Shöbitz et al., 1994). Once activated, the microglia proliferate and are recruited to the site of the lesion, where they display increased expression of several immune surface molecules (MHC class I and II antigens) (Gehrmann, Matsumoto & Kreutzberg, 1995). These markers serve an essential function in that they are involved in the recruitment of phagocytic glial cells to the injured area.

Involvement of cytokines and microglia in central nitric oxide production

There are three waves of gene expression following central insults such as ischemia (Iadecola, 1997). First is the early response of genes such as *c-fos*, *c-jun* and *zif-268*, which trigger activator proteins. The second wave of gene expression involves the production of heat shock proteins (HSP). In ischemic animals, these proteins are expressed in regions where cerebral blood flow has decreased below 50% of normal. It is postulated though that the HSP response occurs only in injured cells that remain viable after the insult. The third wave of gene expression encodes for the inflammatory cytokines, such as IL-1 β , IL-6 and TNF- α (Iadecola, 1997). Once produced, IL-1 β and TNF- α , in particular, may promote NMDA-mediated excitotoxic damage in neurons because of their effects on nitric oxide (NO) production (Rothwell & Strijbos, 1995).

Nitric oxide production is exclusively regulated by the enzyme nitric oxide synthase (NOS), which converts L-arginine to citrulline and NO (Bolaños, Almeida, Stewart, Peuchen, Land, Clark & Heales, 1997; Choi, 1992). The inducible form of NOS (iNOS) can be produced by microglia and astrocytes following stimulation with LPS or cytokines, such as IL-1 β (Rothwell & Hopkins, 1995). In the presence of oxygen, NO is rapidly converted to other reactive intermediates, including nitrite, nitrate and the dangerous oxidant, peroxynitrite (Choi, 1992). While NO modulates some aspects of immune functioning, such as cytokine expression and prostaglandin release, for the most part it signals the death of cells by apoptosis (Bolaños et al., 1997).

It appears that both IL-1 β and TNF- α promote central release of NO (Bonmann, Suschek, Sranger & Kolb-Bachofen, 1997; de Bock et al., 1996; Iadecola, 1997; Lee, Dickson, Liu, & Brosnan, 1993; Rothwell & Relton, 1993; Rothwell & Strijbos, 1995). Thus, it is not surprising that following stimulation with LPS, cultured rat and human cells secrete TNF- α as well as iNOS. In addition, TNF- α and IL-1 β synergistically enhance central NO release (Rothwell & Strijbos, 1995; Vincent & Van Dam, 1997). While there is much evidence to suggest that IL-1 β and TNF- α have adverse effects on neurons, in some instances cytokines may also serve to protect neurons against degeneration (Fagan & Gage, 1990).

Genetic Variations in Responsivity

It has been shown repeatedly that the immune and HPA responses of an animal depend substantially on its genetic makeup (Lu et al., 1998). Likewise, while early environmental experiences affect HPA functioning during adulthood, such effects may also be dependent on genetic factors (Zaharia, Kulczycki, Shanks, Meaney & Anisman,

1996). Furthermore, there is recent evidence that two strains of rats, bred originally for their susceptibility to amygdala kindling (FAST and SLOW kindling development), exhibit profound differences in behavioural tasks reflecting emotionality (Mohapel & McIntyre, in press), and HPA responses to psychogenic versus neurogenic stressors (Anisman et al, 1997). Also, during those same stressor events, the two strains exhibited marked variation in their immune response, particularly macrophage activity. Thus, an examination of such strains could provide additional evidence for genetic variation in an individual's susceptibility to the interaction between a stressful environment, immune reactions and subsequent development of the brain.

Materials and methods

EXPERIMENT #1: EFFECTS OF ISOLATION IN FAST VS. SLOW RATS

Procedure

Experimental Slow-strain rat pups of randomized sex, aged 6 (n=20), 12 (n=14) and 21 (n=6) days were used to determine the impact that isolation has on BBB continuity (see Appendix a). To make this determination, we either (1) isolated several pups of each litter from the dam for 3 hours, or (2) provided no treatment (i.e., littermate pups remained with dam during this time). Isolated pups from each time period were placed on heated pads (to avoid cold-induced stress) with their 'same treatment' littermates. At the end of 3 hours, the isolated animals [aged 6 (n=10), 12 (n=7) and 21 (n=3) days] were returned to their mothers for 24 hours. An equal number of non-isolated, sibling control rats [aged 6 (n=10), 12 (n=7) and 21 (n=3) days] were used.

Fast-strain rat pups, both isolated and sibling controls, aged 6 (n=7), 12 (n=11) & 21 (n=12) days, were treated in the exactly same manner and for the same duration as their Slow cohorts (see Appendix a).

At the end of the 24 hr period, all pups (experimental and controls) were taken from their home cage and overdosed with sodium pentobarbital (approx. 100 mg/kg). In volumes appropriate for their ages, the rats were perfused with 20, 40 or 60 ml of 0.9% saline. Identical volumes of Lana's perfusate (14% picric acid and 20% paraformaldehyde in 100mM PB) were then applied in order to fix protein activity without destroying immune reactivity. Brains were postfixed for 90 minutes in Lana's fixative before being transferred to bottles containing 15% sucrose in PB. The brains remained in the sucrose solution for 3 or more days before sectioning on a cryostat.

Histology

Whole brains were Fast frozen using compressed CO₂, then sliced in at -21 °C in 18 µm sections on a Reichart-Jung Model-2800 cryostat. Brains were carefully mounted on frosted glass slides, then transferred to slide boxes and stored in a -80 °C freezer.

To process tissue for immunoglobulin G, slides were removed from the freezer and allowed to dry in a processing chamber with 80% relative humidity for 15 minutes. All of the following activities were performed in this processing chamber. Slides were first rinsed with 100 mM PBS (15 minutes). After removal of excess PBS, a 1:200 solution of biotinylated sheep anti-rat antiserum (Amersham) in 3% Triton-X (Sigma) in PBS (10mM) was applied to the slides and allowed to react for 1 ½ hours. Subsequently, slides were again rinsed with PBS (15 minutes) before being covered with a 1:200 solution of streptavidin Cy3 conjugate (Sigma) in 3% Triton-X (Sigma) in PBS (10mM) for 45 minutes. After a final rinse in PBS (100 mM), slides were carefully coverslipped using a 40% glycerol in PBS (100mM) solution as an anti-fade binding agent, and were then placed back in the freezer.

Figures 1, 2 and 3 provide coronal sections of the juvenile rat brain, depicting the specific brain regions that were of interest in the present investigation. In particular, Figures 1 & 2 show the nuclei of interest in both the anterior and posterior rat brain respectively. The hippocampal subfields including the dentate gyrus are highlighted in Figure 3.

Figure 1.

Nuclei of interest in the anterior brain

Figure 2.

Nuclei of interest in the posterior brain

EXPERIMENT # 2: EFFECTS OF ISOLATION AND LPS IN SLOW-STRAIN RATS.

Procedure

Slow-strain rat pups aged 6 (n=29), 12 (n=19) & 21 (n=12) days were used to determine the effects that isolation and LPS injection have on BBB continuity (see Appendix b). The conditions that were effected in this study were (1) isolation from the dam plus LPS injection, (2) isolation from the dam and saline injection, and (3) a no treatment control (pups left with the dam during the sibling isolation experience). Animals in the first condition were injected with LPS derived from *E. coli* (026:B6; 1 mg/kg) in 0.9% saline (n=25) and placed with their similarly treated littermates on heated pads for 3 hours before being returned to their dams. Rat pups in the second condition were injected with 1 mg/kg of 0.9% saline and isolated with their saline littermates for 3 hours before being returned to their mothers. The controls were not manipulated. After 24 hours, the pups in all 3 conditions were removed and perfused in a manner identical to the procedure described in experiment 1. In addition, all brains were prepared for IgG assessment in a process identical to that described in experiment 1.

Results

EXPERIMENT #1

SLOW CONTROL ANIMALS

In the majority of 6 day Slow control animals (14 out of 15), IgG positive neurons were found along the length of the cingulate crest (Table 1). About half of these IgG neurons were found bilaterally, and at densities greater than 25 neurons per square micrometer. As often as not, cells in hippocampal subfields, CA1 (n=9) and CA2 (n=8), expressed IgG's, whereas CA3 (n ≈ 13) and dentate gyrus (n=14) neurons were found to be immunopositive in most cases. Neurons staining for IgG were found both unilaterally and bilaterally in the CA1 subfield, most often in concentrations greater than 25 neurons/ μm^2 . Likewise, IgG positive neurons in the CA2 subfield were found both unilaterally and bilaterally in equal numbers, yet densities of 1-25 neurons/ μm^2 were as common as densities of greater than 25 neurons/ μm^2 . When present, IgG positive neurons in the CA3 subfields were always found in concentrations greater than 25 neurons/ μm^2 . Half of the animals expressed IgG positive neurons in the hypothalamus (n=7) and the majority of animals also expressed IgG immunoreactivity in the dorsal thalamus (n=11). Interestingly, dorsal thalamic immunoreactivity was found to be exclusively bilateral, although in varying densities, while immunoreactive neurons in the ventral division were unilateral. While most animals had IgG positive neurons in the olfactory nucleus (n=12), none showed activity in the amygdala. As a point of interest, in the majority of animals olfactory nucleus immunoreactivity was dense and bilateral.

In contrast to the 6 day old animals, the 12 day Slow controls rats showed little IgG activity (Table 2). Only a few animals showed unilateral immunoreactivity along the cingulate crest. Of the limbic structures, only the CA2 subfield of the hippocampus

Table 1

Number of 6 day old control Slow rats (out of 15) in experiment 1 showing 3 different levels of cell numbers expressing IgGs (0, 1-25, >25) in several different brain structures¹.

Brain Structures	Unilateral	Bilateral	0	1 to 25	> 25
Anterior Cingulate Crest	6	8	1	2	12
Posterior Cingulate Crest	5	8	2	4	9
Olfactory Bulb	2	10	3	3	9
Dorsal Thalamus	0	11	4	4	7
Ventral Thalamus	5	2	8	5	2
Hippocampus: CA 1	1	8	6	1	8
Hippocampus: CA 2	4	4	7	5	3
Hippocampus: CA 3a	5	4	6	2	7
Hippocampus: CA 3b	4	9	2	1	12
Hippocampus: CA 3c	3	12	0	1	14
Dentate Gyrus	2	12	1	2	12

¹ When effects were noted in the olfactory bulb, it was highly likely that IgG staining was evident in other regions; however, staining in the hippocampus and cingulate were not correlated with one another. This also applies to Tables 2-18.

Table 2

Number of 12 day old control Slow rats (out of 9) in experiment 1 showing 3 different levels of cell numbers expressing IgGs (0, 1-25, >25) in several different brain structures.

Brain Structures	Unilateral	Bilateral	0	1 to 25	> 25
Anterior Cingulate Crest	2	0	7	1	1
Posterior Cingulate Crest	1	0	8	0	1
Olfactory Bulb	0	0	9	0	0
Dorsal Thalamus	0	0	9	0	0
Ventral Thalamus	0	0	9	0	0
Hippocampus: CA 1	0	0	9	0	0
Hippocampus: CA 2	0	2	7	2	0
Hippocampus: CA 3a	0	0	9	0	0
Hippocampus: CA 3b	0	0	9	0	0
Hippocampus: CA 3c	0	0	9	0	0
Dentate Gyrus	0	7	7	1	1

exhibited any IgG immunoreactivity (n=2). There were no other immunopositive brain structures in the 12 day Slow animals.

The important baseline condition which provides a comparison for later experimental procedures is the 21-day Slow control rats (n=4) which were found to have no IgG positive neurons in any brain structures (Table 3).

FAST CONTROL ANIMALS

Half of the eight 6 day control Fast animals exhibited IgG immunoreactivity along the cingulate crest (Table 4). While anterior cingulate staining did not seem to show overall lateralization, in the posterior cingulate, IgG expression was almost always bilateral and diffuse. Five animals displayed IgG positive neurons in the hippocampal CA1 subfield, while in CA2, 6 of the 8 rats were found to be immunoreactive. On average, as many animals as not showed IgG immunoreactivity in the three divisions of the CA3 subfield. Of these, none had any marked hemispheric preference. Four of the eight animals displayed IgG's in the dentate gyrus, of which two expressed the IgG's unilaterally. The lateralized expression of these two animals was diffuse (between 1 and 25 neurons/ μm^2). Only one of the 8 animals had any IgG expression in the hypothalamus, and this was found to be unilateral and at a concentration of >25 neurons/ μm^2 . Five of the eight rats were found to demonstrate IgG immunoreactivity in the dorsal thalamus. There was an overwhelming, largely bilateral, response in the olfactory nucleus with 7 of the 8 animals showing dense IgG staining (greater than 25 neurons/ μm^2). This contrasted with the amygdala which showed no significant IgG activity.

Table 3

Number of 21 day old control Slow rats (out of 4) in experiment 1 showing 3 different levels of cell numbers expressing IgGs (0, 1-25,>25) in several different brain structures.

Brain Structures	Unilateral	Bilateral	0	1 to 25	> 25
Anterior Cingulate Crest	0	0	4	0	0
Posterior Cingulate Crest	0	0	4	0	0
Olfactory Bulb	0	0	4	0	0
Dorsal Thalamus	0	0	4	0	0
Ventral Thalamus	0	0	4	0	0
Hippocampus: CA 1	0	0	4	0	0
Hippocampus: CA 2	0	0	4	0	0
Hippocampus: CA 3a	0	0	4	0	0
Hippocampus: CA 3b	0	0	4	0	0
Hippocampus: CA 3c	0	0	4	0	0
Dentate Gyrus	0	0	4	0	0

Table 4

Number of 6 day old control Fast rats (out of 8) in experiment 1 showing 3 different levels of cell numbers expressing IgGs (0, 1-25, >25) in several different brain structures.

Brain Structures	Unilateral	Bilateral	0	1 to 25	> 25
Anterior Cingulate Crest	2	1	5	1	2
Posterior Cingulate Crest	1	4	3	4	1
Olfactory Bulb	1	6	1	1	6
Dorsal Thalamus	1	4	3	2	3
Ventral Thalamus	1	0	7	0	1
Hippocampus: CA 1	1	4	3	1	4
Hippocampus: CA 2	1	5	2	4	2
Hippocampus: CA 3a	3	1	4	2	2
Hippocampus: CA 3b	2	0	6	0	2
Hippocampus: CA 3c	3	3	2	2	4
Dentate Gyrus	2	2	4	2	2

Among the 12 day control Fast animals, there was little IgG expression in the anterior cingulate crest (3 of 9), while only 2 of the nine animals had any appreciable expression in the posterior cingulate (Table 5). Immunoreactivity in the cingulate was diffuse as a whole. Immunoglobulin G expression in the CA1 and CA3a layers of the hippocampus, while sparse was found to be present. Although no IgG's were present in the CA2 layer, both the CA3b and CA3c subfields had moderate expression ($\approx 50\%$ of animals expressing IgG's). Likewise, nearly half of the animals expressed IgG's in the dentate gyrus ($n=4$). The ventral and dorsal thalamus had strong, bilateral IgG expression in 2 of the nine animals. Just over half of the nine 12 day Fast rats were found to have IgG positive neurons in the olfactory nucleus (bilaterally expressed in about half of the animals), while none showed expression in the amygdala.

In 21 day Fast control animals, the entire length of the cingulate crest showed no IgG presence (Table 6). As a whole, there were no IgG immunoreactive structures, except in one anomalous animal that showed heavy bilateral IgG staining in the entire hippocampus, the dentate gyrus, the dorsal thalamus and the olfactory nucleus. Additionally, another animal showed weak unilateral staining in CA3b, the dentate and the olfactory nucleus. No animals were found to have IgG's present in the amygdala.

ISOLATED ANIMALS

Relative to controls, separating pups from the dams for 3 hours among the 6 day old animals, irrespective of the strain, resulted in little variability in IgG uptake compared to the control condition (Tables 7 & 8). Contrary to the original hypothesis that IgG uptake would increase as a result of separation from the dam for three hours, among the 6 day old Slow animals, an increase of IgG uptake was restricted to the

Table 5

Number of 12 day old control Fast rats (out of 9) in experiment 1 showing 3 different levels of cell numbers expressing IgGs (0, 1-25, >25) in several different brain structures.

Brain Structures	Unilateral	Bilateral	0	1 to 25	> 25
Anterior Cingulate Crest	2	1	6	1	2
Posterior Cingulate Crest	2	0	7	1	1
Olfactory Bulb	2	3	4	1	4
Dorsal Thalamus	0	2	7	0	2
Ventral Thalamus	0	2	7	0	2
Hippocampus: CA 1	1	0	8	1	0
Hippocampus: CA 2	0	0	9	0	0
Hippocampus: CA 3a	1	0	8	0	1
Hippocampus: CA 3b	3	0	6	2	1
Hippocampus: CA 3c	4	1	4	1	4
Dentate Gyrus	2	2	5	3	1

Table 6

Number of 21 day old control Slow rats (out of 10) in experiment 1 showing 3 different levels of cell numbers expressing IgGs (0, 1-25,>25) in several different brain structures.

Brain Structures	Unilateral	Bilateral	0	1 to 25	> 25
Anterior Cingulate Crest	0	0	10	0	0
Posterior Cingulate Crest	0	0	10	0	0
Olfactory Bulb	1	1	8	2	0
Dorsal Thalamus	0	1	9	0	1
Ventral Thalamus	0	0	10	0	0
Hippocampus: CA 1	0	1	9	0	1
Hippocampus: CA 2	0	1	9	0	1
Hippocampus: CA 3a	0	1	9	0	1
Hippocampus: CA 3b	1	1	8	0	2
Hippocampus: CA 3c	0	1	9	0	1
Dentate Gyrus	1	1	8	1	1

Table 7

Number of 6 day old isolated Slow rats (out of 11) in experiment 1 showing 3 different levels of cell numbers expressing IgGs (0, 1-25,>25) in several different brain structures.

Brain Structures	Unilateral	Bilateral	0	1 to 25	> 25
Anterior Cingulate Crest	4	6	1	1	9
Posterior Cingulate Crest	4	7	0	2	9
Olfactory Bulb	5	5	1	2	8
Dorsal Thalamus	1	10	0	3	8
Ventral Thalamus	3	6	2	5	4
Hippocampus: CA 1	0	8	3	1	7
Hippocampus: CA 2	1	2	8	0	3
Hippocampus: CA 3a	2	5	4	2	5
Hippocampus: CA 3b	2	4	5	0	6
Hippocampus: CA 3c	0	8	3	0	8
Dentate Gyrus	2	7	2	3	6

Table 8

Number of 6 day old isolated Fast rats (out of 7) in experiment 1 showing 3 different levels of cell numbers expressing IgGs (0, 1-25, >25) in several different brain structures.

Brain Structures	Unilateral	Bilateral	0	1 to 25	> 25
Anterior Cingulate Crest	2	2	3	3	1
Posterior Cingulate Crest	1	4	2	3	2
Olfactory Bulb	0	7	0	0	7
Dorsal Thalamus	0	7	0	6	1
Ventral Thalamus	0	0	7	0	0
Hippocampus: CA 1	5	1	1	4	2
Hippocampus: CA 2	2	3	2	5	0
Hippocampus: CA 3a	2	2	3	2	2
Hippocampus: CA 3b	1	0	6	0	1
Hippocampus: CA 3c	2	3	2	2	3
Dentate Gyrus	1	3	3	3	1

hippocampal CA3c subfield [$X^2(1) = 4.62$, $p < 0.01$]. Among the 12 and 21 day old animals (see Tables 9 -12) separation from the dam was without effect with respect to IgG expression.

A COMPARISON BETWEEN FAST AND SLOW STRAINS

Considering that all brains were precisely oriented in a brain-blocking device and were sectioned similarly, qualitative observations of both the Slow and Fast animals indicate that the hippocampus of Slow animals appears to develop more Slowly than that of the Fast animals. Interestingly, by Day 21 the gross anatomy of the two strains were indistinguishable from one another, with the exception of the 3rd and lateral ventricles which were larger in the Fast than in the Slow rats. This would imply a difference between the strains in volume of the hippocampus. Furthermore, it was also observed that while IgG's tended to be found dorsally in cells along the anterior callosum in Slow animals, in Fast animals there was a tendency to find IgG positive neurons anteriorly along the medial and ventral aspects of the callosum.

Consistent with our working hypothesis, it was observed that Slow animals exhibited more IgG's in the brain than Fast animals. For instance, the anterior cingulate crest of 6 day Slow animals had significantly [$X^2(1) = 8.44$, $p < 0.01$] more IgG's than their 6 day Fast counterparts (Tables 1 & 4). The posterior cingulate region of the Fast animals did not show any difference in IgG expression from that of the Slow animals. Likewise, IgG expression in hippocampal CA1, CA2 & CA3a subfields of the Slow animals were no different than those of Fast rats. Yet, the Slow rats expressed more IgG's in hippocampal CA3b [$X^2(1) = 8.75$, $p < 0.01$] and CA3c [$X^2(1) = 4.11$, $p < 0.05$] subfields than Fast animals. Not surprisingly, the dentate of 6 day Slow rats was also found to express

Table 9

Number of 12 day old isolated Slow rats (out of 7) in experiment 1 showing 3 different levels of cell numbers expressing IgGs (0, 1-25,>25) in several different brain structures.

Brain Structures	Unilateral	Bilateral	0	1 to 25	> 25
Anterior Cingulate Crest	1	0	6	0	1
Posterior Cingulate Crest	0	0	7	0	0
Olfactory Bulb	0	0	7	0	0
Dorsal Thalamus	1	0	6	1	0
Ventral Thalamus	0	1	6	0	1
Hippocampus: CA 1	0	0	7	0	0
Hippocampus: CA 2	0	1	6	0	1
Hippocampus: CA 3a	0	1	6	0	1
Hippocampus: CA 3b	1	0	6	0	1
Hippocampus: CA 3c	0	1	6	0	1
Dentate Gyrus	0	1	6	0	1

Table 10

Number of 12 day old isolated Fast rats (out of 11) in experiment 1 showing 3 different levels of cell numbers expressing IgGs (0, 1-25,>25) in several different brain structures.

Brain Structures	Unilateral	Bilateral	0	1 to 25	> 25
Anterior Cingulate Crest	3	2	6	2	3
Posterior Cingulate Crest	0	1	10	0	1
Olfactory Bulb	3	1	7	2	2
Dorsal Thalamus	0	0	11	0	0
Ventral Thalamus	3	2	6	4	1
Hippocampus: CA 1	1	0	10	1	0
Hippocampus: CA 2	2	0	9	2	0
Hippocampus: CA 3a	2	0	9	2	0
Hippocampus: CA 3b	3	0	8	2	1
Hippocampus: CA 3c	5	0	6	5	0
Dentate Gyrus	3	0	8	3	0

Table 11

Number of 21 day old isolated Slow rats (out of 3) in experiment 1 showing 3 different levels of cell numbers expressing IgGs (0, 1-25,>25) in several different brain structures.

Brain Structures	Unilateral	Bilateral	0	1 to 25	> 25
Anterior Cingulate Crest	0	0	3	0	0
Posterior Cingulate Crest	0	0	3	0	0
Olfactory Bulb	0	0	3	0	0
Dorsal Thalamus	0	0	3	0	0
Ventral Thalamus	0	0	3	0	0
Hippocampus: CA 1	0	0	3	0	0
Hippocampus: CA 2	0	0	3	0	0
Hippocampus: CA 3a	0	0	3	0	0
Hippocampus: CA 3b	0	0	3	0	0
Hippocampus: CA 3c	0	0	3	0	0
Dentate Gyrus	0	0	3	0	0

Table 12

Number of 21 day old isolated Fast rats (out of 11) in experiment 1 showing 3 different levels of cell numbers expressing IgGs (0, 1-25,>25) in several different brain structures.

Brain Structures	Unilateral	Bilateral	0	1 to 25	> 25
Anterior Cingulate Crest	0	0	11	0	0
Posterior Cingulate Crest	1	0	10	0	1
Olfactory Bulb	0	0	11	0	0
Dorsal Thalamus	0	0	11	0	0
Ventral Thalamus	0	0	11	0	0
Hippocampus: CA 1	0	0	11	0	0
Hippocampus: CA 2	0	0	11	0	0
Hippocampus: CA 3a	0	0	11	0	0
Hippocampus: CA 3b	0	2	9	2	0
Hippocampus: CA 3c	0	0	11	0	0
Dentate Gyrus	0	0	11	0	0

more IgG's than the Fast counterpart [$X^2(1)=5.78$, $p<0.05$]. Both Fast and Slow animals exhibited similar IgG expression in the ventral and dorsal thalamus, as well as the olfactory nucleus and amygdala.

Immunoglobulin G expression in the cingulate crest of 12 day Slow animals was found to be no different from that of the Fast animals (Tables 2 & 5). All hippocampal structures, including the dentate gyrus, were similar in IgG expression in the strains, with the exception of CA3c [$X^2(1)=6.92$, $p<0.01$] where Slow animals exhibited no IgG's. The Slow strain exhibited less olfactory IgG expression than the Fast animals [$X^2(1)=6.92$, $p<0.01$]. The strains were not found to differ from one another with respect to thalamic IgG expression.

Finally, in contrast to the differences seen in the youngest animals, no appreciable immunoglobulin differences were found between the strains in any brain structures in 21 day old animals (Tables 3 & 6).

EXPERIMENT #2

SLOW SALINE VS. CONTROL

As a whole, it was expected that saline injection might be sufficiently stressful to promote upregulation of IgG uptake into neurons. However, sizeable differences of IgG expression between the 6 day saline and control conditions were only evident in a few brain regions (Tables 1 & 13), namely the posterior cingulate [$X^2(1)=8.36$, $p<0.01$], the dentate gyrus [$X^2(1)=4.17$, $p<0.05$] and the hypothalamus [$X^2(1)=6.48$, $p<0.05$].

Among 12 day old saline injected animals, limited variations of IgG expression were observed relative to controls (Tables 2 & 14). The hippocampal CA3a subfield was found to have more IgG's than control brains [$X^2(1)=6.92$, $p<0.01$]. Surprisingly,

Table 13

Number of 6 day old saline injected Slow rats (out of 10) in experiment 2 showing 3 different levels of cell numbers expressing IgGs (0, 1-25,>25) in several different brain structures.

Brain Structures	Unilateral	Bilateral	0	Jan-98	> 25
Anterior Cingulate Crest	1	6	3	3	4
Posterior Cingulate Crest	3	0	7	1	2
Olfactory Bulb	1	9	0	0	10
Dorsal Thalamus	0	4	6	2	2
Ventral Thalamus	0	0	10	0	0
Hippocampus: CA 1	1	6	3	2	5
Hippocampus: CA 2	3	4	3	6	1
Hippocampus: CA 3a	3	0	7	3	0
Hippocampus: CA 3b	3	3	4	1	5
Hippocampus: CA 3c	6	3	1	3	6
Dentate Gyrus	3	3	4	3	3

Table 14

Number of 12 day old saline injected Slow rats (out of 9) in experiment 2 showing 3 different levels of cell numbers expressing IgGs (0, 1-25, >25) in several different brain structures.

Brain Structures	Unilateral	Bilateral	0	1 to 25	> 25
Anterior Cingulate Crest	1	0	8	1	0
Posterior Cingulate Crest	0	0	9	0	0
Olfactory Bulb	0	1	8	0	1
Dorsal Thalamus	0	0	9	0	0
Ventral Thalamus	0	0	9	0	0
Hippocampus: CA 1	0	1	8	0	1
Hippocampus: CA 2	1	2	6	3	0
Hippocampus: CA 3a	2	3	4	4	1
Hippocampus: CA 3b	1	1	7	0	2
Hippocampus: CA 3c	1	0	8	0	1
Dentate Gyrus	0	0	9	0	0

within the dentate gyrus fewer than expected IgG's were observed [$X^2(1)=6.46$, $p<0.05$].

Finally, although few brains of 21 day old animals were assessed, it was clear that little IgG expression was evident in these brains, and thus differences between the strains were absent at this age (Tables 3 & 15).

SLOW SALINE VS. LPS

Qualitative observations made during the course of the study indicated that the 6 day old pups were less affected by the LPS treatment than the older animals. For instance, the younger animals tended to exhibit fewer signs of distress and maintained their body colour. In effect, the sickness behaviour common in adult LPS treated animals was modest in the 12 and 21 day old animals, and was largely absent in the 6 day olds. Thus it was expected that perhaps any central effects of LPS might likewise be tempered in the youngest age group.

Supporting the supposition that 6 day animals may have mechanisms in place to deal with immunological challenges, six day old animals injected with LPS were only found to have more IgG's than salines in the posterior cingulate [$X^2(1)=7.08$, $p<0.01$] as well as the ventral [$X^2(1)=6.88$, $p<0.01$] and dorsal thalamus [$X^2(1)=4.96$, $p<0.05$] (Tables 13 & 16). It had been expected that pronounced effects of LPS would be evident at hypothalamic sites given the restricted brain permeability present at the OVLT. Surprisingly, there was little neuronal staining in the hypothalamic region. That said, there appeared to be a non-artifactual lightening of the ventral brainstem which could be indicative of immunoglobulin presence in the extracellular medium.

Table 15

Number of 21 day old saline injected Slow rats (out of 4) in experiment 2 showing 3 different levels of cell numbers expressing IgGs (0, 1-25, >25) in several different brain structures.

Brain Structures	Unilateral	Bilateral	0	1 to 25	> 25
Anterior Cingulate Crest	0	0	4	0	0
Posterior Cingulate Crest	0	0	4	0	0
Olfactory Bulb	0	0	4	0	0
Dorsal Thalamus	0	1	3	0	1
Ventral Thalamus	0	0	4	0	0
Hippocampus: CA 1	1	0	3	1	0
Hippocampus: CA 2	0	0	4	0	0
Hippocampus: CA 3a	1	0	3	1	0
Hippocampus: CA 3b	0	0	4	0	0
Hippocampus: CA 3c	0	0	4	0	0
Dentate Gyrus	1	0	3	1	0

Table 16

Number of 6 day old LPS injected Slow rats (out of 13) in experiment 2 showing 3 different levels of cell numbers expressing IgGs (0, 1-25,>25) in several different brain structures.

Brain Structures	Unilateral	Bilateral	0	1 to 25	> 25
Anterior Cingulate Crest	1	7	5	3	5
Posterior Cingulate Crest	0	11	2	7	4
Olfactory Bulb	0	11	2	0	11
Dorsal Thalamus	0	11	2	2	9
Ventral Thalamus	1	5	6	2	4
Hippocampus: CA 1	0	11	2	1	10
Hippocampus: CA 2	4	8	1	10	2
Hippocampus: CA 3a	2	5	6	5	2
Hippocampus: CA 3b	5	7	1	4	8
Hippocampus: CA 3c	1	8	4	0	9
Dentate Gyrus	1	10	2	2	9

Twelve day old LPS injected animals provide strong support for the hypothesis that IgG presence increases after endotoxin injection. With the exception of CA2, CA3b and the hypothalamus, all CNS structures of the LPS animals examined in the present investigation exhibited significant increases of IgG expression (Tables 14 & 17). Specifically, the anterior [$X^2(1)=8.10$, $p<0.01$] and posterior [$X^2(1)=14.4$, $p<0.001$] cingulate crest of LPS injected animals were both found to have significantly more IgG's than their saline counterparts (Figure 4). Likewise, the CA1 [$X^2(1)=10.89$, $p<0.001$], CA3a [$X^2(1)=5.14$, $p<0.05$] and CA3c [$X^2(1)=10.89$, $p<0.001$] subfields of the hippocampus (including the dentate [$X^2(1)=14.40$, $p<0.001$]) of LPS injected rats expressed a strong, primarily bilateral IgG response beyond that observed in the saline controls (Figure 5). Last, IgG expression in the dorsal thalamus [$X^2(1)=18.00$, $p<0.001$] and olfactory nucleus [$X^2(1)=8.10$, $p<0.01$] of LPS injected animals was found to be significantly greater than that of the saline controls.

The brains of 21 day LPS injected animals also demonstrated significant increases in IgG expression over saline controls, with the exception of hippocampal CA2, CA3a & CA3b subfields, and the hypothalamus Tables (15 & 18). The brain areas of LPS animals that did show increases in IgG expression beyond that of saline animals included both the anterior [$X^2(1)=9.00$, $p<0.01$] and posterior cingulate crest [$X^2(1)=9.00$, $p<0.01$], the hippocampal subfields CA1 [$X^2(1)=5.63$, $p<0.05$], CA3c [$X^2(1)=5.76$, $p<0.05$] & dentate gyrus [$X^2(1)=5.63$, $p<0.05$], the dorsal thalamus [$X^2(1)=5.63$, $p<0.05$] and last, the olfactory nucleus [$X^2(1)=9.00$, $p<0.01$].

Figure 4.

Photomicrograph of IgG immunoreactive cells in the cingulate crest of an LPS injected

Slow rat.

Figure 5.

Photomicrograph of IgG immunoreactive cells in the dorsal hippocampus of an LPS injected Slow rat.

Table 17

Number of 12 day old LPS injected Slow rats (out of 9) in experiment 2 showing 3 different levels of cell numbers expressing IgGs (0, 1-25,>25) in several different brain structures.

Brain Structures	Unilateral	Bilateral	0	1 to 25	> 25
Anterior Cingulate Crest	1	6	2	4	3
Posterior Cingulate Crest	1	7	1	2	6
Olfactory Bulb	0	7	2	0	7
Dorsal Thalamus	0	9	0	2	7
Ventral Thalamus	0	3	6	3	0
Hippocampus: CA 1	1	7	1	2	6
Hippocampus: CA 2	1	6	2	4	3
Hippocampus: CA 3a	1	8	0	7	2
Hippocampus: CA 3b	2	4	3	2	4
Hippocampus: CA 3c	1	7	1	2	6
Dentate Gyrus	1	7	1	1	7

Table 18

Number of 21 day old LPS injected Slow rats (out of 5) in experiment 2 showing 3 different levels of cell numbers expressing IgGs (0, 1-25,>25) in several different brain structures.

Brain Structures	Unilateral	Bilateral	0	1 to 25	> 25
Anterior Cingulate Crest	0	5	0	0	5
Posterior Cingulate Crest	1	4	0	0	5
Olfactory Bulb	0	5	0	1	4
Dorsal Thalamus	0	5	0	0	5
Ventral Thalamus	0	3	2	0	3
Hippocampus: CA 1	1	4	0	2	3
Hippocampus: CA 2	0	3	2	3	0
Hippocampus: CA 3a	0	4	1	4	0
Hippocampus: CA 3b	1	2	2	3	0
Hippocampus: CA 3c	2	2	1	2	2
Dentate Gyrus	0	5	0	1	4

Discussion

IgG expression associated with maternal separation

In a recently published report, Upender et al. (1997) indicated that neuronal incorporation of IgG's was an integral part of the developing nervous system. They found that up to and including embryonic day 13 (E13), IgG was incorporated into subplate neurons. The source of these IgG's was assumed to be maternal in that this is the only class of antibody capable of crossing the placenta. While it is possible that neurons are synthesising this protein, it is far more likely that at E13 the as yet incomplete capillary BBB allows for simple uptake and retrograde transport to the cell body. These IgG positive neurons were found to extend pioneering corticofugal axons which provided scaffolding by which neuroblasts were guided to their appropriate layers. Immunolabelling indicated that these IgG positive populations of neurons were located at the anterior olfactory nucleus, the deep layers of the anterior cortex and the hippocampus. Furthermore, between postnatal days 6 and 15 these immunopositive neurons selectively underwent a wave of programmed cell death and were thus sparsely represented at postnatal day 15, and largely absent at postnatal day 21. As might be expected, IgG expression in our Slow control condition in the present investigation mirrored the Upender et al. (1997) findings. Indeed, the majority of control animals in the present study displayed fairly dense bilateral IgG representation in the olfactory bulb, hippocampus and cingulate at Day 6, while at Day 12 there were significantly fewer of the antibodies present. This trend continued to postnatal Day 21 where none of the animals expressed IgG's in any brain region other than the choroid plexus and endothelial cells. Upender et al. (1997) suggested the declining expression of IgG

positive neurons is a natural process wherein targeted neurons are phagocytized by microglia over a period of days. It is interesting that IgG's play a role in natural ontogenetic cell death, as will be discussed later, this apoptotic process may also be initiated by endotoxic insult.

It will be recalled that the HPA axis as a whole is a stress sensitive pathway. In the same fashion, Papez's circuit is thought to be fundamentally involved in subserving emotional responses. Although later works include the amygdala, Papez's original circuit comprises the hippocampus, the thalamus, and the cingulate (Papez, 1937; as cited in Henke, Ray & Sullivan, 1991). It will be recognised that Papez's circuit and the HPA circuit share many common nuclei which, in the present experiment, were examined for IgG accumulation under both control conditions and following acute separation from the dam. In the present investigation, the Slow and Fast strains that were subjected to these treatments were previously found to be differentially sensitive to stressful events, at least with respect to HPA activation (Anisman et al., 1997). As well, these investigators found that stressors more profoundly influenced macrophage activity in Slow animals. It remains to be established whether B cells are present in greater number in Slow rats, thus potentially giving rise to greater circulating IgG concentrations.

Contrary to prediction, 6 day old isolated Slow animals were found not to display increases of IgG expression over that of control animals, with the exception of CA3c. At both Day 12 and at Day 21 there were no differences in IgG expression at all between the strains. Likewise, Fast rats separated from the dam were not found to be significantly different at any age group from their control populations. Knowing that the stress and immune systems utilize several common brain pathways, and that a 3

hour acute isolation experience may be sufficiently powerful to permanently promote changes in stressor reactivity during adulthood, it was expected that IgG's could play a role in the proposed mechanism for the change. It is clear, however, that a single period of isolation was not sufficient to induce such an outcome. It should be underscored, however, that in studies where maternal separation was found to influence CNS processes, these procedures were instituted for several successful days (Meany et al., 1996). Accordingly, it is premature to conclude from the present findings, where only a single separation session was employed, that repeated separation would likewise be ineffective in modifying BBB permeability or central IgG expression.

In contrast to the absence of marked effects attributable to maternal separation, ontogenetic differences between the Fast and Slow strains of rats were evident under control conditions. Aside from the visually apparent difference in ventricle size, quantitative differences were evident with respect to IgG expression in the anterior cingulate crest, CA3b, CA3c and the dentate of 6 day Slow control animals relative to their Fast counterparts. By Day 12 only CA3c and the olfactory bulb were found to differ in IgG immunolabeling, and not surprisingly, at Day 21 there were no differences in IgG representation between the strains. It has been suggested that psychosocial events, particularly those associated with dam-pup interactions, may profoundly influence the response to stressors encountered in adulthood. Among other things, such events influence corticoid cells within the hippocampus, CRH and AVP expression within the hypothalamus, and thus circulating ACTH and corticosterone in response to stressors (Meany et al., 1996; Meany et al., 1986). As already indicated, a single brief period of maternal separation was not sufficient to alter BBB permeability. However, it appeared that at the youngest age tested, IgG expression differed in the Slow and Fast

strains, with the more emotional Slow rats (Mohapel and McIntyre, in press) exhibiting higher IgG levels which may reflect greater accessibility to brain. As the hippocampus plays a fundamental role in the modulation of stress responses (Sapolsky, Krey & McEwen, 1986) it could be postulated that differential IgG expression in this regulatory region among juveniles might somehow serve to alter neuronal functioning, and thus proactively influence adult responses to stressors.

Influence of a bacterial endotoxin on IgG expression.

It has been demonstrated repeatedly that even mild environmental stimuli, including intraperitoneal injection, may have marked neurochemical effects in adult rats (Merali et al., 1998). In fact, it was observed that separated 6 day old rats injected with saline showed increased IgG expression in the posterior cingulate, the dentate gyrus and the hypothalamus. By Day 12 the only brain region in saline injected animals found to have more IgG's than in controls was the CA3a hippocampal subfield. Curiously, the dentate gyrus of the saline animals was found to express fewer IgG's than in control animals. It is unclear why such a paradoxical outcome was obtained, nor is it known whether this was a spurious finding. On the one hand, fully half of the control animals exhibited bilateral dentate gyrus IgG expression, suggesting that this effect was a relatively robust one. On the other hand, it was found that in 12 day old rats, IgG expression in the dentate gyrus did not differ between saline and control animals.

It had been assumed that transient vascular leakage, as reflected by IgG uptake, would be evident in the young, already compromised brain, but would be absent in a somewhat more mature brain. Indeed, it was evident that the effects of saline injection on IgG expression became progressively less pronounced with increasing age. In fact, it

is known that at Day 6 rats have a naturally leaky BBB (Upender et al., 1997) and conceivably, at this age can be rendered still more permeable. With maturation of the vessels, they are less likely to be perturbed by such a 'trivial' stressor.

As discussed earlier, it has been reported that neonatal administration of an endotoxin (on Days 2 and 10) proactively influenced HPA functioning, such that exposure to a stressor during adulthood greatly enhanced plasma corticosterone levels relative to rats that had not received the early life challenge (Shanks et al., 1995). These findings were reminiscent of the effects associated with repeated separation from the dam during the early postnatal period (Meaney et al., 1994). In the present investigation, animals received only a single injection of the endotoxin and only the immediate IgG changes were examined, as opposed to any long term proactive effects. While it was found that LPS induced marked variations of IgG expression in several brain regions, these were most notable in 12 and 21 day old animals, rather than the more immature (6 day old) rats. In contrast to the effects of saline injection, which were marked primarily in 6 day old pups, variations of IgG expression between the saline and LPS conditions were evident in the Day 12 and 21 animals, but were limited at Day 6. At this age, animals injected with LPS displayed more IgG's than saline treated rats only in the posterior cingulate crest and the ventral and dorsal thalamus. However, at Day 12 LPS injected animals show increased IgG expression over the saline controls in the anterior and posterior cingulate crest, CA1, CA3a, CA3c, the dentate gyrus, the dorsal thalamus and the olfactory nucleus. Likewise, both the anterior & posterior cingulate crest, the hippocampal subfields CA1 & CA3c, the dentate gyrus, the dorsal thalamus and the olfactory nucleus of 21 day old rats treated with LPS were found to have significantly more IgG's than saline treated animals.

It is premature to conclude that the progressively greater BBB disruption associated with ageing reflects characteristics of the developing brain. As indicated earlier, the behavioural signs of sickness were far more pronounced in 12 and 21 day old animals than in the 6 day old pups. Thus, it is possible that either a greater immunological response to LPS, or a greater sickness response given an equivalent immunological response occurs in the two older groups, thus leading to greater IgG expression. Obviously, firm conclusions require not only complete dose response curves, but also equating different aged animals with respect to immune changes and sickness responses, and then evaluating BBB permeability. Nevertheless, the relative similarities of IgG expression in Day 6 saline and LPS injected animals is commensurate with the suggestion of Alheim et al. (1997), that near this age rat pups may have mechanisms in place to deal with immunological challenge, likely in the form of antibodies passed from the dam to the pup in her milk. Alternatively, it is known that young rats (4-14 days of age) undergo a stress insensitive period, wherein stressors do not lead to elevated plasma corticosterone levels (Walker, 1995). It is likewise possible that endotoxin challenge in young pups has less of a cortisol effect than in the 12 and 21 day old rats, hence leading to the differential IgG expression. Of course, this does not exclude the possibility that LPS induces different cytokine (e.g. IL-1, TNF- α) effects as a function of age, which in turn differentially impact on prostaglandin E₂ and hence BBB permeability.

The present findings should not be taken as being inconsistent with the previous report that early life endotoxin administration had long term effects on HPA reactivity in response to stressors (Shanks et al., 1995). The endotoxin used in these studies were different, and more importantly, the salmonella-derived LPS was delivered on postnatal

days 2 and 10 in the Shanks et al. (1995) study. It is certainly possible that a challenge on postnatal day 2 may have resulted in vastly different effects from those associated with just a Day 6 challenge. That is, if HPA functioning somehow is involved in mediating the effects of early life trauma, then it would follow that LPS administration before or after the 'stress insensitive' period would have effects distinguishable from those seen at other times.

That is not to say that it is implausible that the differences in stressor effects at the different age groups might not be modulated by pup-dam interactions. It is known that upon reunion from a period of separation, the dam dramatically alters her behaviour towards her pup and engages in more grooming and arched-back nursing (Liu et al., 1998). Hence, a 6 day old pup that demonstrates sickness behaviour due to LPS injection may evoke a different response from the dam than its other siblings. This in turn could lead to changes in adult behaviours. Nevertheless, this scenario appears unlikely in the present investigation in that the rats at all age groups did not exhibit obvious sickness behaviours that would engender special treatment from the dam.

Given the possible causes for long term change, it is not surprising then that the greatest IgG activity in the LPS injected animals fell along the nuclei forming the HPA axis. Immunoglobulin G in these brain areas may have been upregulated due to the susceptibility to BBB disruption during the stress response. In effect, the possibility is being offered that the altered BBB permeability may be secondary to the stress associated with endotoxin challenge. In fact, the increased IgG levels were not restricted to hypothalamic sites, but were also present in other stress sensitive nuclei comprising Papez's circuit. If one excludes the overlap between the HPA axis and Papez's circuit, the cingulate crest and olfactory nucleus are the only non-HPA axis

structures that showed significant increases of IgGs relative to saline treated animals. Thus, it is possible that there happens to be a particular vulnerability to endothelial disruption in these structures and that the IgG accumulation happens to fall along Papez's established circuit.

Two major questions stem from the present investigation. Specifically, what role do immunoglobulins ordinarily play in the brain, and further, what is the significance of their presence in the brain of endotoxically stressed animals? It is, of course, well established that IgG acts an antibody, attracted to antigens including bacteria, viruses and toxins circulating in the blood and lymph fluids. This class of protein readily crosses the walls of blood vessels and activates monocytes upon contact. From a developmental standpoint the function of IgG's in the brain can be deduced by observing what kind of effect it's presence has on the surrounding immunological machinery. Along these lines, Ferrer et al. (1992) found that macrophages and microglia were seen to be concentrated in the subplate and ventricular zone following birth. It will be recalled that these areas contain high concentrations of IgG's within the neurons and are known to undergo heavy cell loss during the first few postnatal weeks in rats (Upende et al., 1997). It was further demonstrated (Upende, Leckman & Neigle, 1996) that macrophages and microglia are often found adjacent to IgG-containing neurons in the cerebral cortex during cell death. Thus, this might infer that after some triggering event, presumably birth, the presence of IgG's in neurons somehow signal local monocytes that the host cell is now considered to be non-self, thus subject to phagocytosis. Accordingly, LPS injection, and subsequent uptake of IgG's into neurons, triggers the cell to display these same non-self features which then attract local monocytes. In turn, these monocytes dispose of the cell and alter the anatomical landscape, perhaps permanently. This then,

could be one of the mechanisms responsible for protracted change in the endotoxically challenged juvenile.

Conclusion

It was found that IgG's were present in many brain areas in Slow control animals at 6 & 12, but not 21 days of age. Acute isolation in Slow and Fast animals was not found to engender changes in BBB permeability as measured by IgG accumulation in neurons. Nevertheless, patterns of expression show that as a whole Day 6 & 12 Slow rats had a more IgG's than their Fast counterparts. By 21 days of age antibody expression in pups from the two strains appeared to be uniformly absent.

Since isolation itself did not cause differences in IgG expression, it would appear that the IgGs alone are not responsible for known long term changes associated with acute separation. Conversely, differences in IgG expression between the strains might reflect some of the distinct behavioural differences observed elsewhere in the adults.

In experiment 2, saline injections did not promote significant changes in the normal pattern of IgG expression in Slow rats from all 3 age groups. Conversely, exposure to LPS caused large increases in IgG expression at days 12 and 21, but not day 6, compared to control values, suggesting that the younger rats are somehow 'resistant' to the effects of the endotoxin. Thus, although the acute stress of isolation alone did not alter IgG expression, perhaps in combination with an endotoxic challenge, it might have contributed to the dramatic age-specific increase in expression that was observed.

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APPENDICES

Appendix (a)

Summary of Animal numbers in experiment #1: Effects of isolation in Fast vs. Slow
rat pups

Slow-Strain Rats			Fast-Strain Rats		
Ages (days)	Isolated (# of pups)	Control (# of pups)	Ages (days)	Isolated (# of pups)	Control (# of pups)
6	11	10	6	7	8
12	7	7	12	11	9
21	3	4	21	11	10

Appendix (b)

Summary of animal numbers in experiment #2: Effects of isolation and LPS in Slow-
strain animals

Slow-Strain animals			
Ages (days)	Control (# of pups)	Saline (# of pups)	LPS (# of pups)
6	10	10	13
12	7	9	9
21	4	4	5

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