Intranasal VEGF-A and VEGF-E in a modified Levine model of stroke

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Abstract

VEGF (Vascular endothelial growth factor)-A has been shown to successfully enter the brain via the intranasal pathway and improve symptoms following focal ischemia in rats. However VEGF-A promotes inflammation and vascular permeability which are believed to contribute to the damage following stroke. VEGF-E has been shown to produce reduced inflammation and vascular permeability while still stimulating similar levels of angiogenesis. We aimed to compare intranasal VEGF-A and VEGF-E doses in a modified Levine model of stroke. VEGF was delivered on days 3, 4 & 5 following hypoxic ischemia. Infarct was measured on day 14 using cresyl violet stain. Behavioural assessments were performed before hypoxic ischemia and on day 1, 7 and 14. We found that VEGF-A and VEGF-E did not affect behavioural scores or infarct volume. However, VEGF-E (20μg) reduced weight loss at day 14 after stroke. These findings suggest that intranasal VEGF-A and VEGF–E are not effective in the modified Levine model used in this study.

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Table of Contents

List of Abbreviations

BDNF: brain derived neurotrophic factor

CCAO: common carotid artery occlusion

Flk: fetal liver kinase

Flt: fms-like tyrosine kinase

KDR: kinase insert domain-containing receptor

MCAO: middle cerebral artery occlusion

NMDA: *N*-Methyl-D-aspartate

OCT: Optimal cutting temperature

PD: postnatal day

r-tPA: recombinant tissue-plasminogen activator

SGZ: subgranular zone

SVZ: subventricular zone

VEGF: vascular endothelial growth factor

VEGFR: Vascular endothelial growth factor receptor

WHO: World Health Organisation

Introduction

1.1. Stroke Epidemiology

The World Health Organisation (WHO) defines stroke as "rapidly developed clinical signs of focal or global disturbance of cerebral function, lasting more than 24 hours or until death, with no apparent non-vascular cause" (WHO MONICA Project Principal Investigators, 1988). Stroke is currently the third highest cause of death in New Zealand (after heart disease and cancer), and a leading cause of disability (Tobias, 2001; Wellington: Ministry of Health, 2009). This is consistent with stroke statistics in other countries (Murray & Lopez, 1997). An Auckland study found the incidence of stroke in New Zealand to be 833 in 100,000 for people over 15 (Bonita et al., 1997). Age plays a significant factor in stroke, with 70% of strokes occurring in people over 65 and only 5% occurring before 45 (Wellington: Ministry of Health, 2002). As New Zealand is currently an aging population (Anderson & Hussey, 2000), this is of concern as the incidence of stroke is predicted to increase. Overall the rates of stroke mortality have been declining globally (Bonita, Stewart, & Beaglehole, 1990). However the increase in the number of stroke survivors is likely to further increase the prevalence of stroke and its burden (Kuller, 2000).

While stroke represents the third highest cause of death, approximately 75% of people who have a stroke survive. Of these, approximately 30% will not make a full recovery and 20% are left with severe neurological disabilities to the point that assistance is required to perform daily activities (Bonita et al., 1997). These disabilities most commonly involve motor impairment but also include changes in vision, mood, sensory function, language and cognition (Kelly-Hayes et al., 1998). In 1996 there were approximately 7500 people in New

Zealand severely impaired due to stroke. This was estimated to account for 11% of the country's severely disabled population (Wellington: Ministry of Health, 2002).

1.2. Stroke Pathophysiology

The most common form of stroke is ischemic and is caused primarily by cardioembolism, small-artery occlusion or large artery atherosclerosis (Kolominsky-Rabas et al., 2001). This results in an impaired blood supply to a section of the brain, which in turn lead to hypoxia in the core region. Hypoxic cells cannot maintain their membrane potential, leading to an excitotoxic cascade; cells depolarise and release of glutamate leading to over activation of the NMDA (*N*-Methyl-D-aspartate) receptor and a subsequent influx of Ca^{2+} into cells. This facilitates multiple cell death mechanisms (Lo, 2008). Ischemic stroke accounts for approximately 80% of all strokes, with haemorrhagic accounting for the remainder (Kolominsky-Rabas et al., 2001). Haemorrhagic stroke results from the rupturing of a blood vessel in the brain. This is typically due to an aneurysm or a weakened blood vessel. Blood pooling from the haemorrhage leads to an increasing pressure in the brain, which in turn compresses surrounding blood vessels, and can lead to an excitotoxic cascade similar to that seen in ischemic stroke (Hademenos & Massoud, 1997).

In a stroke, the greatest proportion of hypoxic cells dies within minutes due to the aforementioned mechanisms of excitotoxicity. This region is referred to as the ischemic core and today is considered largely unsalvageable. However Astrup et al. (1977) found that, in the area surrounding the ischemic core, cells are unable to produce action potentials. However by returning blood supply to these areas, the cells regained electrical function. They referred to this region as the "penumbra". Astrup et al. (1977) suggested that cells in this region may be in a lethargic state, having not experienced complete electrical failure.

However without intervention the infarcted tissue expands into the penumbra in the hours and days following stroke (Furlan et al., 1996; Donnan & Davis, 2002). The penumbra is the primary target of modern stroke research.

1.3. Stroke Treatments

In 1995, a clinical trial demonstrated the efficacy of the thrombolytic agent recombinant tissue-plasminogen activator (rt-PA) in ischemic stroke if given within 3 hours of stroke. rt-PA is contraindicated in haemorrhagic stroke, as it increases the risk of intracerebral haemorrhage (NINDS rt-PA Stroke Study Group, 1995). In 1996 rt-PA was approved for use in the treatment of stroke by the United States Food & Drug Administration (FDA) and later for use in New Zealand (NINDS rt-PA Stroke Study Group, 1997; Fink, 2005). However to this day rt-PA remains the only FDA approved pharmacological intervention for acute stroke. Despite being the only approved pharmacological intervention, rt-PA is delivered to only an estimated 3% of ischemic strokes in New Zealand (Child et al., 2011) and 1.1% in the United States (Samson, 2007). This is thought to be primarily due to an inability of patients to reach hospital and receive neuroimaging (to exclude haemorrhagic stroke) within the narrow therapeutic window that rt-PA provides (Kwan et al., 2004). This suggests that treatments which can occur in a wider timeframe and/or are not contraindicated with haemorrhagic stroke may be more viable in the current clinical setting.

While rt-PA has been shown to be effective in human stroke, treatments to directly reduce cell death via neuroprotection have not. Various studies have shown that inhibiting processes that lead to cell death (such as NMDA receptor overactivation or production of matrix metalloproteinases; MMP) are effective at reducing infarct size and improving

functional recovery in animal models of stroke. However none of these have proven effective in human clinical trials. This has led to the suggestion that many of these processes that lead to cell death in the early stages of stroke may be important for triggering endogenous repair mechanisms (Lo, 2008). Indeed while NMDA overactivation is known to be cytotoxic, evidence supports that it promotes neurogenesis following focal ischemia (Arvidsson et al., 2001), and is also linked to neuroprotection and neuroplasticity (Young et al., 1999). Similarly at early stages of stroke injury MMP induces oedema, blood brain barrier (BBB) damage, haemorrhage and neuronal death (Cunningham et al., 2005). However in the delayed phases MMP appears to promote angiogenesis as inhibiting MMP at 7 days after stroke reduces endogenous VEGF-A production, inhibits angiogenesis and leads to increased infarct size (Zhao et al., 2006).

Lo (2008) suggested that this conversion of injury processes to repair processes may underpin the failing of neuroprotective drugs to translate into viable therapeutics for stroke. Potentially there may be a very limited window for these treatments to be effective before they start to interfere with repair. While it is possible to give these treatments immediately after focal ischemia in animals, this is not the case in humans due to delays in arrival at hospital and diagnosis; only 20% of New Zealand stroke patients in 2001 arrived at hospital within 3 hours of symptoms and only 48% arrived within 6 hours (Barber et al., 2004). This suggests that for treatments to be of use in a larger demographic of stroke victims, they would need to be effective at delayed time points. Therefore it is important that research focuses on investigating treatments that augment and facilitate the repair processes in the delayed phase of stroke.

1.4. Angiogenesis

Angiogenesis, the formation of new blood vessels from existing ones, is a process that is essential to enable oxygen to reach tissues that lack blood supply. In vertebrates, blood vessels are formed by layers of endothelial cells (Adams & Alitalo, 2007). Usually these cells are quiescent with only 0.1% dividing at any given time. However under angiogenic stimulation this proliferation can increase several fold (Hobson & Denekamp, 1984). Angiogenesis can be activated by various stimuli including hypoxia (Pugh & Ratcliffe, 2003), inflammation (Halin & Detmar, 2008) and mechanical factors such as stretch (Shiu et al., 2005).

Angiogenesis occurs in a series of overlapping stages. In sprouting angiogenesis (the most common form of angiogenesis), the extracellular matrix of the vascular plexus is degraded proteolytically. Endothelial cells proliferate and migrate chemotactically to form a lumen. The endothelial cells mature and are coated by pericytes and smooth muscle cells. This gives the new blood vessels structural integrity and allows control of blood flow (Risau, 1997; Carmeliet, 2005).

1.5. Vascular Endothelial Growth Factors

One of the most important regulators of angiogenesis is the vascular endothelial growth factor (VEGF) family. The VEGF family has several endogenous members, including VEGF-A, -B, -C & -D and placenta growth factor. Additionally two exogenous members of the VEGF family have recently been discovered: VEGF-E from the parapox Orf virus and VEGF-F from the venom of *Trimeresurus flavoviridis* (Olsson et al., 2006; Shibuya, 2009a). These ligands activate different combinations of the membrane-bound tyrosine kinase receptors; Vascular

endothelial growth factor receptor (VEGFR)-1 (also referred to as fms-like tyrosine kinase, Flt-1), VEGFR-2 (also referred to as kinase insert domain-containing receptor, KDR, in humans and fetal liver kinase, Flk-1, in mice) and VEGFR-3. VEGFR-3 is primarily involved in lymphangiogenesis (Olsson et al., 2006) and therefore will not be discussed here.

1.6. VEGF-A

VEGF-A (often referred to as merely VEGF) was the first member of the VEGF family to be discovered (Leung et al., 1989). VEGF-A is a highly conserved homodimeric glycoprotein of 34-46 kDa in size (Thomas, 1996). It is encoded by a single gene located at chromosome 6p21.3 (Vincenti et al., 1996) and can be differentially spliced to yield one of several isoforms. These isoforms are numbered based on the number of amino acids they contain and in humans include $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$ (Tischer et al., 1991), $VEGF_{206}$ (Houck et al., 1991) and less common variants $VEGF_{145}$ (Poltorak et al., 1997), $VEGF_{162}$ (Lange et al., 2003), and $VEGF_{183}$ (Jingjing et al., 1999). Interestingly an isoform $VEGF_{165b}$ was identified, which appears to have an anti-angiogenic effect (Bates et al., 2002). In mice the VEGF isoforms are one amino acid shorter than their corresponding human isoform (Olsson et al., 2006).

VEGF-A mRNA expression is induced by a wide variety of factors including HIF-1, epidermal growth factor, transforming growth factor-α &-β, keratinocyte growth factor, insulin-like growth factor-1, fibroblast growth factor, platelet-derived growth factor, gonadotrophin, thyroid stimulating hormone, adrenocorticotropic hormone, progestin, interleukin-1-α and interleukin-6 (Ferrara, 2004). VEGF-A binds to the tyrosine-kinase receptors VEGFR-1 and VEGFR-2 with high affinity (de Vries et al., 1992; Millauer et al., 1993). Additionally VEGF-A has been shown to bind to heparin (Ferrara & Henzel, 1989),

integrins (Vlahakis et al., 2007), neuropilin-1 (Soker et al., 1998) and neuropilin-2 (Gluzman-Poltorak et al., 2000).

1.7. Downstream effects of VEGF-A

VEGF-A contributes to all phases of angiogenesis (Bahramsoltani et al., 2010). VEGF-A has been shown to be a potent mitogen for endothelial cells in multiple *in vivo* models (Plouët et al., 1989; Leung et al., 1989). VEGF-A also induces expression of the serine proteases urokinase-type plasminogen activator and tissue-type plasminogen activator (Pepper et al., 1991) and the metalloprotease interstitial collagenase (Unemori et al., 1992). These compounds facilitate angiogenic invasion, the process by which the extracellular matrix of the vascular plexus is broken down allowing migration of endothelial cells (Moscatelli & Rifkin, 1988).

VEGF-A also facilitates endothelial migration. Endothelial cells have been shown to migrate along gradients of VEGF-A *in vitro* (Barkefors et al., 2008). This is consistent with the high level of VEGFR-2 mRNA expression found in migrating tip cells (Gerhardt et al., 2003). As the concentration gradient will peak at the area of VEGF-A production, such as hypoxic tissue, this provides some spatial specificity to the angiogenesis induced by VEGF-A.

Additionally, VEGF is known to induce vascular permeability (Senger et al., 1983) via the activation of vasicular-vacuolar organelles located between endothelial cells, fenestrations and the opening of endothelial cell junctions (Feng et al., 1999; Feng et al., 2000). Endothelial cells typically form tight junctions separating blood and its constituents from the surrounding tissues. VEGF-A has been shown to promote the association of focal adhesion kinase and cadherens (Chen et al., 2012), and the interaction of these proteins is

known to facilitate to the opening of the tight junctions between endothelial cells (Dejana et al., 2008; Quadri, 2012). Nitric oxide is also thought to be a mediator of VEGF-A induced vascular permeability as inhibition of nitric oxide synthase reduces VEGF-A's ability to induce vascular permeability (Tilton et al., 1999). It has also recently been shown that neuropilin-1 also plays an important role in VEGF-A induced vascular permeability and it has been suggested that this may be the primary receptor by which VEGF-A induces vascular permeability (Becker et al., 2005; Acevedo et al., 2008).

It has been proposed that this vascular permeability may be important to angiogenesis (Dvorak et al., 1995). Indeed vascular permeability has been shown to allow leakage of plasma proteins, leading to the formation of a new extracellular matrix that would more easily enable migration of endothelial cells (Dvorak et al., 1979). Vascular permeability may be an important step in angiogenesis, but there is debate as to this (Ferrara, 2004). For instance knockout of Src results in normal VEGF-A induced angiogenesis but without VEGF-A induced vascular permeability, suggesting that vascular permeability is not necessary for angiogenesis (Eliceiri et al., 1999).

VEGF-A has also been linked to inflammatory responses, as it promotes monocyte activation and migration across layers of collagen and endothelial cells (Clauss et al., 1990) and production of proinflammatory cytokines and chemokines (Hao et al., 2009). These inflammatory processes have been shown to precede angiogenesis (Croll et al., 2004). There is some suggestion that this inflammation may promote angiogenesis as targeted inactivation of monocytes inhibits VEGF-A induced angiogenesis (Ishida et al., 2003). VEGF-A is also known to upregulate ICAM-1 (Radisavljevic et al., 2000), and inhibition of ICAM-1 has been shown to inhibit angiogenesis (Sakurai et al., 2003).

VEGF-A also prevents apoptosis of endothelial cells (Alon et al., 1995; Gerber et al., 1998a; Gerber et al., 1998b). This effect is thought to be produced via the induction of survival factor expression as VEGF-A increases the expression of Bcl-2, A1 (Gerber et al., 1998a), X-linked inhibitor of apoptosis protein, survivin (Tran et al., 1999) and poly(adenosine diphosphate-ribose) polymerase (Hörmann et al., 2011). Additionally VEGF-A has been linked to neuroprotection. Following induced seizures VEGF-A and its mRNA are upregulated in neurons and glia (Newton et al., 2003; Nicoletti et al., 2008), and VEGF-A has been shown to protect *in vitro* neurons from excitotoxicity and ischemia (Matsuzaki et al., 2001; Svensson et al., 2002; Jin et al., 2001).

VEGF-A has also been linked to mitogenesis of neuronal stem cells and progenitor cells (also referred to as neurogenesis). Two of the primary areas, in which neurogenesis occurs, are the subventricular zone (SVZ) and the subgranular zone (SGZ) of the hyippocampal dentate gyrus. These regions are closely associated with microvasculature (and endothelial cells) and it is believed that VEGF-A and other factors released from these endothelial cells are involved in regulating neurogenesis (Madri, 2009). VEGF-A has been shown to induce neurogenesis *in vitro,* while intracerebroventricular (ICV) VEGF-A induces neurogenesis in the SVZ and SGZ *in vivo* in both ischemic and non-ischemic conditions (Jin et al., 2002; Sun et al., 2003). VEGF-A has also been linked to neurogenesis in several disease states. VEGF-A is responsible for antidepressant induced neurogenesis (Warner-Schmidt & Duman, 2007), while reduced neurogenesis in animal models of chronic stress has been linked to reduced VEGF-A expression and impaired neurogenesis (Heine et al., 2005).

There are even suggestions that VEGF-A induces neuroplasticity. VEGF-A overexpression has been shown to facilitate learning in rats in the Morris water maze (Cao

et al., 2004). VEGF-A has also been shown to enhance long term potentiation (Licht et al., 2011), a biological process that is thought to be involved in memory and learning (Lynch, 2004). It is commonly thought that this facilitated learning is tightly coupled with neurogenesis (Leuner et al., 2006). However recent evidence suggests that VEGF-A induced plasticity occurs before newly made neurons would be functional, and that neurogenesis in the absence of VEGF-A did not facilitate learning (Licht et al., 2011). These findings suggest that VEGF-A may have a more direct role in neuroplasticity.

1.8. VEGF-E

Recently VEGF homologues have been characterised from strains NZ2, NZ7 and D1701 of the Orf virus, type species of the parapoxvirus genus of the Poxvirus family (Lyttle et al., 1994; Wise et al., 1999; Meyer et al., 1999; Ogawa et al., 1998). These homologues have been named VEGF-E, with a subscript noting the strain of origin (e.g. $VEGF_{NZ2}$; Shibuya, 2009b). Despite a low homology with VEGF-A (22 to 27% and 16 to 23% for VEGF-E_{NZ2} and VEGF-E_{NZ7} respectively; Lyttle et al., 1994), VEGF-E has similar angiogenic abilities to those of VEGF-A (Wise et al., 2003; Zheng et al., 2006). As does VEGF-A, VEGF-E activates VEGFR-2. However it does not interact with VEGFR-1 or VEGFR-3 making its binding profile unlike any other member of the VEGF family (Ogawa et al., 1998; Wise et al., 1999).

1.9. VEGF Receptor 2

VEGFR-2 is thought to be the primary receptor responsible for mediating the downstream effects of VEGF-A (Matsumoto & Claesson-Welsh, 2001). Unlike VEGFR-1, VEGFR-2 produces a strong phosphorylation signal in response to VEGF-A (Waltenberger et al., 1994). Because of this there has been a focus on dissecting VEGFR-2 signalling for its potential use as a therapeutic (Olsson et al., 2006).

The primary evidence that VEGFR-2 is the main receptor responsible for angiogenesis has come from knockout studies. Knockout of VEGFR-2 and is embryonically lethal due to impaired vasculogenesis (Shalaby et al., 1995). However knockout of the intracellular tyrosine kinase component of VEGFR-1 is not, resulting in normal vasculature (Hiratsuka et al., 1998). This suggests that VEGFR-1 phosphorylation is not required for angiogenesis, and that VEGFR-2 is predominantly responsible. As VEGF-A promotes all stages of angiogenesis it may therefore be presumed that all necessary steps are mediated by VEGFR-2.

VEGFR-2 activates the Raf-MEK-MAP kinase pathway through PLC-γ. It is believed that this pathway is responsible for endothelial cell mitogenesis as inhibition of MEK 1/2 inhibits proliferation (Takahashi et al., 1999). VEGFR-2 has also been implicated in endothelial cell migration. Gerhardt et al. (2003) found that VEGFR-2 expression was increased in migrating tip cells. They also found that VEGFR-2 neutralising antibodies inhibited endothelial cell migration, while VEGFR-1 neutralising antibodies did not. It should be noted that VEGFR-1 has been identified as a positive modulator of endothelial cell migration (Kearney et al., 2004), however these findings support a more predominant role of VEGFR-2. Additionally, activation of VEGFR-2 by VEGF-A leads to the phosphatidylinositol 3'-kinase (PI3-K)/Akt-dependent activation of integrins (Byzova et al., 2000) and integrins promote endothelial cell migration and survival during angiogenesis (Avraamides et al., 2008).

VEGFR-2 is also thought to be responsible for the majority of neuronal effects of VEGF-A. VEGFR-2 is expressed on neurons and glia (Luciano et al., 2010). The neuroprotective effects of VEGF-A in response to hypoxia or serum withdrawal are, at least

in part, due to activation of the PI3-K/Akt/nuclear factor- κB (NF-κB) pathway via VEGFR-2 (Jin et al., 2000a; Jin, et al., 2000b). Similarly, newly formed neurons express VEGFR-2 but not VEGFR-1 and inhibition of VEGFR-2 reduces neurogenesis (Jin et al., 2002).

1.10. VEGF Receptor 1

VEGFR-1 was originally thought to act as a "decoy" receptor by binding VEGF-A, thereby preventing VEGFR-2 from binding (Park et al., 1994). While VEGFR-1 has an affinity more than 10 times higher than that of VEGFR-2 for VEGF-A, it shows a much weaker phosphorylation signal (Waltenberger et al., 1994). Also while knockout of VEGFR-1 is embryonically lethal due to a disorganised vascular endothelium (Fong et al., 1995), as mentioned earlier, knockout of the intracellular tyrosine kinase domain of VEGFR-1 is not and results in normal development of vasculature (Hiratsuka et al., 1998). This decoy role is further supported by the existence of an endogenously produced soluble VEGFR-1 (sVEGFR-1), which lacks the transmembrane and intracellular tyrosine kinase domains. sVEGFR-1 binds to VEGF-A with high affinity and inhibits its mitogenic effect on endothelial cells *in vitro* (Kendall & Thomas, 1993). sVEGFR-1 has been shown to be anti-angiogenic in the retina (Honda et al., 2000). These findings suggest that it is not VEGFR-1's activity that is required for angiogenesis, but rather its ability to bind VEGF-A, and to thereby indirectly moderate VEGFR-2 activation.

Other findings further support VEGFR-1's involvement in inflammation. VEGFR-1, but not VEGFR-2, is expressed on monocytes. VEGF-A and PlGF (VEGFR-1 specific) both induce monocyte tissue production and chemotaxis at similar levels (Clauss et al., 1996; Barleon et al., 1996). These effects are dependent on the PI3-K/Akt signalling pathway (Tchaikovski et

al., 2008). Additionally, while producing normal vasculature, knockout of the VEGFR-1 tyrosine kinase domain results in suppressed macrophage migration (Hiratsuka et al., 1998).

While ligands that activate VEGFR-2 alone can induce vascular permeability as shown by studies utilizing VEGF-E (Wise et al., 1999; Ogawa et al., 1998), there is mounting evidence of VEGFR-1's role in vascular permeability. The anti-angiogenic $VEGF_{165b}$ induces vascular permeability through the VEGFR-1 receptor but not VEGFR-2 (Glass et al., 2006). A mutated form of VEGF-A, with reduced ability to bind to VEGFR-2, has been shown to induce similar levels of vascular permeability to that of wild type VEGF-A (Stacker et al., 1999). VEGF-F is another exogenous member of the VEGF family that was recently discovered in the venom of *Trimeresurus flavoviridis.* Like VEGF-A, VEGF-F binds to both VEGFR-1 and VEGFR-2. VEGF-F activates similar levels of VEGFR-1 autophosphorylation to that VEGF-A. However, VEGF-F activates approximately 10% of the VEGFR-2 autophosphorylation to that of VEGF-A. Interestingly VEGF-F leads to similar levels of vascular permeability as VEGF-A, but only 10% of the mitogenicity. This has led to the suggestion that vascular permeability is increased by simultaneous activation of VEGFR-1 and VEGFR-2 (Takahashi, et al. 2004). Recently it was found that endothelial nitric oxide synthase is induced by convergent signalling from VEGFR-1 and VEGFR-2 (Ahmad et al., 2006). As noted earlier, nitric oxide synthase appears to be critical to the vascular permeability effects of VEGF-A (Tilton et al., 1999). This further supports that VEGFR-1 and VEGFR-2 increase vascular permeability synergistically.

VEGF-E, as also noted earlier, induces levels of angiogenesis similar to those of VEGF-A, further indicates the importance of VEGFR-2 in angiogenesis (Zheng et al., 2006). What makes VEGF-E interesting is that it has been shown in therapeutic studies to produce less

vascular permeability and inflammation than VEGF-A (Kiba et al., 2003; Zheng et al., 2006; Zheng et al., 2007). The inability of VEGF-E to bind to VEGFR-1, combined with this reduced vascular permeability and inflammation, provides further support that these functions are mediated via VEGFR-1. It should be noted however that the isoform of VEGF-E used in these studies was NZ-7, which does not bind to neuropilin-1 (Zheng et al., 2006). As neuropilin-1 has been implicated as a key mediator of vascular permeability (Becker et al., 2005; Acevedo et al., 2008), the reduced vascular permeability may be due to this as opposed to an inability to bind to VEGFR-1.

1.11. VEGF-A and Stroke

It has been shown that VEGF-A and its associated receptors are upregulated in response to focal ischemia and that this expression persists for weeks after the event (Hayashi et al., 1997; Plate et al., 1999; Zhang et al., 2002). VEGF-A is also known to induce angiogenesis, neurogenesis (Wang et al., 2009) and neuroprotection in ischemic conditions (Wei et al., 2001; Jin et al., 2002; Matsuzaki et al., 2001). This has led to a widely-held belief that VEGF represents one of the endogenous repair mechanisms following stroke. Indeed several studies have supported this, as (1) VEGF-A antagonism, via antibodies, increases infarct volume following focal ischemia in rats (Bao et al., 1999); (2) VEGF-A administration or increased expression decreases infarct volume (Hayashi et al., 1998; Zhang et al., 2000; Sun et al., 2003; Wang et al., 2005; Kilic et al., 2006; Kaya et al., 2005; Wang et al., 2007; Wang et al., 2009; Yang et al., 2009a; Yang et al., 2010); and (3) VEGF-A improves behavioural scores following focal ischemia in rats and mice (Zhang et al., 2000; Sun et al., 2003; Zhu et al., 2005;Kaya et al., 2005; Wang et al., 2006; Wang et al., 2007; Yang et al., 2009a; Yang et al., 2010). All of these treatments were given in the days after the induction

of focal ischemia, rather than immediately. This would offer a clinical window of opportunity which is generous compared to the only currently available treatment for stroke, rt-PA, which needs to be administered within the first three hours after stroke to be neuroprotective (NINDS rt-PA Stroke Study Group, 1995).

The effectiveness of VEGF-A treatment appears to be highly dependent on the time of delivery. Van Bruggen et al. (1999) found that VEGF-A antagonism via antibodies leads to decreased infarct size in mice following focal ischemia. This conflicts with the results of Bao et al. (1999) who found that VEGF-A antagonism resulted in larger infarcts in rats. Apart from the obvious difference in species, the time of administration also differed. Van Bruggen et al. (1999) administered the antibody 12-16 hours prior to surgery, while Bao et al. (1999) delivered the antibody later with daily doses for 7 days following surgery.

Zhang et al., (2000) looked at the importance of the time of exogenous VEGF-A administration. They found that administration at 1 hour following surgery increased BBB leakage and infarct size, while administration at 48 hours following surgery increased behavioural function and did not increase BBB leakage in the contralateral hemisphere. This is consistent with studies which have administered the VEGF in the days following the focal ischemia, and have found exogenous VEGF-A to be protective (Sun et al., 2003; Zhu et al., 2005; Wang et al., 2006; Wang et al., 2007; Wang et al., 2009; Yang et al., 2009a; Yang et al., 2010). Impressively a few of these studies supported the conclusion that VEGF-A was effective even when given 3 days after the ischemic incident (Zhu et al., 2005; Yang et al., 2009a; Yang et al., 2010). On the other hand, some reports have shown VEGF-A to be protective immediately after induction of ischemia (Hayashi et al., 1998; Kaya et al., 2005; Wang et al., 2005; Kilic et al., 2006). However these last three papers measured infarct sizes

1 day following focal ischemia, potentially an insufficient time for any VEGF-A induced damage via inflammation or oedema to be evident.

The findings in the previous paragraph also highlight the fact that, while VEGF-A appears to have some effects that tend towards helping repair following stroke, it can also induce effects that can exacerbate stroke. The two primary mechanisms by which VEGF-A is believed to induce harmful effects are via its ability firstly to induce vascular permeability and secondly to induce inflammation. VEGF-A induced vascular permeability is believed to contribute to oedema and BBB leakage following ischemia (Weis, 2004; Ruiz de Almodovaret al., 2009) and VEGF-A-induced oedema and BBB leakage have been shown to contribute to ischemic damage (van Bruggen et al., 1999; Schoch et al., 2002). With respect to inflammation, Manoonkitiwongsa et al. (2006) showed that doses of VEGF that induce angiogenesis also lead to increased macrophage density and damaged neuropil. These effects may potentially be interfering with the therapeutic effects of VEGF-A, leading to a suboptimal treatment.

As mentioned earlier, VEGF-A's effects on inflammation and vascular permeability appear to be mediated via VEGFR-1 activation. This supports a conclusion that VEGF-E may be a more viable treatment in stroke because it does not activate VEGFR-1 and it has already been shown to induce less inflammation and vascular permeability than VEGF-A. VEGF-E nevertheless induces comparable levels of angiogenesis to VEGF-A in therapeutic studies (Zheng et al., 2006; Zheng et al., 2007).

1.12. Intranasal Delivery

The method of delivery of proteins to the brain has been a major limitation in their potential as treatments of CNS disorders. This is in large part due to the BBB. The BBB is comprised of a layer of endothelial cells surrounding the cerebral capillaries (Bradbury, 1993). These cells are connected via tight junctions, separating blood from the brain. This severely limits the ability of compounds to cross from the blood into the brain. While hydrophobic compounds and some small proteins can still cross the blood brain barrier, these are also limited in their ability to enter the brain.

It has been shown that VEGF-A can be delivered to the brain indirectly via intravenous (IV) delivery (Yang et al., 2009b) and IV VEGF has been shown to be effective in a stroke model (Zhang et al., 2000). There is potential that VEGF-A is more effective than VEGF-E at crossing the BBB as it would be expected to promote higher levels of BBB permeability. However this is an unfavourable outcome especially in stroke, as mentioned earlier. Additionally IV administration of VEGF-A requires high doses. This is of concern as VEGFR-1 and VEGFR-2 are expressed throughout the body (Witmer et al., 2002), meaning that there will likely be unwanted systemic effects. For instance IV VEGF-A in rats induces an increase in heart rate and a reduction in cardiac output and mean arterial blood pressure (Yang et al., 1996).

One way to bypass the BBB is to deliver the compound to the brain directly. In animal studies this has been achieved for VEGF using ICV injection (Sun et al., 2003; Kaya et al., 2005; Feng et al., 2008), surgical implant (Zhu et al., 2005) and topical application (Hayashi et al., 1998). These techniques have been used successfully with VEGF-A in stroke models. The techniques provide protein directly to the brain, and therefore reduce

peripheral effects of the protein. However these techniques are all highly invasive - a hole must be drilled in the skull. In the case of ICV, a hole must also be drilled into the brain. This leads to inflammation as foreign bodies come in contact with the brain, and even the risk of infection. This acts as a confounding factor in stroke studies, and also disincentives the use of these methods of delivery in more clinical settings.

Recently intranasal (IN) delivery has garnered support as a non-invasive, direct way to deliver protein to the brain, bypassing the BBB completely (Hanson & Frey, 2008). Evidence that proteins could enter the brain via IN delivery was noted as early as 1971. Kristensson & Olsson (1971) found that horseradish peroxidase (HRP) and albumin could be delivered to the rat brain by natural aspiration into the nasal cavity. Thorne et al., (1995) furthered this by finding that concentrations in the olfactory bulb from IN delivery were over 100 fold that of IV delivery suggesting that this pathway was highly specific. These findings supported that IN delivery provided direct delivery of protein to the brain, as well as being far more efficient than IV delivery. This provides both potentially more cost effective treatment as less drug is required to reach a desired concentration, while also reducing peripheral concentrations of the drug and thereby reducing unwanted side effects. These factors combined with the non-invasive nature of IN delivery make it potentially viable for a clinical setting.

In more recent years, IN delivery has been demonstrated to be effective in rodents for a wide range of proteins including nerve growth factor (Chen et al., 1998), vasoactive intestinal peptide (Gozes et al., 1996), brain derived neurotrophic factor, ciliary neurotrophic factor, neurotrophin-4/5 (Alcalá-Barraza et al., 2010), erythropoietin and insulin growth factor (Fletcher et al., 2009). Additionally studies have supported that

intranasal delivery of protein is viable in humans using arginine-vasopressin (Pietrowsky et al., 1996a), cholecystokinin-8 (Pietrowsky et al., 1996b), insulin (Kern et al., 1999), melanocyte-stimulating hormone & adrenocorticotropin (Smolnik et al., 1999). Of particular note, VEGF-A has already been successfully delivered via the IN route. Yang et al. (2009b), using the same methods as that of Thorne et al. (1995), showed that [^{125}I]-labelled human VEGF-A 165 entered the brain via IN delivery.

1.13. Intranasal VEGF-A in stroke

In the past few years the Yang group have demonstrated the efficacy of intranasally delivered VEGF-A in a rat model of stroke. After initially confirming that VEGF-A can be delivered intranasally (Yang et al., 2009b), they went on to test intranasal VEGF-A in rats that have received MCAO (Yang et al., 2009a; Yang et al., 2010). The VEGF-A was delivered on days 3, 4 and 5 after focal ischemia. Behavioural were performed before focal ischemia (day) 0 and 1, 7 and 14 days after focal ischemia while the infarct was measured on day 14 after focal ischemia. They found that VEGF-A was both effective at reducing infarct size and behavioural impairments. They also showed that intranasal VEGF-A resulted in increased angiogenesis at the ischemic boundary.

1.14. Current Aims

In the present study we aimed to compare the efficacy of intranasal VEGF-A and VEGF-E in a surgical modified Levine model of stroke. Intranasal VEGF-A, VEGF-E and PBS (Phosphate buffered saline) were delivered on days 3, 4 and 5 after carotid ligation surgery. Infarct volume was measured at day 14 after surgery, while behavioural tests were performed before the surgery and on days 1, 7, and 14 after the surgery. It was

hypothesized that VEGF-A and VEGF-E would reduce infarct size and improve behavioural scores. Additionally it was hypothesized that VEGF-E would be more effective than VEGF-A in these respects due to reduced inflammation and oedema.

Unless stated otherwise, all chemicals were obtained from Sigma–Aldrich, St. Louis, MO, USA. All surgical materials used were acquired from Southern Medical Products Ltd., Dunedin, New Zealand. Statistics were performed using GraphPad Prism TM.

2.1 Protein production

Stable 293EBNA cell lines secreting the FLAG-tagged VEGF-E from orf virus NZ2 or the FLAG-tagged VEGF-A (murine isoform 164) had previously been constructed by transfection with the pAPEX plasmid containing the VEGF genes and selection with hygromycin-B (Wise et al., 2003). The 293EBNA cells were grown until confluent in selection medium in T175 cm2 tissue culture flasks (Falcon) at 37°C with 5% $CO^2$for 3 days at 37°C with 5% CO_2. The culture supernatant was then collected and cellular debris was removed by centrifugation at 1,200 rcf for 5 min.

The FLAG-tagged VEGFs were purified by anti-FLAG (M2) affinity chromatography. Clarified supernatant collected from the 293EBNA cells was adjusted to pH 7.0 and 6 mL of 5M NaCl was added per 200 mL of supernatant. Anti-FLAG M2-Agarose (Sigma) (1 mL) was then added per 200 mL of culture supernatant and the mixture was incubated at 4°C for 16 h with rotation. The agarose was then collected by centrifugation at 2,500 rpm for 5 min at 4°C, before the supernatant was removed and stored at -80^{O}C for additional rounds of purification. The agarose was resuspended in 10 mL of the remaining supernatant and transferred to a Poly-Prep® Chromatography Column (Bio-Rad Laboratories). The column was washed three times with 10 mL of ice-cold TBS-T and one further wash where the column was capped and rotated for 10 min at 4°C. FLAG-peptide was prepared to working

concentration (50 µg/mL) in 4 mL of cold TBS-T and added to the column, rotated for 10 min at 4°C and the eluate collected. The eluate was concentrated and buffer exchanged into PBS using a centrifugal concentrator (Amicon Ultracel centrifugal filters, Millipore Corporation) according to the manufacturer's instructions. Where required batches of VEGF protein were combined and concentrated further using the centrifugal concentrator.

Proteins were resolved by SDS-PAGE, stained with Coomassie blue then the bands were quantitated, by comparison with control proteins of known concentration, using a densitometer and the Quantity One Program (Bio-Rad Laboratories) (Wise et al., 2003). A quantitative ELISA was also performed in which 96-well Maxisorp immunoplates (Nunc) were incubated with serial dilutions of the FLAG-tagged VEGFs, and control proteins of known concentration, in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCo_3$, pH 9.6) for 16 hours at 4^{o}C then in blocking buffer (PBS containing 1% BSA and 0.01% Tween20) for 1 h at 37 ^{o}C. Plates were washed between steps with PBS containing 0.005% Tween20. The coated FLAG-tagged proteins were detected by horseradish peroxidase-conjugated M2 (anti-FLAG) antibody, developed with tetramethylbenzidine (TMB) reagent (BD Bioscience) and quantified by measuring the absorbance at 450 nm.

2.2 Animals and Treatments

All animal use was performed under the guidelines of the University of Otago Animal Ethics Committee. Postnatal day (PD) 21 male Sprague Dawley rats were ordered from the Hercus/Taieri breeding facility. Rats were housed in the Department of Pharmacology for 5 days to allow them to acclimatise to their surroundings. On PD 26 the rats were randomly divided into 5 treatment groups: VEGF-E (20 µg), VEGF-E (60 µg), VEGF-A (60 µg), PBS, and

Sham. A pilot study also included VEGF-E (200 µg). However VEGF-E appeared to increase hypoxic-ischemia induced deficits at this concentration (data not shown).

2.3 Surgery

A modified Levine model was used as a model of stroke. The surgery was performed on PD 26 as described previously (Clarkson et al., 2005). Animals were anaesthetized by gaseous halothane, and a small incision made slightly above and lateral to the sternum. Body temperature was maintained using a heating pad and a rectal thermometer. Using blunt dissection, the left common carotid artery was revealed and tightly occluded by two 5-0 silk surgical sutures a few mm apart. In the case of sham surgery the left common carotid artery (CCAO) was revealed but not occluded. The opening was then injected with 100 µL of 20 mg/mL of lignocaine hydrochloride for local pain relief and then closed with surgical clips. Animals (excluding Shams) were then given 2 hours to recover before being placed in the hypoxic chamber (33±1^{o}C; 100% humidity; 8% O_2-92% N_2) for 1 hour. Animals were then returned to their cages.

While this model is not a traditional model of stroke due to the hypoxia component, it was chosen for a couple of reasons. Firstly the model had already been well established in our laboratory. The Levine model produces unilateral infarcts similar to that of the MCAO model (Gunn et al., 1989) and results in motor deficits in the forelimb contralateral to the infarct while not impairing the left forelimb (Tomimatsu et al., 2002). Additionally the modified Levine model has a low mortality rate (≈10%) in our lab while models such as the MCAO model are associated with high mortality rates, reaching up to 80% in some studies (Aspey et al., 2000; Aoki et al., 2001).

2.4 Behavioural Assessment

Behavioural assessment was performed on PD 26, PD 27, PD 33 and PD 40 (Days 0 (before CCAO), 1, 7 and 14 following CCAO). In order to access behavioural outcomes, a battery of behavioural tests was utilised. This was to ensure a comprehensive evaluation of the effectiveness of the VEGF isoforms. The tests included the vibrissae-elicited forelimb response test, the foot-fault test and the forelimb-use asymmetry test. These tests are all commonly utilized in stroke research (Kleim et al., 2007). Behavioural tests were performed and analysed with the experimenter blind to treatment condition.

2.5 Vibrissae-elicited forelimb placing test

The vibrissae-elicited forelimb placing test was performed as previously described (Hua et al., 2002). The rat was held by the torso, allowing free movement of the forelimbs, parallel to the edge of a desk. The rat was then lowered so that its vibrissae came in contact with the desk. The expected response was for the rat to extend its closest forelimb to touch the desk. This was performed 10 times for each forelimb and the number of trials in which there was an appropriate limb extension was recorded.

2.6 Forelimb use asymmetry test

The forelimb use asymmetry test was performed as previously described (Hua et al., 2002). Individual rats were placed in a transparent cylinder (20 cm in diameter, 30 cm in height) for ten minutes. During this time a video camera was used recorded the rat's movements with a mirror to enable the recording of movements facing away from the camera. The number of times the animal used impaired (contralateral/right), unimpaired and both forelimbs to mount the wall of the cylinder was recorded and converted into

proportions. Severity of impairment was indicated by an increased bias to use the unimpaired forelimb.

2.7 Foot fault test

Lastly the foot fault test (Originally described by Hernandez & Schallert, 1988) was performed. Animals were placed on an elevated grid wire floor (approximately 45 cm by 30 cm). The grid was composed of 6 cm^2 gaps. Rats tend to use the grid to balance themselves. Sometimes a limb will miss the grid and fall between the wires. This is identified as a foot fault. Foot faults were then calculated as a percentage of the total steps. In normal animals these foot faults tend to be symmetrical. Animals that have received focal ischemia to one side of the brain show an increased number of foot faults on the contralateral side.

2.8 Intranasal Delivery

Intranasal delivery was performed on PD 29, 30 and 31 (Day 3, 4 and 5 following CCAO). The most common method of intranasal delivery, originally developed by Thorne et al. (1995), involves anaesthesia. However we chose to perform the technique in awake animals as it was decided that the benefits of control did not outweigh the potentially harmful and confounding effects of the anaesthetic. This is not innovative as it has already been shown that the technique can be effective in awake animals (Kristensson & Olsson, 1971; van Velthoven et al., 2010). 5mg/kg of diazepam, while not being enough to induce sleep, was used to facilitate handling. Doses of 3 and 4 mg/kg of diazepam were initially tried but animals would sneeze and move. Animals were then wrapped in a towel to restrain their neck and limb and held in a supine position. A total of 100 µL of PBS (vehicle), alone or with VEGF-E (20 µg), VEGF-E (60 µg), VEGF-A (60 µg), was then delivered via a micropipette in 10 µL aliquots to each nostril every 2 minutes over a total of 10 minutes. The widely used

method of (Thorne et al., 1995) requires 20 minutes, so this time reduction was achieved by delivering to both nostrils every 2 minutes rather than alternating nostrils every 2 minutes. This was done to minimize stress to the animals.

2.9 Histology

Animals were sacrificed on day 14 (PND 40) post CCAO using transcardial perfusion. 100 mg/kg of pentobarbital was injected intraperitoneally. The animal was then restrained and foot twitch response monitored. When the animal was deeply anaesthetized, the chest cavity was opened and a catheter inserted into the left ventricle. The animal was then perfused via gravity with 0.01 M PBS followed by 4% formalin in phosphate buffer. The brain was then dissected and refrigerated in 4% formalin in phosphate buffer for 24 hours followed by 30% sucrose solution for 48 hours. The brain was then frozen in OCT (optimal cutting temperature) compound at -20^{o}C.

Frozen OCT brains were mounted in a cryotome and sliced into 50 µm coronal sections, taking a section every 1 mm. The sections were placed in distilled water to remove the OCT and free floated onto poly-L-lisine coated glass microscope slides (Sigma). The slides were then left to dry. Slides were placed for 30 minutes in 0.1% cresyl violet; 30 seconds in tap water; 30 seconds in distilled water; 1 minute in 50% ethanol; 1 minute in 70% ethanol; 1 minute in 90% ethanol; and lastly 1 minute in 100% ethanol. The slides were then placed in 4% xylene for 4 minutes. Slides were then dried, applied with DPX mountant, and coverslipped.

Sections were scanned onto a computer and analysed using image processing software (ImageJ). Infarct was measured using the indirect method (Swanson et al., 1990;

Lin et al., 1993). This method compares the amount of viable tissue in both hemispheres, rather than directly measuring the infarct. This reduces error associated with directly measuring the infarct. The following formula was used: LI = TR – LN, where LI = infarct volume in the left hemisphere, TR = total tissue in the right hemisphere and LN = non-infarcted tissue in the left hemisphere. After the infarct area was calculated for each section from an individual rat, the infarct areas for each section were added to yield an infarct volume in mm^3 (as the sections were 1 mm apart).

2.10 Statistical Analysis

Histological differences were analyzed using a Kruskal-Wallis test with a Dunn's multiple comparisons post-hoc test to compare for differences between treatment groups. Behavioural results were compared using repeated measures two way ANOVA with a Bonferroni post-hoc test for differences between groups. Results are presented as mean ± SEM. Statistical siginificance was set at $p<0.05$.

Results

3.1. Histology

Histological studies were performed on day 14 after hypoxic ischemia to quantify the size of infarction. The results of histological studies are shown in *Figure 1*. There was a significant effect of treatment group ($p = 0.210$; Kruskal Wallis Statistic = 11.55). The most important finding was that there was not a significant difference between the PBS and Sham groups ($p>0.05$). This suggests that the stroke was not severe enough and/or too variable in the current study. No significant differences were detected between any of the groups that received CCAO (PBS, VEGF-E 20 µg, VEGF-E 60 µg & VEGF-A 60µg) suggesting no significant effect of treatment on infarct volume. The VEGF-E 20 µg group did however show significantly higher infarct volumes compared to the Sham group ($p<0.05$). This was due to the hypoxic ischemia rather than being an effect of VEGF-E as there was not a significant difference between VEGF-E and PBS ($p>0.05$).

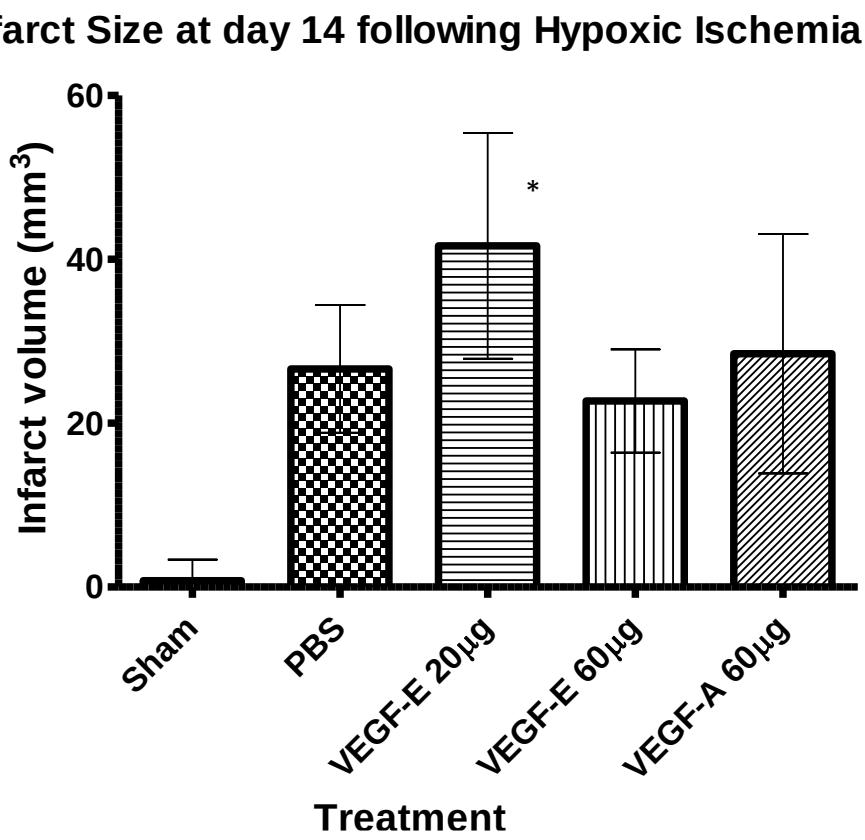


Figure 1. Assessment of infarct size was performed on day 14 after CCAO using cresyl violet stain. * = p<0.05 vs Sham. n=8 per treatment group. Data are presented as mean ± SEM.

3.2. Forelimb Placing Task

The forelimb placing task was used to investigate the sensorimotor impairment and proprioception following hypoxic ischemia. In this test an optimal score is ten out of ten correct forelimb placements, while the lower the score, the more severe the sensorimotor impairment. The results of the unimpaired (left) forelimb were consistently 10 out of 10 in all animals and so data were not presented. The results of the forelimb placement are shown in *Figure 2*. There was a highly significant effect of treatment (p=0.0007; F = 6.246) and time (p<0.0001; 54.11) on the correct number of right forelimb placements. There was also a significant interaction effect between treatment and time (p<0.0001; F = 5.617).

All groups showed significantly reduced correct forelimb placements when compared with Sham at day 1 after hypoxic ischemia ($p<0.01$), supporting a significant effect of hypoxic-ischemia on the number of forelimb placements. However there was a significant variation in forelimb placements on day 1 to the point that VEGF-A 60µg showed a significantly lower number of correct forelimb placements compared to the PBS group ($p<0.05$). VEGF-A 60 µg also showed significantly reduced correct forelimb placements when compared with PBS ($p<0.01$), VEGF-E 20 µg ($p<0.05$) and VEGF-E 60 µg ($p<0.01$) at 7 days after hypoxic-ischemia and PBS ($p<0.05$) and VEGF-E 60 µg ($p<0.05$) at day 14. These differences are likely to be due to VEGF-A receiving a more severe initial impairment rather than being due to a treatment effect of VEGF-A. All other treatment groups were not significantly different from Sham at day 7 or 14 ($p>0.05$). As this includes the PBS control group, this suggests that recovery occurred independently of treatment.

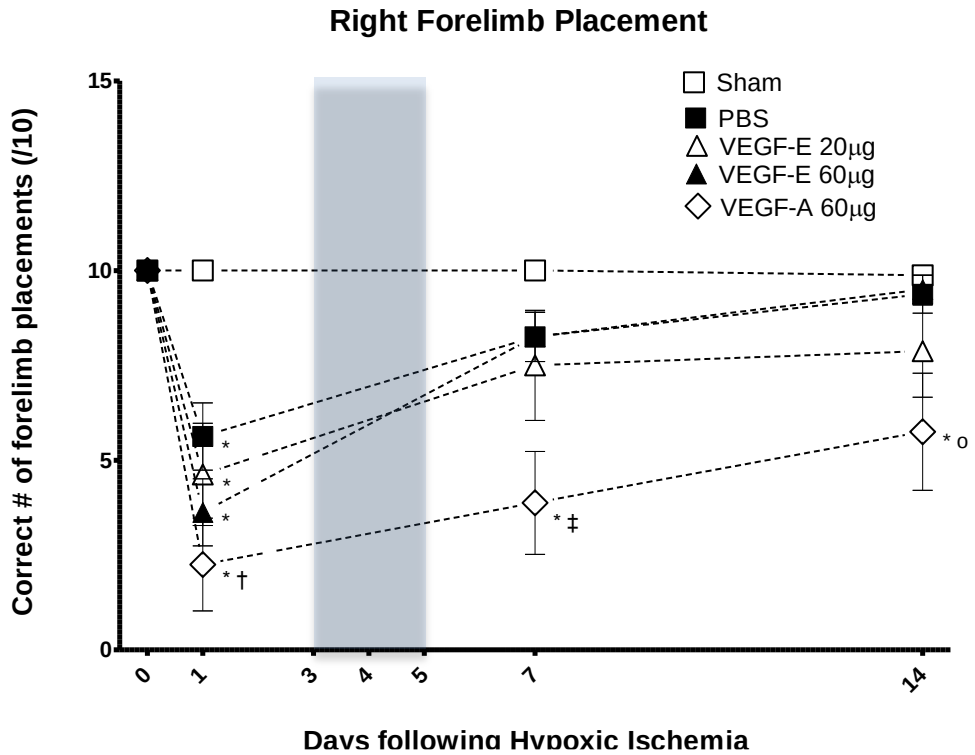


Figure 2. The vibrissae elicited forelimb placing task was performed before CCAO (day 0) and on days 1, 7 and 14 following CCAO. Intranasal treatment occurred on days 3, 4 and 5 (as indicated by the shaded area. * = $p<0.05$ vs Sham. † = $p<0.05$ vs PBS.‡ = $p<0.05$ vs PBS, VEGF-E 20μg & VEGF-E 60μg. ^{o} = $p<0.05$ vs PBS & VEGF-E 60μg. n=8 per treatment group. Data are presented as mean ± SEM.

3.3 Forelimb Use Asymmetry Test

The forelimb use asymmetry test was used to assess the changes in forelimb use following hypoxic ischemia. The results of the forelimb use assymetry test are shown in *Figures 3, 4 & 5*. There was no significant effect of treatment ($p = 0.4523$; $F = 0.94$) or time after hypoxic ischemia ($p = 0.3154$; $F = 1.195$) for use of the right forelimb (*Figure 3.*). For use of the left forelimb (*Figure 4.*) there was a significant effect of time after hypoxic ischemia ($p = 0.0024$; $F = 5.119$) but not treatment ($p = 0.4251$; $F = 0.9914$). However, VEGF-E 60 μg resulted in significantly higher use of the unimpaired (left) forelimb at day 14 when compared with the Sham group ($p<0.05$) suggesting a greater impairment of the impaired (right) limb. This was not significantly different from the PBS group suggesting that it was not an effect of treatment. For the simultaneous use of forelimbs (*Figure 5.*),

there was no significant effect of treatment (p =0.9907; F = 0.06989) or time after hypoxic ischemia (p = 0.1774; F = 1.673).

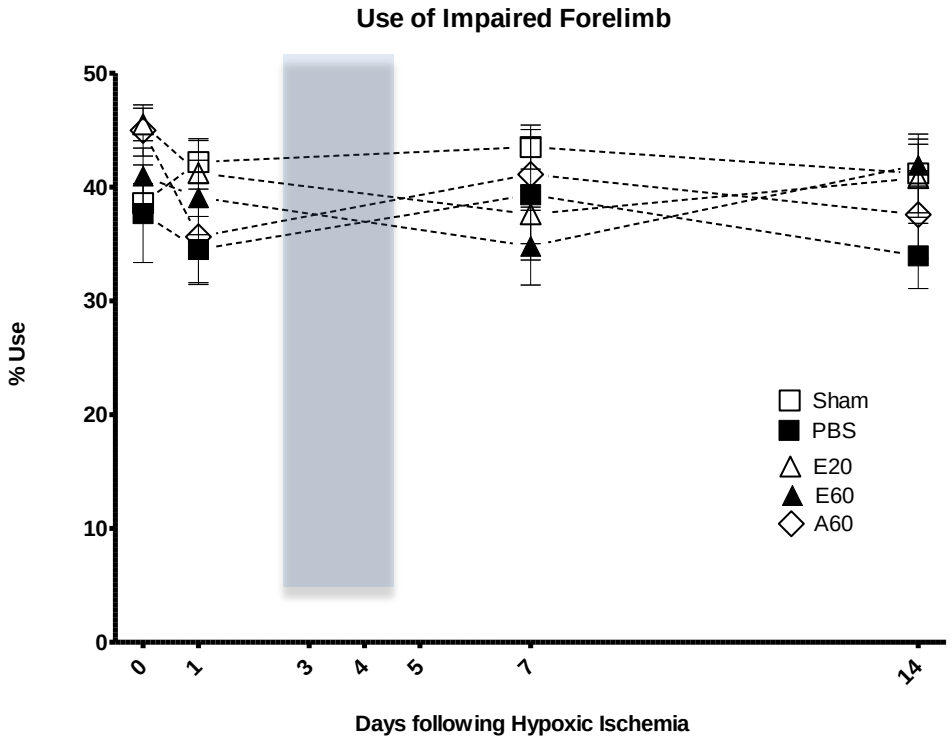


*Figure 3.*The forelimb use asymmetry test was performed before CCAO (day 0) and on days 1, 7 and 14 following CCAO. The percentage of use of the impaired (right) forelimb was calculated. Intranasal treatment occurred on days 3, 4 and 5 (as indicated by the shaded area. n=8 per treatment group. Data is presented as mean ± SEM.

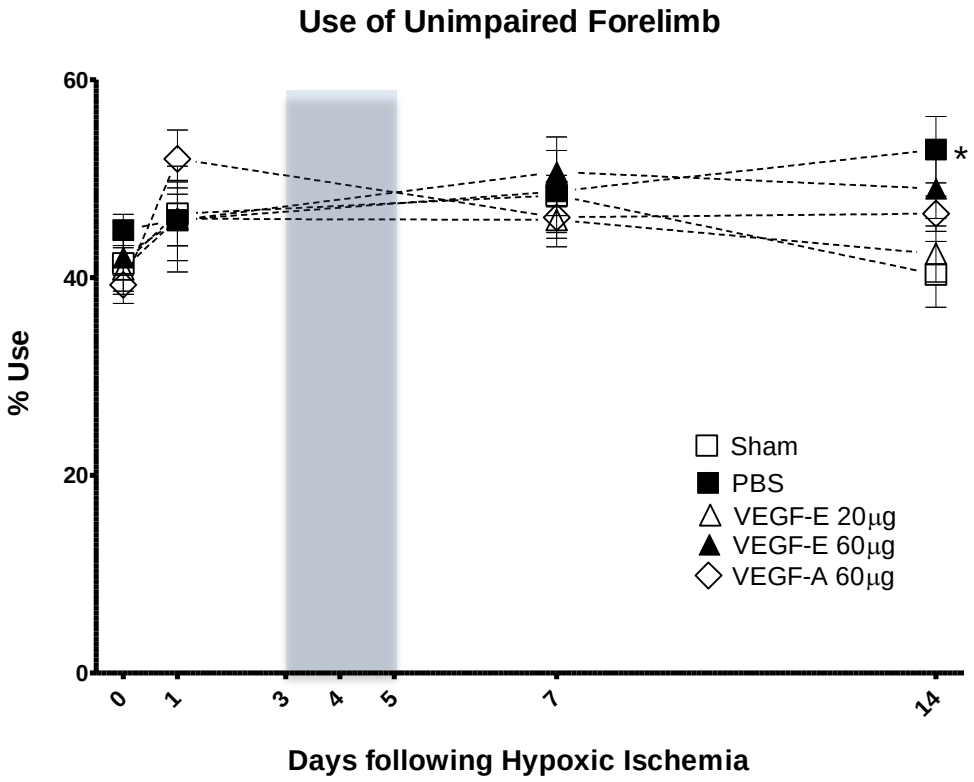


*Figure 4.*The forelimb use asymmetry test was performed before CCAO (day 0) and on days 1, 7 and 14 following CCAO. The percentage of use of the unimpaired (left) forelimb was calculated. Intranasal treatment occurred on days 3, 4 and 5 (as indicated by the shaded area). *=p<0.05 v Sham. n=8 per treatment group. Data are presented as mean ± SEM.

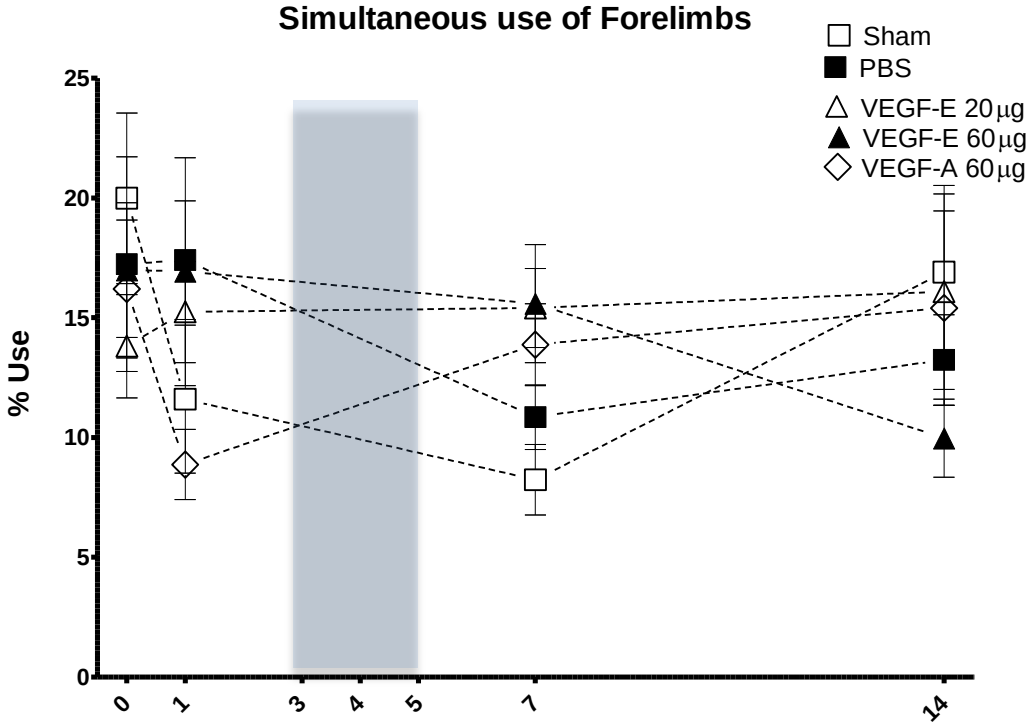


*Figure 5.*The forelimb use asymmetry test was performed before CCAO (day 0) and on days 1, 7 and 14 following CCAO. Intranasal treatment occurred on days 3, 4 and 5 (as indicated by the shaded area). n=8 per treatment group. Data are presented as mean ± SEM.

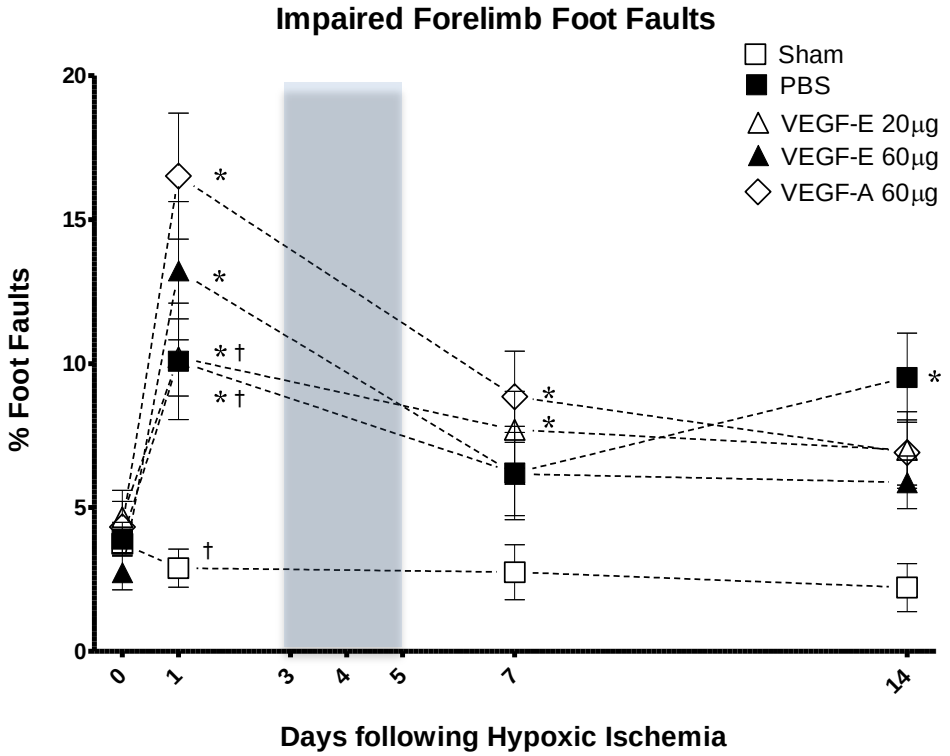
3.4. Foot Faults

The footfault test was used to assess the There was a significant effect of treatment ($p<0.0001$; $F = 10.13$) and time after hypoxic ischemia ($p<0.0001$; $F = 22.76$) for right forelimb foot faults (*Figure 6.*). There was also a significant interaction effect between treatment and time after hypoxic ischemia ($p = 0.0005$; $F = 3.265$). At day 1 there was a significantly higher proportion of right forelimb foot faults for all treatment groups when compared with the Sham group. This supports that the hypoxic ischemia had a significant effect on right forelimb foot faults. However there was also a significantly higher proportion of right forelimb foot faults for VEGF-A 60µg when compared with VEGF-E 20µg ($p<0.01$) and PBS groups ($p<0.01$). This suggests that the VEGF-A 60µg group had more severe deficits induced by the hypoxic ischemia, as this occurred prior to any treatment.

At day 7 there was a significantly higher proportion of right forelimb foot faults for VEGF-A 60µg ($p<0.01$) and VEGF-E 20µg ($p<0.05$). At day 14 there was a significantly higher proportion of right forelimb foot faults for PBS ($p<0.001$). No other significant differences were detected between treatment groups ($p<0.05$). These data do not support a protective effect of treatment as none of the treatment groups scored significantly better than the PBS group on day 7 or 14.

For left forelimb foot faults (*Figure 7.*) there was no significant effect of treatment ($p = 0.5345$; $F = 0.7983$) or time after hypoxic ischemia ($p = 6.143$; $F = 0.6033$). This supports that the hypoxic ischemia had no effect on left forelimb foot faults. This was expected as the left forelimb's motor cortex was contralateral to the CCAO.

A



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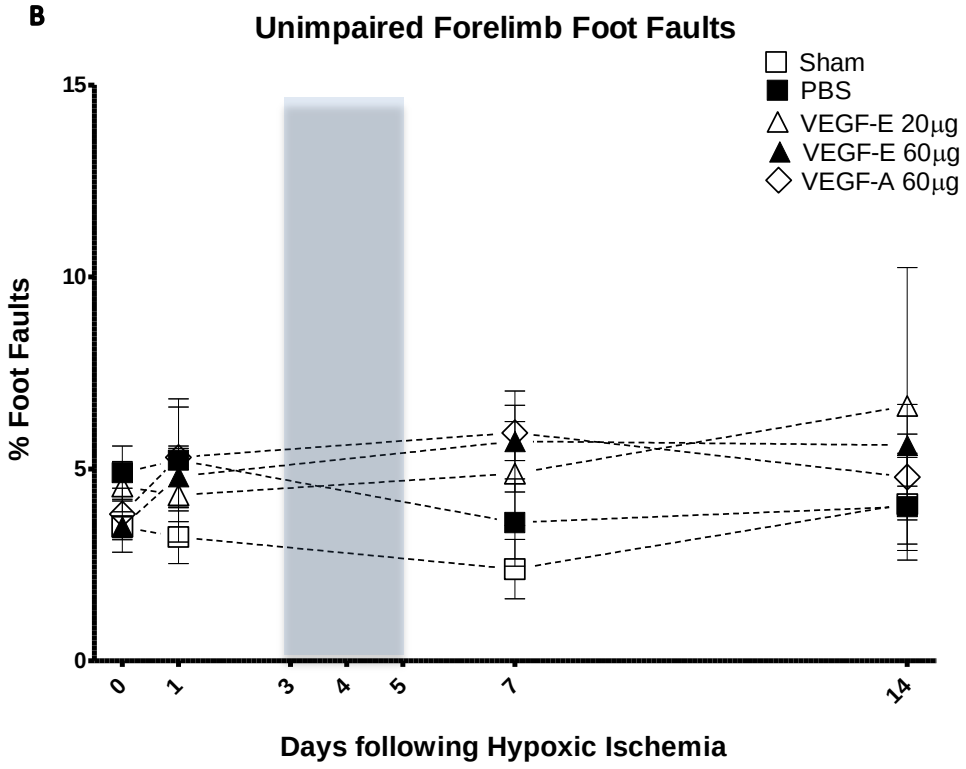

Figure 6. The foot fault test was performed before hypoxic ischemia (day 0) and on days 1, 7 and 14 following CCAO. The amount of right (impaired) & left (unimpaired) forelimb foot faults were recorded as a percentage of the total steps taken. Intranasal treatment occurred on days 3, 4 and 5 as indicated by the shaded region. No significant differences were detected ($p>0.05$). n=8 per treatment group. * = $p<0.05$ vs Sham. † = $p<0.05$ vs A60. Data are presented as mean ± SEM.

3.5. Locomotion

The number of forelimb steps taken per minute was recorded in the foot fault test before (day 0) and on days 1, 7 and 14 after hypoxic ischemia. There was a significant effect of time after hypoxic ischemia ($p<0.0001$; $F = 34.35$) but not treatment ($p = 0.7736$; 0.4473) or interaction between time and treatment ($p = 0.0877$; $F = 1.655$). At day 7 after hypoxic ischemia, VEGF-A 60µg resulted in significantly higher motor activity compared to PBS ($p<0.05$). No other significant differences were detected.

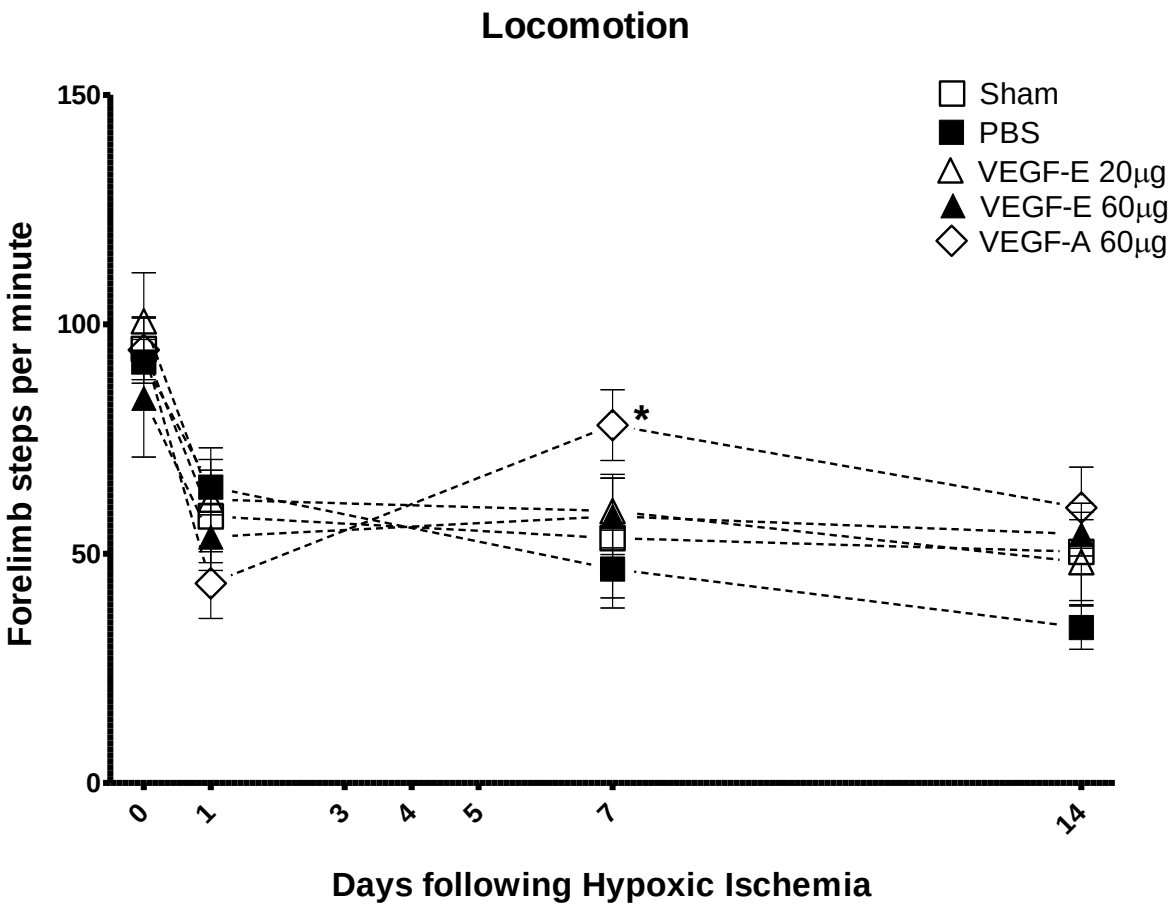

Figure 8. The foot fault test was performed on before hypoxic ischemia (day 0) and on days 1, 7 and 14 following CCAO. The number of forelimb steps taken was measured. Intranasal treatment occurred on days 3, 4 and 5 as indicated by the shaded region. * = $p<0.05$ vs PBS. n=8 per treatment group. Data are presented as mean ± SEM.

3.6. Weight

Weight was measured before hypoxic ischemia (day 0) and on days 1, 2, 3 and 14 after hypoxic ischemia and normalized as a percentage of starting weight. There was a significant effect of time after hypoxic ischemia ($p<0.0001$; $F = 2600$) but not treatment ($p = 0.0217$; $F = 3.29$; *Figure 9.*). No significant differences were detected before hypoxic ischemia (day 0) or on days 1, 2 and 3 after hypoxic ischemia. At day 14 after hypoxic ischemia, Sham, PBS, VEGF-E 20µg & VEGF-E 60µg ($p<0.05$) showed significantly higher weights when compared with the VEGF-A group.

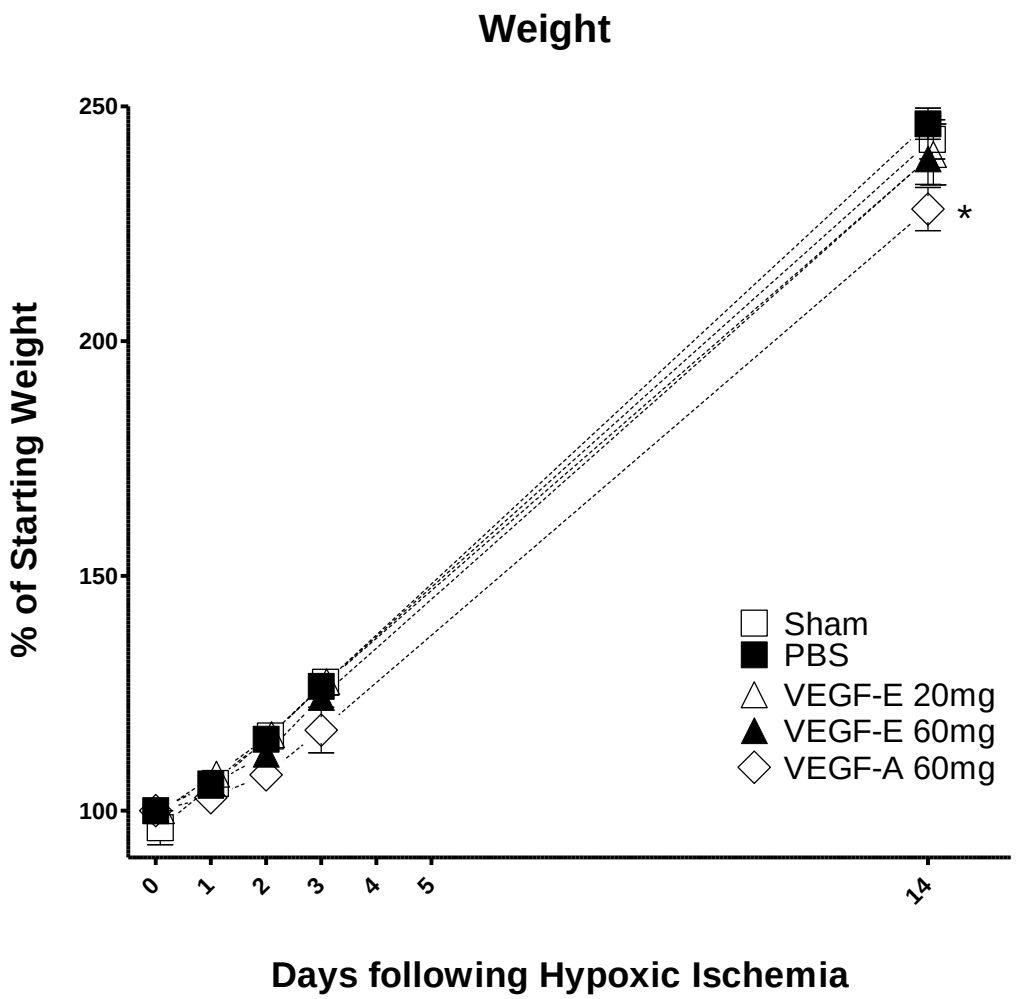

Figure 8. Weight was measured before hypoxic ischemia (day 0) and on days 1, 2, 3 and 14 after hypoxic ischemia and was normalized as a percentage of starting weight. * = $p<0.05$ vs PBS, Sham, VEGF-E 20µg & VEGF-E 60µg. n=8 per treatment group. Data are presented as mean ± SEM.

Discussion

Using a combination of behavioural and histological studies we aimed to test and compare the efficacy of intranasal VEGF-E and VEGF-A to reduce deficits in a modified Levine model of stroke. Our hypothesis that VEGF-A and VEGF-E would reduce infarct sizes was not supported as none of the VEGF treated groups were significantly different from the PBS group. Similarly, the hypothesis that VEGF-A and VEGF-E would improve behavioural recovery was not supported. For the forelimb use asymmetry test and the foot fault test, none of the VEGF groups were significantly different from the PBS group on days 7 or 14. VEGF-A 60 µg did show a significant increase in foot faults at day 1; However this occurred before any intranasal delivery and therefore is likely to be due to differences in the severity of hypoxic ischemia induced deficits in this group.

In the forelimb placement task, the VEGF-A 60 µg group showed a significantly lower number of correct right forelimb placements at day 7 days after hypoxic ischemia. However VEGF-A also showed a significantly lower number of correct right forelimb placements at 1 day after hypoxic ischemia. This, taken in conjunction with the finding that VEGF-A 60 µg showed significantly higher foot faults, supports that the VEGF-A group suffered greater behavioural impairments prior to any intranasal treatment.

There was a minor improvement in that VEGF-A showed a significantly higher level of motor activity at day 7 although differences were not evident at day 14 suggesting that they were temporary. This effect is surprising given that VEGF-A had larger pre-treatment deficits than the other groups. Previous studies have found that increased VEGF-A expression in the brain and ICV VEGF-A do not significantly alter locomotor activity (Cao et al., 2004; Nicoletti et al., 2008). Interestingly locomotive data for VEGF-A in stroke models was unable to be

found despite several studies testing behavioural deficits. As this effect did not occur in the VEGF-E groups, this increased locomotion may be due to excessive activation of VEGFR-1.

The findings of the current study do not support the efficacy of intranasal VEGF-A in a stroke model seen by Yang et al. (2009a;2010). The current study was largely based around these papers, using the same species, doses and timing of doses. However there were several critical differences between the studies. Probably the primary difference being that Yang used MCAO while we used a modified Levine model. The primary differences are the use of global hypoxia, high temperature and younger animals. Additionally they used human VEGF-A_{165}, while we used mouse $VEGF_{164}$. This should not have been of major consequence because the isoforms are equivalent and were both being used in rats. We also changed the intranasal protocol to be done in awake animals rather than anaesthetized animals. This also involved reducing the time between doses to reduce the overall length of the procedure (from 20 minutes to 10 minutes) and therefore stress. This may have reduced absorption of the proteins.

Studies using rats in MCAO, such as the intranasal VEGF-A study by Yang et al. (2009a), typically use mature males ranging from between 200 and 250g in weight (Rupadevi et al., 2011), which equates to approximately 2 months of age. However the current model used PD26 rats. This leads to the question of whether the age impacts on the validity of the model as a stroke model, and also as to whether it impacts on the effectiveness of VEGF. It has recently been shown that VEGF-A shows reduced angiogenesis and neurogenesis in older animals. Additionally VEGFR-2 upregulation in response to VEGF-A is also reduced in aged animals (Gao et al., 2009). Aged rats, despite having higher mortality rates, show significantly reduced oedema following MCAO (Liu et al., 2009). This

would be consistent with a reduced functionality of VEGF-A and inversely suggests that younger animals have stronger VEGF-A mediated repair following ischemia. Assuming this is the case it is possible that augmenting an already highly functional VEGF-A system via exogenous VEGF-A or VEGF-E could potentially increase inflammation and oedema more so than in older rats, resulting in a reduced net benefit. Despite this, it has been shown that VEGF-A can be neuroprotective in the PD7 Levine model (Feng et al., 2008). The study by Feng et al. (2008) found similar concentrations of ICV VEGF-A to be neuroprotective to that of another study that also used ICV VEGF-A in adult rats (Sun et al., 2003) suggesting that age may not significantly affect the effective dose range of VEGF-A (or VEGF–E).

Another potentially important factor is temperature. In the current modified Levine model, hypoxia occurs at a temperature of 33±1 ^{o}C. This was originally done by Rice et al. (2004) to keep the temperature consistent. This consistency is important as body temperature is thought to have an impact on the severity of stroke. Hyperthermic patients have a higher mortality while hypothermic patients have a lower mortality (Wang et al., 2000), and hypothermia has beem shown to be neuroprotective and is receiving focus as a potential treatment for ischemia (van der Worp et al., 2010). It has also been demonstrated recently that increasing the temperature 4 ^{o}C during hypoxia resulted in greater infarction and behavioural deficits following hypoxic ischemia while the opposite was achieved at lower temperatures (Mishima et al., 2004). These results suggest that the temperature maintenance in the current model plays a significant role in the production of deficits following hypoxic ischemia. Therefore this may contribute to differences between this model and MCAO. Additionally it is likely that even within the 2^{o}C range of the current model, that a large amount of variation will occur as a result of temperature.

One limitation of the current study was that we did not definitively show that VEGF-E is capable of entering the brain via the intranasal route. The exogenous VEGF-A in the present study would have been identifiable from endogenous VEGF-A due to the associated FLAG octapeptide. This was not attempted as any residual protein would be expected to be degraded by 9 days after the last intranasal delivery. Protein has been shown to be degraded at approximately 0.8% per hour in the brains of PD 30 rats (Dunlop et al., 1978). Such an experiment would have required a separate group of animals. Therefore, it remains to be confirmed whether VEGF-E can enter the CNS via the intranasal route.

Despite the lack of evidence in the current study, the amount of support for the intranasal pathway allowing delivery of proteins to the brain is significant. A large number of compounds of varying sizes have all been shown to enter the brain quickly and effectively (Talegaonkar & Mishra, 2004). Indeed recently it has been shown that even bacteria and stem cells can invade the brain via the intranasal route (Sjölinder & Jonsson, 2010; van Velthoven et al., 2010). It would therefore be reasonable to assume that the same is true of VEGF-E, a protein of similar size and charge to that of VEGF-A which has already been shown to enter the brain via the intranasal pathway (Yang et al., 2009b).

Regarding the ineffectiveness of VEGF-E in the current study, it is important to consider that while VEGF-E does not activate VEGFR-1, the endogenous VEGF-A induced by the ischemia will be. As mentioned earlier there is evidence that vascular permeability involves both VEGFR-1 and VEGFR-2 activation. Assuming this is the case it is possible that the simultaneous activity of both endogenous VEGF-A and exogenous VEGF-E in the current experiment may lead to higher levels of vascular permeability than VEGF-E alone. This could negate the beneficial effects of VEGF-E and explain the lack of beneficial effects. It would be

interesting to look into the effects of VEGF-E on vascular permeability in a system devoid of VEGF-A.

The current study was also limited by high variability. One problem with the current modified Levine model is that it has been linked to high variability in deficits between individuals (Rivers, Sutherland, & Ashton, 2011), with the Levine model in one study yielding infarcts ranging from 0-87.5% of the hemisphere ipsilateral to CCAO (Saeed et al., 1993). This is believed to be due to individual differences in anatomical and physiological ability to compensate for the reduced oxygen supply (Brown, 1966; Rivers et al., 2011). Rivers et al. (2011) recently identified that seizure scores during the hypoxia provide better indicator of ischemic damage than the length of hypoxia. Animals were removed from the chamber after a tonic clonic seizure occurred. They found that there was a significant reduction in the variability of infarct sizes when compared with animals that received a set time of hypoxia. This method would be favourable as an increased statistical power may have better reflected any differences between treatment groups.

It is also important to consider that the treatments may also not have occurred at the optimum time. While the studies of Yang et al. (2009a; 2010) do support intranasal VEGF-A at days 3-5, it is possible that earlier treatment would be more effective as no time course study was undertaken. This is particularly the case with VEGF-E as it induces less inflammation and oedema than VEGF-A, and these may be the reasons why VEGF-A is ineffective immediately after ischemia. It will be important for future studies to identify the optimal time that VEGF-A and VEGF-E can be effective, and where this crossover between neurotoxicity and repair occurs, as this will not only improve our knowledge of the viability

of VEGF-A and VEGF-E as potential treatments in stroke, but also potentially give further insights into the progression of ischemic damage.

The current study did not support an efficacy of intranasal VEGF-A or VEGF-E to reduce infarct size or improve behavioural recovery. Overall the current results do not support VEGF-A or VEGF-E as a treatment for stroke. As we are the second group to attempt intranasal VEGF-A and the first to attempt intranasal VEGF-E it is likely that these results are due to the differences in experimental design compared with that of Yang et al. (2009a; 2010) and/or the high variability observed in the current study. It will be important for further studies to validate the efficacy of VEGF-A and VEGF-E in stroke models; specifically to confirm VEGF-E uptake via intranasal delivery, the optimal time of delivery and optimal dosing.

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