Development of vascular regulation in the zebrafish embryo.

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Development of vascular regulation in the zebrafish embryo.

by

Nabila Bahrami

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
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ABSTRACT

The vascular system is placed under enormous stress at the onset of cardiac contractility and blood flow. Nascent blood vessel tubes initially consist of a thin endothelial wall and rapidly acquire support from mural cells (pericytes and vascular smooth muscle cells; vSMCs). Following their association with vessels, mural cells acquire vasoactive ability (contraction and relaxation). However, we have little information as to when this vasoactivity first develops, and the extent to which each mural cell type contributes to vascular tone regulation during development. For the first time in an in vivo system, we highlight the dynamic changes in mural cell vasoactivity during development. We assess mural cell vasoactivity in the early zebrafish (Danio rerio) cerebral vasculature in response to pharmacological agents. We determine that pericyte-covered vessels constrict and dilate at 4 days post fertilization (dpf) but not at 6 dpf. The prostaglandin EP4 receptor contributes to pericyte-covered vessel dilation at 4 dpf. In contrast, vSMC-covered vessels constrict but do not dilate at 4 dpf. At 6 dpf, vSMC-covered vessels continue to constrict but only dilate from a pre-constricted state. Using genetic ablation, we demonstrate that mural cell contraction and relaxation is an active response by pericytes and vSMCs. Thus, we show that both pericytes and vSMCs have the ability to regulate cerebral vascular tone but at different stages of development. Pericytes are involved in regulating vessel diameters prior to the maturation of the vSMCs. Once vSMCs mature, pericytes are no longer active, and only vSMCs mediate vasomotor activity in the developing embryonic brain of zebrafish. The onset of vSMC vasoactivity corresponds to the development of increased neuronal activity and neurovascular coupling.
ACKNOWLEDGEMENTS

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<tbody>
<tr>
<td>acta2</td>
<td>α-smooth muscle actin</td>
</tr>
<tr>
<td>AC</td>
<td>adenyl cyclase</td>
</tr>
<tr>
<td>AH23848 hemicalcium salt hydrate</td>
<td>PGE$_2$ EP$_4$ receptor antagonist</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BA</td>
<td>basilar artery</td>
</tr>
<tr>
<td>BBB</td>
<td>blood brain barrier</td>
</tr>
<tr>
<td>BK$_{Ca}$</td>
<td>big conductance Ca$<em>{2+}$-activated K$</em>+$/channel</td>
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<tr>
<td>bmp3</td>
<td>bone morphogenetic protein 3</td>
</tr>
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<td>CaDI</td>
<td>caudal division of the internal carotid</td>
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<td>chondroitin sulfate proteoglycan 4</td>
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<td>CSVD</td>
<td>cerebral small vessel disease</td>
</tr>
<tr>
<td>CtAs</td>
<td>central arteries</td>
</tr>
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<td>DA</td>
<td>dorsal aorta</td>
</tr>
<tr>
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<td>diacylglycerol</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
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<td>dpf</td>
<td>days post fertilization</td>
</tr>
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<td>endothelial cell</td>
</tr>
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<td>endothelial nitric oxide synthase</td>
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<td>prostaglandin E$_2$ receptor</td>
</tr>
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<td>FGPs</td>
<td>fluorescent granular perithelial cells</td>
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<tr>
<td>FoxF2</td>
<td>forkhead box F2</td>
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<td>GDP</td>
<td>guanosine diphosphate</td>
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<td>G protein coupled receptor</td>
</tr>
<tr>
<td>GS</td>
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<td>GTP</td>
<td>guanosine triphosphate</td>
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<tr>
<td>GWAS</td>
<td>genome wide association study</td>
</tr>
<tr>
<td>hpf</td>
<td>hours post fertilization</td>
</tr>
<tr>
<td>IP$_3$</td>
<td>inositol 1,4,5-trisphosphate</td>
</tr>
<tr>
<td>ISVs</td>
<td>intersegmental vessels</td>
</tr>
<tr>
<td>K$_{ATP}$</td>
<td>ATP-sensitive K$_+$ channel</td>
</tr>
<tr>
<td>K$_i$</td>
<td>inward rectifying K$_+$ channel</td>
</tr>
<tr>
<td>K$_v$</td>
<td>voltage-gated K$_+$ channel</td>
</tr>
<tr>
<td>MLC</td>
<td>myosin light chain</td>
</tr>
<tr>
<td>MLCK</td>
<td>myosin light chain kinase</td>
</tr>
<tr>
<td>MLCP</td>
<td>myosin light chain phosphatase</td>
</tr>
<tr>
<td>Mtz</td>
<td>metronidazole</td>
</tr>
<tr>
<td>NA</td>
<td>(−)-norepinephrine or noradrenaline</td>
</tr>
<tr>
<td>ng2</td>
<td>neural/glial antigen 2</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NONOate</td>
<td>diethylamine NONOate sodium salt hydrate</td>
</tr>
<tr>
<td>notch3</td>
<td>notch receptor 3</td>
</tr>
<tr>
<td>NSC</td>
<td>non-specific cation channel</td>
</tr>
<tr>
<td>NTR</td>
<td>nitroreductase</td>
</tr>
<tr>
<td>PCV</td>
<td>posterior cardinal vein</td>
</tr>
<tr>
<td>pdgfrβ</td>
<td>platelet-derived growth factor receptor β</td>
</tr>
<tr>
<td>PE</td>
<td>(R)-(−)-phenylephrine hydrochloride</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PGE&lt;sub&gt;2&lt;/sub&gt;</td>
<td>prostaglandin E&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>PGI&lt;sub&gt;2&lt;/sub&gt;</td>
<td>prostaglandin I&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>PHBC</td>
<td>primary hindbrain channels</td>
</tr>
<tr>
<td>PHS</td>
<td>primary head sinus</td>
</tr>
<tr>
<td>PIP&lt;sub&gt;2&lt;/sub&gt;</td>
<td>phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>Akt</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PKG</td>
<td>protein kinase G</td>
</tr>
<tr>
<td>PLC&lt;sub&gt;β&lt;/sub&gt;</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>PVMS</td>
<td>perivascular macrophages</td>
</tr>
<tr>
<td>RBC</td>
<td>red blood cell</td>
</tr>
<tr>
<td>ROCS</td>
<td>receptor-operated Ca&lt;sup&gt;2+&lt;/sup&gt; channels</td>
</tr>
<tr>
<td>ROCK</td>
<td>Rho-associated protein kinase</td>
</tr>
<tr>
<td>SERCA</td>
<td>sarco/endoplasmic reticulum Ca&lt;sup&gt;2+&lt;/sup&gt; ATPase</td>
</tr>
<tr>
<td>sGC</td>
<td>soluble guanylyl cyclase</td>
</tr>
<tr>
<td>SHH</td>
<td>sonic hedgehog signaling</td>
</tr>
<tr>
<td>SK&lt;sub&gt;Ca&lt;/sub&gt;</td>
<td>small conductance Ca&lt;sup&gt;2+&lt;/sup&gt;-activate K&lt;sup&gt;+&lt;/sup&gt; channel</td>
</tr>
<tr>
<td>SNAP</td>
<td>S-Nitroso-N-acetyl-DL-penicillamine</td>
</tr>
<tr>
<td>SNP</td>
<td>sodium nitroprusside dihydrate</td>
</tr>
<tr>
<td>SOC</td>
<td>store operated channel</td>
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<tr>
<td>tagln/sm2</td>
<td>transgelin</td>
</tr>
<tr>
<td>tagln2</td>
<td>transgelin 2</td>
</tr>
<tr>
<td>Tg</td>
<td>transgene</td>
</tr>
<tr>
<td>TRP</td>
<td>transient receptor potential</td>
</tr>
<tr>
<td>TGFβ</td>
<td>transforming growth factor-β</td>
</tr>
<tr>
<td>U46618</td>
<td>thromboxane A&lt;sub&gt;2&lt;/sub&gt; mimic</td>
</tr>
<tr>
<td>VDCC</td>
<td>L-type voltage dependent Ca&lt;sup&gt;2+&lt;/sup&gt; channel</td>
</tr>
<tr>
<td>vSMC</td>
<td>vascular smooth muscle cell</td>
</tr>
</tbody>
</table>
CHAPTER ONE: BACKGROUND AND INTRODUCTION

The introduction of this thesis contains text published as “Chapter 4: Pericyte Biology in Zebrafish” from the book “Pericyte Biology – Novel Concepts” as part of “Advances in Experimental Medicine and Biology, volume 110” published in 2019 in Springer series with myself and Dr. Sarah Childs as co-authors. The content of the book chapter is reprinted/adapted by permission from RightsLink Printable License: SPi Global, Springer, Pericyte Biology – Novel Concepts, Chapter 4: Pericyte Biology in Zebrafish by Nabila Bahrami and Sarah J Childs (© 2019).
1.1 Development of cerebral vessels in zebrafish

1.1.1 Prior to the start of neurovascular coupling

The brain requires roughly 20% of the cardiac output to carry out its function (Joutel and Faraci, 2014). Further, changes in neuronal activity are accompanied by local changes in blood flow, a process known as neurovascular coupling. The perfusion of cerebral parenchyma depends on the activity of endothelial and mural cells that make up the cerebral vasculature (Joutel and Faraci, 2014; Santoro et al., 2009). However, how blood flow is regulated, and the role of mural cells involved in regulating vascular tone prior to the start of neurovascular coupling remains unknown. We are focused on understanding the role of mural cells in regulating cerebral vessel diameters prior to the start of neurovascular coupling. Understanding the contractile property of mural cells in the early stages of development is crucial to elucidating the onset and mechanism of neurovascular coupling.

1.1.2 Vasculogenesis

In zebrafish, vascular endothelial cells (ECs) arise from the lateral mesoderm and become specified at the end of gastrulation (approximately 10 hours post fertilization; hpf; Reischauer et al., 2016; Stainier et al., 1995). First, angioblasts with an arterial identity migrate medially to initiate the formation of the dorsal aorta (DA) in the trunk (Fouquet et al., 1997; Jin, 2005; Kohli et al., 2013; Quillien et al., 2014). This is followed by the medial migration of venous-fated angioblasts to form the posterior cardinal vein (PCV; Lawson and Weinstein, 2002; Jin, 2005; Kohli et al., 2013; Quillien et al., 2014). In the process
of vasculogenesis, the first vessels that form, lumenize and connect with the heart to start a rudimentary blood flow (Xu and Cleaver, 2011). Secondary angiogenic sprouts then emerge from these vasculogenic vessels to vascularize the brain (Bussmann et al., 2011; Ulrich et al., 2016; Vanhollebeke et al., 2015), heart (Harrison et al., 2015), gut (Goi and Childs, 2016; Hen et al., 2015; Koenig et al., 2016) and trunk (Jin, 2005), among other organs.

1.1.3 Vascular mural cells stabilize cerebral vessels

A shift in focus from understanding EC biology to the genesis and maturation of vascular mural cells is providing new insights into the process of angiogenesis. In the brain, the vascular network is stabilized by perivascular cells, which include pericytes, vascular smooth muscle cells (vSMCs), astrocytes, microglia, perivascular macrophages (PVMs) and fluorescent granular perithelial cells (FGPs; originally known as Mato cells due to their first description by Masao Mato in 1979; Gaengel et al., 2009; Galanternik et al., 2017; Mato, 1979; Williams et al., 2001). Vascular mural cells, which are pericytes and vSMCs, interact with the abluminal surface of ECs to provide stability and integrity to vessels (Gaengel et al., 2009).

Although there are strong similarities between pericytes and vSMCs in terms of gene expression, there are also key differences in terms of gene expression, morphology and presence on the vessels (Hirschi and D’Amore, 1996). Once attached to the endothelium, pericytes are generally solitary cells. Their morphology varies along the vascular tree, but
they all have a rounded cell body with long processes, and attach to the longitudinal axis of capillaries (Figure 1; Armulik et al., 2005; Armulik et al., 2011; Attwell et al., 2016; Gaengel et al., 2009; Hartmann et al., 2015). Pericytes share the basement membrane with ECs, and the two cell types physically contact each other via gap junctions, adhesion plaques, and peg and socket connections (Armulik et al., 2005; Armulik et al., 2011; Sweeney and Foldes, 2018). In contrast, vSMCs wrap perpendicularly around the vessels. They are connected to ECs via myoendothelial gap junctions, allowing for direct signals from one cell to the other (Borysova et al., 2018; Heberlein et al., 2009; Straub et al., 2014). Vessel size determines the degree of coverage by vSMCs, as they form multi-layers around larger vessels and a single layer around smaller vessels (Santoro et al., 2009).
Figure 1: Mural cell coverage of cerebral vessels.
Pericytes are solitary cells that appear on the smaller cerebral vessels where they extend their long processes far along the surface of the capillary endothelium. vSMCs appear as bands and wrap around larger cerebral vessels such as the arteries and arterioles.
1.1.4 Developmental origin of head vascular mural cells

In zebrafish, mural cells of the forebrain and midbrain are of a different lineage than those of the hindbrain. Through the use of double morpholino knockdown experiments (Wang et al., 2011), knockdown experiments (Wang et al., 2014; Whitesell et al., 2014) and morphant zebrafish (Ando et al., 2016), mural cells of the forebrain and midbrain are known to be neural crest derived. The use of “switch” reports, activated by the expression of Cre recombinase, under lineage-specific promoters (neural crest or lateral mesoderm) have been used to further confirm that the neural crest gives rise to forebrain and midbrain vSMCs (Ando et al., 2016; Cavanaugh et al., 2015) while the mesoderm gives rise to hindbrain pericytes (Ando et al., 2016).

1.1.5 Spatiotemporal expression of vascular mural cell markers

In general, the markers platelet-derived growth factor receptor β (pdgfrβ), notch receptor 3 (notch3), neural/glial antigen 2 (ng2) or chondroitin sulfate proteoglycan 4 (cspg4) are used to identify pericytes. The markers α-smooth muscle actin (acta2), transgelin (tagln/sm2) and transgelin 2 (tagln2) are used to identify smooth muscle cells.

Pdgfrβ serves as one of the earliest markers of pericytes in zebrafish (Figure 2; Ando et al., 2016; Wang et al., 2014). In mammals, the Pdgfbb ligand of the platelet-derived growth factor (PDGF) signaling pathway is expressed in the endothelium and signals via its receptor, Pdgfrβ, in pericytes to promote pericyte recruitment to the blood vessels (Hirschi et al., 1998; Leveen et al., 1993). In the zebrafish head, in situ hybridization reveals pdgfrβ expression in the neural crest at 20 hpf (French et al., 2014). At 48 hpf,
cells expressing the pericyte markers *pdgfrβ* and *notch3* appear around the larger arteries and veins of the brain, including the basilar artery (BA) and the primary hindbrain channels (PHBC; Wang et al., 2014). This early pericyte population also proliferates. At 48 hpf, there are ~20 *pdgfrβ*+ve cells associating with the larger arteries while at 56 hpf, this number increases to ~50 *pdgfrβ*+ve cells (Wang et al., 2014). These pericyte numbers are consistent with increasing pericyte coverage through development.
Figure 2: Spatiotemporal expression of vascular mural cell markers.

Early vascular mural cell marker *pdgfrb* can be seen at the 8-somite stage in the neural crest. At 17 hpf, it also appears in the base of the brain. By 2 dpf, markers *pdgfrβ* and *notch3* begin to be specified as pericycle markers and appear around the larger arteries and veins of the brain, including the BA and PHBC. At roughly 2.5 dpf, *pdgfrβ* marks only pericytes. As for vSMCs, at around 2 dpf, *tagln2* can be seen in the brain in but becomes difficult to detect by 3 dpf. The vSMC specific marker, *acta2*, turns on at roughly 3.5 dpf and continues to mark vSMCs as the fish grows.

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More recently, transgenic lines have been used to track pericyte appearance on cerebral vessels since they allow for a much earlier detection. These lines allow for \( \text{pdgfr} \beta \) expression at the 8-somite stage in the neural crest and at 17 hpf, in the base of the brain (Ando et al., 2016). In the hindbrain, transgenic \( \text{pdgfr} \beta^{+ve} \) cells are seen at 60 hpf, a time when arteries are being formed at the base of the brain (Ando et al., 2016). Pericyte coverage of the hindbrain central arteries (CtAs) is progressive, with few weakly \( \text{pdgfr} \beta^{+} \)-positive cells sitting ventrally to the forming CtAs and adjacent to BA in early development. These cells then move along the vessels and proliferate to cover the CtAs by 120 hpf (Figure 3).

**Figure 3: Progressive pericyte coverage of cerebral vessels in zebrafish.**

\( \text{pdgfrb}^{-} \) cells appear on the BA and the PHBC at 48 hpf. By 60 hpf, \( \text{pdgfr} \beta^{+} \) cells begin to move onto the cerebral CtAs, and their number also increases. By 120 hpf, \( \text{pdgfr} \beta^{+} \) cells cover all the CtAs.

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Although zebrafish cerebral pericytes and smooth muscle cells develop in parallel with one another, cerebral smooth muscle cells are first visualized later than cerebral pericytes. In situ hybridization reveals tagln2 expression in the head at 48 hpf, but then becomes difficult to detect by 72 hpf (Wang et al., 2014). tagln expression is also difficult to visualize in the early brain using the transgenic zebrafish TgBAC(tagln:EGFP). Thus, in order to examine vSMCs, a smooth muscle actin line was generated (Tg(acta2:EGFP); Whitesell et al., 2014). In this transgenic line, acta2:EGFP expression does not overlap with the expression of any pericyte marker, confirming that acta2 only marks vSMCs. This Tg(acta2:EGFP) line was then used to detect vSMC expression in the zebrafish head. In the ventral head, this line is expressed at around 3-3.5 dpf while in the dorsal head, only a few Tg(acta2:EGFP) or acta2+ve cells can be seen to associate with the cerebral vessels by 4 dpf (Whitesell et al., 2014). By 7 and 11 dpf, acta2 expression begins to appear around the larger cerebral vessels and continues throughout the zebrafish life (Whitesell et al., 2014).

1.2 Signaling pathways involved in mural cell development, differentiation and recruitment

1.2.1 Sonic hedgehog signaling (SHH) is required for pericyte development

There are multiple pathways involved in mural cell development and recruitment to the vessels. SHH (Kallakuri et al., 2015; Lamont et al., 2010) and transforming growth factor-β (TGFβ)/bone morphogenic protein 3 (bmp3; Lei et al., 2017) signaling pathways are
involved in mural cell development. Compromised SHH results in decreased or a lack of perivascular mural cells on the brain vessels (Lamont et al., 2010). Compromised TGFβ/BMP signaling results in hemorrhage and a leakage in the blood brain barrier (BBB) due to an impairment in pericycle coverage (Lei et al., 2017).

1.2.2 Notch and PDGF signalling are required for mural cell recruitment to the endothelium

It is well established that notch1 is expressed in the endothelium and serves to recruit pericytes to the endothelial lining of blood vessels. Pericytes express the Notch3 receptor and vSMCs are likely to also express notch3 (Wang et al., 2014). Notch3 mutants possess a curved body axis and at 3 dpf, a small percentage of the notch3 mutants present with hemorrhage in the brain, either with a small focal hemorrhage or with a broad hemorrhage in the brain ventricles (Wang et al., 2014). Further, hemorrhage in these mutants is accompanied by defects in the BBB as these notch3 mutants have a reduced pericyte population but no defects in the endothelium (Wang et al., 2014). Thus, the effect of Notch3 signaling in pericytes is to positively regulate pericycle numbers and therefore stabilize the BBB (Wang et al., 2014).

In mammals, PDGFs have multiple ligands (PDGF-AA, PDGF-BB, PDGF-CC and PDGF-DD) and two receptors, which are PDGFRα and PDGFRβ (Pitulescu and Adams, 2014). ECs express the ligand (PDGF-BB) in tip cells while mural cells express the receptor, PDGFRβ. In mice, pdgfrβ ablation results in mural cell deficiency (Gaengel et al., 2009). In zebrafish, pdgfrβ genetic loss of function mutations or inhibition of the Pdgfrβ receptor,
using small molecule inhibitors such as AG1296, also result in decreased mural cell numbers (Ando et al., 2016; Wang et al., 2014). Thus, Pdgfrβ signalling is involved in the proliferation and recruitment of mural cells but is not essential for their specification.

1.3 Pericyte recruitment to the endothelium

1.3.1 Are hemodynamic forces required to recruit pericytes to the endothelium?

The process of mural cell recruitment to the endothelium is poorly understood, and there are conflicting reports. In zebrafish, when blood flow initiates at approximately 26 hpf, it induces shear stress on the arterial endothelium. In this low-flow state, shear stress causes deflection of endothelial cilia that function as mechanosensors and induces the recruitment of mural cells to the endothelium (Chen et al., 2017; Goetz et al., 2014). However, another study found that mural cell recruitment is flow-independent around the BA and CtAs of the brain (Ando et al., 2016). Also, a lack of blood flow did not affect mural cell development on the DA and intersegmental vessels (ISVs; Ando et al., 2016). Our understanding of mural cell recruitment is still evolving.

1.3.2 Mural cell migration following association with vessels

Once associated with the ECs, pericytes of the cerebral arteries extend their processes along EC junctions to cover blood vessels (Ando et al., 2016). Normally, it is the tips of pericyte processes and not the entire length of the processes that attach to the inter-EC junctions (Ando et al., 2016). To migrate, pericytes use the stiffness of endothelial
junctions as scaffolding, with their processes preceding along the junction before relocating their entire cell bodies along the scaffold (Ando et al., 2016). In contrast, smooth muscle cells of the ventral head move only minimally once the acta2 marker is expressed (Whitesell et al., 2014).

1.4 Contractile machinery in vascular mural cells
A rise in intracellular calcium concentration and cell depolarization drives contraction in both mural cell types (Figures 4 and 5). During relaxation, a decrease in intracellular calcium concentration and cell hyperpolarization occur (Figures 6 and 7).

1.4.1 Mechanism of contraction in vascular mural cells
In vSMCs, when agonists such as norepinephrine/noradrenaline (NA) and phenylephrine (PE) bind to their $\alpha_1$ adrenergic G-protein coupled receptor (GPCR) on the cell membrane, it results an increase in intracellular calcium concentration (Figure 4; Liu and Khalil, 2018; Webb, 2003). These receptors are coupled to guanosine triphosphate (GTP) binding protein Gq. Receptor-ligand signalling activates phospholipase C (PLC$\beta$), which stimulates the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP$_2$) into inositol 1,4,5-trisphosphate (IP$_3$) and diacylglycerol (DAG; Liu and Khalil, 2018; Webb, 2003). Water soluble IP$_3$ diffuses into the cytosol and binds to its receptors on the sarcoplasmic reticulum causing calcium release (Liu and Khalil, 2018; Webb, 2003). Unlike IP$_3$, DAG is water insoluble and remains on the cell membrane, where it activates protein kinase C (PKC; Liu and Khalil, 2018; Webb, 2003). PKC inhibits the activity of potassium channels, preventing cell hyperpolarization, and induces the activity of calcium channels (Liu and
Khalil, 2018; Webb, 2003). Specifically, calcium enters the cell via L-type voltage dependent Ca$_{2+}$ channels (VDCCs), receptor-operated Ca$_{2+}$ channels (ROCS), transient receptor potential (TRP), store operated channel (SOC), stretch activated calcium channels and non-specific calcium leak channels (Liu and Khalil, 2018; Webb, 2003; Zhao et al., 2015). NA is known to activate VDCC (Liu and Khalil, 2018). As for potassium channels, large conductance Ca$_{2+}$ activated K$^+$ channels (BK$_{Ca}$) are predominant in vSMCs. PKC signalling inhibits the activity of BK$_{Ca}$, ATP-sensitive K$^+$ channels (K$_{ATP}$) and voltage-gated K$^+$ channels (K$_v$; Liu and Khalil, 2018; Webb, 2003). PKC further inhibits the activity of guanylyl cyclase (GC), a key player in vSMC relaxation (Liu and Khalil, 2018).

Overall, an increase in intracellular calcium results in calcium binding to calmodulin (CaM), which then activates myosin light chain (MLC) kinase (MLCK; Liu and Khalil, 2018; Webb, 2003). MLCK phosphorylates MLC and results in actin-myosin cross-bridge formation, causing vSMC contraction (Figure 4; Liu and Khalil, 2018; Webb, 2003).
Figure 4: Mechanism of contraction in vSMCs.

The binding of vasoconstrictors such as NA and PE to their receptors on the surface of vSMCs activates PLCβ, which hydrolyzes PIP₂ into IP₃ and DAG. IP₃ promotes calcium release from the sarcoplasmic reticulum while DAG activates PKC. PKC activates calcium channels and inhibits potassium channels to cause cell depolarization and prevent cell hyperpolarization. This results in the phosphorylation of MLC and subsequent cross-bridge formation between actin and myosin, resulting in vSMC contraction. The thick arrows illustrate substantial activity.
The phosphorylated state of MLC is regulated by myosin light chain phosphatase (MLCP), which is regulated by Rho kinase activity (Liu and Khalil, 2018; Webb, 2003). When Rho-associated protein kinase (ROCK) is active, in its GTP state, it phosphorylates the myosin binding unit of MLCP, which is MYPT1, causing it to become inactive (Liu and Khalil, 2018; Webb, 2003). In an inactive state, MLCP cannot dephosphorylate MLC, resulting in actin-myosin cross-bridge formation and vSMC contraction (Liu and Khalil, 2018; Webb, 2003). However, when ROCK is inactive, in its guanosine diphosphate (GDP) form, it cannot phosphorylate the myosin binding unit of MLCP (Liu and Khalil, 2018; Webb, 2003). This results in an active MLCP, which dephosphorylates MLC and brings about the end of contraction (Liu and Khalil, 2018; Webb, 2003).

In pericytes, contraction occurs in a similar manner with VDCC being the major player in increasing intracellular calcium concentration (Figure 5). Vasoconstrictors will either cause depolarization via direct activation of VDCC, or by first activating non-specific cation channels (NSC), which then activate VDCC (Hamilton et al., 2010). When calcium enters pericytes via VDCC and NSC, it activates Ca\textsubscript{2+}-activated Cl\textsuperscript{−} channels (Cl\textsubscript{Ca}) to open, allowing for chloride to leave the cell, further promoting depolarization via VDCC activity (Hamilton et al., 2010). Extracellular adenosine triphosphate (ATP) also activates VDCC by binding to the P2X\textsubscript{7} receptor. ATP further causes calcium entry into the cell via P2X\textsubscript{7} and P2Y\textsubscript{4} receptors, both of which are calcium permeable (Hamilton et al., 2010). Following an increase in intracellular calcium level, the mechanism of pericyte contraction is similar to that of vSMCs (Hamilton et al., 2010).
Figure 5: Mechanism of contraction in pericytes.

The binding of vasoconstrictors, or agonists, to their receptors on the surface of pericytes can activate VDCC directly or indirectly. VDCC activity results in an increase in intracellular calcium concentration. This rise in calcium promotes the interaction of calcium with CaM, which activates MLCK. Activated MLCK phosphorylates MLC, which results in actin and myosin cross-bridge formation and pericyte contraction. MLCP activity is regulated by ROCK. Active ROCK will promote contraction by phosphorylating MLCP and causing it to become inactive. In its inactive state, ROCK cannot phosphorylate MLCP. Thus, MLCP dephosphorylates MLC, bringing about the end of phosphorylation. The dotted arrows indicate mechanisms that are unknown in pericytes but are well-established in other cell types.
1.4.2 Mechanism of relaxation in vascular mural cells

In endothelial cells, nitric oxide (NO) is produced by endothelial nitric oxide synthase (eNOS) and is the major form of NOS regulating vascular function (Zhao et al., 2015). eNOS stimulation can occur in two ways, both of which depend on calcium. In one mechanism, when agonists, such as acetylcholine, bradykinin, and histamine among others, bind to their receptors on the endothelial surface, it causes an increase in endothelial intracellular calcium concentration (Figure 6; Zhao et al., 2015). Calcium binds to CaM, and this complex then binds to the CaM-binding domain of eNOS, activating eNOS (Zhao et al., 2015). In the second mechanism, stimuli such as shear stress, hormones, and growth factors among others, stimulate eNOS via phosphorylation. Phosphorylation of eNOS increases its sensitivity for calcium, which results in its activation. When eNOS is phosphorylated at the Ser1177 site via protein kinase A (PKA) and protein kinase B (Akt), it is stimulated (Zhao et al., 2015). Phosphorylation at the Thr495 site has an inhibitory effect (Zhao et al., 2015). Stimuli such as hemodynamic stress induce eNOS activity via phosphorylation at the Ser635 site in a PKA independent manner (Zhao et al., 2015). The stimulated eNOS eNOS activity generates NO, which diffuses across the endothelium to vSMCs (Zhao et al., 2015).

In vSMCs, NO acts on soluble GC (sGC) to promote the formation of cyclic guanosine monophosphate (cGMP; Hamilton et al., 2010; Zhao et al., 2015). cGMP activates PKG, which stimulates the sarco/endoplasmic reticulum Ca\textsuperscript{2+} ATPase (SERCA) to reuptake calcium and promotes the expulsion of calcium from vSMCs (Zhao et al., 2015). The reuptake of calcium back into the sarcoplasmic reticulum is ATP dependent and functions
via the Ca\textsubscript{2+}/Mg\textsubscript{2+} ATPase, which is also present on the plasma membrane (Webb, 2003). When the Ca\textsubscript{2+}/Mg\textsubscript{2+} ATPase is phosphorylated, it binds to two Ca\textsubscript{2+} ions and moves them back into the sarcoplasmic reticulum or out of the cell (Webb, 2003). There is also a Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger present on the plasma membrane, which works to remove calcium from vSMCs (Webb, 2003). NO can also independently stimulate SERCA (Zhao et al., 2015). All this machinery works to decrease intracellular calcium, uncoupling calcium from CaM, and preventing the activation of MLCK. Further, PKG increases the activity of MLCP, resulting in relaxation (Figure 6; Webb, 2003).
Figure 6: Mechanism of relaxation in vSMCs.
eNOS activity results in NO production. NO diffuses into the vSMC where it acts on sGC. This results in the activation of PKG, which decreases intracellular calcium concentration, resulting in vSMC relaxation.
As for pericytes, vasodilators such as prostaglandin E2 (PGE₂) bind to their receptors on the cell surface to elicit vasodilation by activating potassium channels (Figure 7). This receptor-ligand signalling activates adenylyl cyclase (AC), which activates PKA by increasing cyclic adenosine monophosphate levels (cAMP; Hamilton et al., 2010). cAMP can also activate PKG since intracellular cAMP levels are relatively higher than that of cGMP. Thus, PKG becomes sensitive to cAMP via autophosphorylation (Hamilton et al., 2010). This agonist-receptor mechanism is complementary to the NO induced pericyte relaxation mechanism.

PKG is also responsive to NO, which similar to vSMCs, activates sCG and increases cGMP levels (Hamilton et al., 2010). In pericytes, PKG reduces the activity of VDCC by causing pericyte hyperpolarization via increased activity of big and small conductance Ca²⁺-activated K⁺ channels (BKCa and SKCa), inward rectifying channel (Kir), voltage-gated K⁺ channel (Kv) and ATP-sensitive K⁺ channel (KATP; Hamilton et al., 2010). NO also inhibits Cl⁺, reducing calcium entry into the cell (Hamilton et al., 2010). Potassium can also exit the cell via NSC and the P2X₇ receptor (Hamilton et al., 2010). Together, PKA and PKG increase the activity of MLCP, resulting in pericyte relaxation (Figure 7; Hamilton et al., 2010).
Figure 7: Mechanism of relaxation in pericytes.

The presence of NO and the binding of vasodilators, such as PGI$_2$, to their receptors activates PKG. PKG activates potassium channels to hyperpolarize the cell and reduces the activity of VDCC. Together, PKA and PKG activate MLCP, which dephosphorylates MLC, causing pericyte relaxation.
1.5 Vascular mural cells regulate vascular tone

In vitro, in vivo, and ex vivo approaches in the adult mouse model have shown that while there is robust control of vascular diameter by vSMCs, pericytes have more limited and controversial roles. The contraction of pericytes was first largely based on indirect evidence (Díaz-Flores et al., 2009; Hamilton et al., 2010; Puro, 2007). For instance, pericytes were found to contract during ischemia and remain contracted even after reperfusion of the occluded artery (Yemisci et al., 2009). Other studies found that although pericytes are contractile and capable of modulating cerebral blood flow (CBF), they do not play a major role in the process of neurovascular coupling (Fernandez-Klett et al., 2010). However, in some of these early studies, pericytes were identified by their morphology without the use of markers. Using adult rat brain slices and in vivo methods in the mouse brain, Hall et al. found that pericytes rather than vSMCs regulate cerebral vessel diameters and the consequent changes in red blood cell (RBC) velocity (Hall et al., 2014). They found pericyte-covered capillaries to dilate prior to vSMC-covered arterioles. However, others have shown that smooth muscle actin is only present in mural cells with a smooth muscle morphology and not in cells with a pericyte morphology (Hill et al., 2015). Using the adult mouse cerebral cortex, Hill et al. showed that vSMCs provide baseline contractile tone, and that only depolarization of vSMCs results in alterations of vessel diameter and the regulation of CBF (Hill et al., 2015). It is important to note that hemodynamic variability within the microcirculation may account for some of these differences in findings (Gould et al., 2017). Overall, with the adult mouse model, there is no consensus on mural cell vasoactivity and the corresponding changes in CBF regulation.
We are using zebrafish to study the function of mural cells in regulating vascular tone during development. While cerebral pericytes and vSMCs develop at different times, they are also of different developmental origins depending on cerebral anatomical location. Consequently, there may be differences between cerebral anatomical locations and mural cell function. We have few insights into vascular tone regulation in the zebrafish, and most of the data is from the trunk. The dorsal aorta (DA) acquires tone between 48 and 80 hpf and the diameter of the DA varies inversely with vSMC number (Stratman et al., 2017). Furthermore, \textit{p}dgfr\textit{\beta} regulates the development of vascular tone in the zebrafish trunk (Stratman et al., 2017), likely through regulating mural cell differentiation. However, there is no information about the acquisition of vasoactivity in brain vessels during development. Here, we test the contractile properties of brain mural cells in zebrafish and determine the corresponding changes in cerebral vessel diameters during development.

**Hypothesis:** Both pericytes and vSMCs mediate vascular tone.
CHAPTER TWO: MATERIALS AND METHODS
2.1 Zebrafish husbandry and fish strains

All experimental procedures were approved by the University of Calgary’s Animal Care Committee (Protocol AC17-0189). Zebrafish embryos were maintained at 28.5°C and in E3 medium in the dark (Westerfield, 1995). Transgenic lines used include: TgBAC(pdgfrβ:GFP)ca41 (Whitesell et al., 2019), TgBAC(pdgfrβ:Gal4)ca42 (Whitesell et al., 2019), Tg(kdrl:mCherry)ci5 (Proulx et al., 2010), Tg(acta2:GFP)ca7 (Whitesell et al., 2014), Tg(acta2:Gal4FF)ca62 (Whitesell et al., 2019), Tg(flk:GFP)la116 (Choi et al., 2007), Tg(UAS:NTR-mCherry)c264 (Davison et al., 2007), and Tg(GFAP:GFP)mi2001 (Bernardos and Raymond, 2006).

2.2 Pharmacological agents and nitroreductase (NTR) ablation

All chemicals were obtained from Sigma Aldrich (St. Louis, MO). Sodium nitroprusside dihydrate (SNP; 71778) was used at 1 mM. S-Nitroso-N-acetyl-DL-penicillamine (SNAP; N3398) was used at 100 μM. Diethylamine NONOate sodium salt hydrate (NONOate; D184) was used at 5 μM. I-(−)-Phenylephrine hydrochloride (PE; P6126) was used at 10 μM. (−)-Norepinephrine (NA; A7257) was used at 2 μM. The prostaglandin E2 receptor subtype 4 (EP4) antagonist, AH23848 hemicalcium salt hydrate (AH23848; A8227), was used at 10 μM. All vasoactive agents were dissolved in E3 fish medium except for AH23848, which was dissolved in 0.1% dimethyl sulfoxide (DMSO) in E3.

For ablation experiments, metronidazole (Mtz; M3761) was used at 50 μM for 3 dpf mural cell ablation. At 5 dpf, 50 μM was used for vSMC ablation and 5 mM was used for pericyte ablation.
2.3 Heart rate measurements

Dose response curves were carried out to determine optimal drug concentrations. For each optimal drug concentration identified, the effect on heart rate was assessed. Heart rate was counted manually for a period of one minute. For each vasoactive agent, 5-6 zebrafish were exposed individually to either E3 fish medium or the vasoactive agent dissolved in E3 medium.

2.4 Microscopy and image analysis

Zebrafish were mounted on glass bottom petri dishes (MatTek, Ashland, MA, Cat. No. P50G-0-30-F), using 0.8% low melt agarose (Invitrogen (Carlsbad, CA) 16520-050), which was dissolved in E3 fish medium. Confocal imaging was conducted with a Zeiss LSM 700 AxioObserver confocal microscope. All images were obtained with the 488 nm and 555 nm lasers, with a pinhole of 1 airy unit (AU). All images were 12-bit with slice intervals of 1-2 μm, line step of 2, with a 10X (NA 0.25 Ph1) or 20X (NA 0.8) objective and either 512x512 or 1024x1024 in frame size. The scaling in xyz directions (per pixel) ranged from 0.09 μm x 0.09 μm x 1.00 μm to 0.56 μm x 0.56 μm x 1.00 μm. The z-stacks varied in range from 35 -240 slices depending on the image. Images were processed using Zen Blue and or ImageJ/Fiji. In ImageJ/Fiji, maximum intensity projection was carried out on the confocal images for all images prior to any measurements.

Vessel diameters were measured at positions where there was an association of a mural cell with the endothelium. The vessel diameter was measured from the outer diameter of
the endothelium. Three measurements were obtained from each mural cell-endothelium association region and averaged to obtain a vessel diameter. All vessel diameter measurements were paired before and after drug treatment.

2.4 Statistics

All data were graphed as normalized to baseline measurements unless otherwise indicated. GraphPad Prism7 was used to carry out all statistics using either a paired two-tailed t-test, unpaired two-tailed t-test or repeated measures ANOVA with $P \leq 0.05 = \ast$, $P \leq 0.005 = \ast\ast$, $P \leq 0.0005 = \ast\ast\ast$. From the repeated measures ANOVAs, $p$ values from Dunnett’s test are reported unless otherwise indicated. When comparing average vessel diameter at each minute, either Tukey’s or Sidak’s multiple comparison tests are carried, which is indicated in the text. All data are represented as mean ± standard deviation (SD).
CHAPTER THREE: RESULTS
3.1 Pericytes and vSMCs appear on cerebral vessels of different diameters

Pericytes and vSMCs are first visualized in the zebrafish brain at 48 hpf and around 80 hpf respectively with their abundance increasing over time (French et al., 2004; Wang et al., 2004). We examine mural cell activity at two stages of development: 4 dpf, when vSMCs start to express *acta2*, and when *pdgfrβ* expressing pericytes have been present for about 2 days, and 6 dpf, when vSMCs have been present for about 2 days. First, we wanted to confirm the identified stereotypical locations for pericytes and vSMCs along the cerebral vessels. At 4 dpf, pericytes are present on smaller vessels with diameters ≤6.5 μm (Figure 8A and B), typically the brain CtAs. vSMCs cover the larger vessels that are ≥9.5 μm in diameter (Figure 8A and F). Early vSMC-covered vessels (at 4 dpf) include the caudal division of the internal carotid (CaDI) and the BA. Cerebral vessels with diameters between 6.5μm and 9.5μm have variable coverage by both pericytes and vSMCs and were excluded from further analysis as we could not clearly separate functions of mural cells in this size range of vessels.

In the zebrafish brain at 4 dpf, pericytes appear sporadically along vessels with long processes that extend along the surface of the endothelium (Figure 8C- E‴). vSMCs appear on only a few brain vessels and form a continuous sheet around the vessel (Figure 8G- I‴). By 6 dpf, the total numbers of pericytes and vSMCs is higher than at 4 dpf. There are more pericytes than vSMCs at both dpf (Figure 9A-B). At 6 dpf, vessels with diameters ≤6.5 μm continue to be primarily covered by pericytes while those with diameters of ≥9.5 μm are primarily covered by vSMCs. At 6 dpf, pericyte processes are in close proximity and appear to contact each other (Figure 9C-F‴). vSMC coverage of the cerebral
endothelium also extends to more dorsal vessels over time (Figure 9G-J’’).
Figure 8: Mural cell morphology and coverage of midbrain cerebral vessels at 4 dpf.

A) Occurrence of mural cell types as a function of vessel diameter. Pericyte are predominantly present on vessels with diameters of ≤6.5 μm (n=220 vessels), while vSMCs appear on larger cerebral vessels (≥9.5 μm; n=119 vessels). Vessels with a diameter of 6.5 μm< and >9.5 μm are covered by a mix of pericytes and vSMCs. B) Lateral image of cerebral vessel pericyte coverage at 4 dpf. C-E””) Insets of vessels outlined in B. The circles highlight pericyte cell bodies while the arrows point to their processes. F) Lateral image of cerebral vessel vSMC coverage at 4 dpf, highlighting vSMC on the BA and CaDI. G-I) Insets of vessels in F. Scale bars represent 50 μm for B and F, and 10 μm for C-E”” and G-I””. A, P, V and D refer to anterior, posterior, ventral and dorsal.
Figure 9: Mural cell morphology and coverage of midbrain cerebral vessels at 6 dpf.

A) A model of pericyte and vSMC locations on the 6 dpf cerebral vessels. PHS refers to primary head sinus. B) Pericyte are mostly found on vessels with diameters of ≤6.5 μm (n=215 vessels), while vSMCs appear on the larger cerebral vessels (≥9.5 μm; n=130 vessels). Cerebral vessels with diameters of 6.5 μm< and >9.5 μm are covered by a mix of pericytes and vSMCs. C) Lateral image of cerebral vessel pericyte coverage at 6 dpf. D-F’’) Insets of vessels in C highlighting contact between the processes of different pericytes. The circles highlight pericyte cell bodies while the arrows point to their processes. G) Lateral image of cerebral vessel vSMC coverage at 6 dpf, particularly on the BA and CaDI. H-J’’) Insets of the vessels in G showing extensive vSMC coverage. Scale bars represent 50 μm for C and G, and 10 μm for D-F’’ and H-J’’). A, P, V and D refer to anterior, posterior, ventral and dorsal.
3.2 Early contraction of pericytes to noradrenaline is lost when vSMCs mature

To determine if mural cells are capable of contraction during early development, we utilized $\alpha_1$ adrenergic agonists (Figures 4 and 5). We carried out dose-response curves for vasoconstricting agents and considered published concentration usage in zebrafish. As a lack appears to act as a confounding variable, we selected 2 $\mu$M of NA to use for experiments (Figure 10A-D). As changes in heart rate might affect vascular dynamics, we determined that heart rate was unchanged with this concentration (Figure 11A). To determine changes in vessel diameter, the cerebral vasculature was imaged before dosing (0 min) and repeatedly every minute after NA treatment (1-4 minutes; Figure 12A). At 4 dpf, pericyte-covered vessels constrict in response to NA within the first minute of treatment ($P=0.0001$; Figure 12B and D-H). Constriction persists for the duration of NA treatment and over time, vessels constrict further. The average pericyte-covered vessel diameters at 0, 1, 2, 3, and 4 minutes were 5.3 $\mu$m ± 0.7, 5.1 $\mu$m ± 0.7, 5.0 $\mu$m ± 0.7, 5.0 $\mu$m ± 0.8 and 5.0 $\mu$m ± 0.8 respectively. We note that of the 60 vessels studied, 31 vessels constrict consistently throughout the experimental period whereas the other pericyte-covered vessels behave inconsistently and demonstrate both dilation and constriction. Surprisingly, we found that at 6 dpf, pericyte-covered vessels no longer constricted to NA (Figure 12N). The average pericyte-covered vessel diameter at 0, 1, 2, 3 and 4 minutes were 5.1 $\mu$m ± 0.8, 5.2 $\mu$m ± 0.9, 5.0 $\mu$m ± 0.9, 5.2 $\mu$m ± 0.8 and 5.1 $\mu$m ± 0.8 respectively.

Larger vSMC-covered vessels also constrict in response to NA at 4 dpf, typically after 3 minutes of exposure ($P=0.006$; Figure 12C and I-M). The average vSMC-covered vessel diameters at 0, 1, 2, 3, and 4 minutes were 11.5 $\mu$m ± 1.2, 11.3 $\mu$m ± 1.5, 11.0 $\mu$m ± 1.4,
10.0 μm ± 1.6, and 9.6 μm ± 1.8 respectively. All measured vessels constrict by 3 minutes and remain constricted. At 6 dpf, the vessels constrict faster than they did at 4 dpf, responding within the first minute, and remaining significantly constricted for the duration of NA exposure (P=0.0001; Figure 12O and P-T). The average vSMC-covered vessel diameters at 0, 1, 2, 3 and 4 minutes were as follows: 11.8 μm ± 1.9, 11.3 μm ± 2.0, 11.2 μm ± 1.7, 11.1 μm ± 1.7, and 10.8 μm ± 1.8 respectively. Kymographs show that over time, a pericyte-covered vessel does not significantly decrease in diameter (Figure 12U), while a vSMC-covered vessel decreases in diameter (Figure 12V).

Thus, both pericyte and vSMC-covered vessels constrict at 4 dpf, with pericytes responding faster. However, at 6 dpf, only vSMC contraction occurs and it occurs faster relative to 4 dpf, suggesting that vSMCs have differentiated further. However, the response of vSMCs is variable; of 32 measured vessels at 6 dpf, only 13 constrict consistently over the experimental period, suggesting that myogenic response is still maturing in many vSMCs.
Figure 10: Dose response curves of α1 adrenergic receptor agonists NA and PE at 6 dpf.

A) Cerebral vessels constrict to 1 µM of NA within the first minute (P=0.05; n=9 measurements from 4 embryos) but do not constrict to 2 µM (B, P=0.106; n=4 measurements from 4 embryos), 10 µM (C, P=0.074; n=3 measurements from 3 embryos) or 1 mM (D, P=0.117; n=5 measurements from 4 embryos). Cerebral vessels constrict in response to 10 µM of PE (F, P=0.026; n=6 measurements from 6 embryos) but not to 1 µM (E, P=0.452; n=6 measurements from 2 embryos) or 100 µM (G, P=0.353; n=3 measurements from 3 embryos).

Significance was determined by an ANOVA with p< 0.005 = **.
Figure 11: Vasoactive agents do not affect heart rate.

Heart rate was not affected by 2 µM of NA (A, P=0.193; 9 embryos) and 10 µM of PE (B, P=0.114; 7 embryos) at 4 dpf or 1 mM of SNP at 6 dpf (C, P=0.106; 17 embryos). Significance was determined by one-way ANOVA for A and B and a paired two tailed t-test for C.
Figure 12: Both pericytes and vSMCs contract in response to NA at 4 dpf but only vSMCs contract at 6 dpf.

A) Schematic of the experimental timeline. Imaging occurs prior to addition of 2 µM NA (0 min) and at one-minute intervals over 4 minutes after drug addition. B) At 4 dpf, pericyte-covered vessels constrict in response to NA within the first minute (P≤0.0001; n=60 measurements from 14 embryos). C) At 4 dpf, vSMC-covered vessels constrict within 3 minutes of treatment (P=0.006; n=10 measurements from 9 embryos). D-H) Images of pericyte-covered vessel constriction. I-M) Images of vSMC-covered vessel constriction. N) At 6 dpf, pericyte-covered vessels do not constrict in response to NA (P=0.019; n=46 measurements from 15 embryos). O) At 6 dpf, vSMC-covered vessels constrict in response to NA (P=0.0001; n=32 measurements from 25 embryos). P-T) Images of vSMC-covered vessel constriction at 6 dpf. U) Kymograph showing the lack of diameter change over time from a pericyte covered vessel over a 5-minute period. V) Kymograph of a vSMC-covered vessel constricting over a 6-minute period. Arrows mark the start of NA treatment in U and V.

Scale bars on D-G, I-L, and P-S represent 10 µm. Significance was determined by ANOVA with P≤ 0.05 = *, P≤ 0.005 = ** and P≤ 0.0005 = ***.
3.3 Early contraction of pericytes to phenylephrine is lost when vSMCs mature

We used a second α₁ adrenergic agonist, PE, to test whether it would cause a similar contractile behavior in mural cells as NA (Figures 4 and 5). A dose response curve identified 10 µM as the optimal concentration (Figure 10E-G), with no effect on heart rate (Figure 11B). Similar to NA administration, pericyte-covered vessels constrict in response to PE at 4 dpf but not at 6 dpf (Figure 13). At 4 dpf, the average pericyte-covered vessel diameters at 0, 1, 2, 3, and 4 minutes were 5.2 µm ± 0.9, 5.2 µm ± 0.8, 5.1 µm ± 0.9, 5.1 µm ± 0.9, 5.1 µm ± 0.8 and 5.0 ± µm 0.8 respectively. These vessels constrict within 3 minutes and maintained this to 5 minutes (P=0.007; Figure 13B and C-G). Also, like NA, only a sub-population of pericyte-covered vessels constrict consistently (35 of 55 vessels constrict at both 3 and 5 minutes). At 6 dpf, similar to NA, pericyte-covered vessels do not constrict to PE (Figure 13H). At 6 dpf, the average pericyte-covered vessel diameters at 0, 1, 2, 3, 4 and 5 minutes were 4.7 µm ± 0.8, 4.7 µm ± 0.8, 4.7 µm ± 0.7, 4.7 µm ± 0.8, 4.8 µm ± 0.8 and 4.7 µm ± 0.9 respectively.

vSMCs contract consistently at both 4 and 6 dpf in response to PE (Figure 13). At 4 dpf, vSMC-covered vessels constrict within the first minute (P=0.0001; Figure 13I-N), which is faster than the pericyte-covered vessels. The response of vSMCs to PE is also faster than their response to NA at 4 dpf (Figures 12C and 13I). At 4 dpf, vSMC-covered vessel diameters at 0, 1, 3, 4 and 5 minutes were 12.0 µm ± 1.4, 10.9 µm ± 1.1, 11.0 µm ± 1.0, 10.7 µm ± 0.9, 10.6 µm ± 1.0 and 10.5 µm ± 1.0 respectively. These vessels constrict to a similar extent for the duration of PE exposure (14/16 vessel constrict). At 6 dpf, vSMC-covered vessels constrict and their average vessel diameters at 0, 1, 2, 3, 4 and 5 minutes
were 11.7 µm ± 1.6, 11.0 µm ± 1.3, 10.8 µm ± 1.5, 10.7 µm ± 1.6, 10.8 µm ± 1.5 and 10.7 µm ± 1.6 respectively (P=0.0001; 27/38 vessels; Figure 13O-T).

Thus, each mural cell responds similarly to each of the vasoconstrictors in this study (NA and PE). At 4 dpf, both pericytes and vSMCs contract in response to these agents whereas at 6 dpf, only vSMCs contract. This highlights the activity of pericytes at the earlier stage, and the dominance of vSMCs at the later stage.
Figure 13: Both pericytes and vSMCs contract in response to PE at 4 dpf but only vSMCs contract at 6 dpf.

A) Schematic of the experimental timeline. Imaging occurs prior to addition of 10 µM PE (0 min) and at one-minute intervals over 5 minutes after drug addition. B) At 4 dpf, pericyte-covered vessels constrict in response to PE (P=0.007; n=55 measurements from 15 embryos). C-G) An example of pericyte-covered vessel constriction at 4 dpf. H) Pericyte-covered vessels do not constrict at 6 dpf (P=0.552; n=68 measurements from 15 embryos). I) vSMC-covered vessels constrict within the first minute of PE treatment at 4 dpf (P=0.0001; n=16 paired measurements from 15 embryos). J-N) An example of vSMC-covered vessel constriction at 4 dpf. O) At 6 dpf, vSMC-covered vessels constrict in response to PE (P=0.0001; n=38 paired measurements from 22 embryos). P-T) An example of the vSMC-covered vessel constriction at 6 dpf.

Scale bars represent 10 µm in C-F, J-M, and P-S. Significance was determined by ANOVA with P≤ 0.05 = *, P≤ 0.005 = ** and P≤ 0.0005 = ***.
3.4 Pericytes and vSMCs actively contract to constrict cerebral vessels.

Genetic ablation of specific lineages was used to determine whether mural cells actively regulate cerebral vessel diameters. We utilized transgenic lines expressing Gal4 under the *pdgfrβ* (Ando et al., 2016; Asakawa and Kawakami, 2009) or *acta2* promoters (Asakawa and Kawakami, 2009; Whitesell et al., 2014) for pericytes and vSMCs respectively. Selective ablation of each mural cell lineage was possible by crossing transgenic lines expressing the NTR gene under a UAS promoter and exposing embryos to Mtz for 24 hours. After ablation, zebrafish were exposed to vasoconstricting agents to determine changes in vessel diameter. Ablation started at 3 dpf for 4 dpf experiments, and at 5 dpf for 6 dpf experiments. The fish had normal morphology (Figure 14A – H) when mural cells were ablated (Figure 14A’-H’).

At 4 dpf, we found constriction of cerebral vessels to be reduced following ablation. At this time, cerebral vessels with diameters of ≤6.5 μm (normally vessels with pericyte coverage), still constrict in response to NA (P=0.001; Figure 15B and F-I). Although they take longer to constrict than in the unablated fish (Figure 12B). The vessel diameters at 0, 1, 2, 3 and 4 minutes were 5.6 μm ± 0.5, 5.6 μm ± 0.6, 5.5 μm ± 0.6, 5.4 μm ± 0.5 and 5.3 μm ± 0.5 respectively. These ≤6.5 μm diameter vessels with ablated pericytes also constrict in response to PE (P=0.0003; Figure 15C and J-M). The average vessel diameters at 0, 1, 2, 3, 4 and 5 minutes were 5.3 μm ± 0.8, 5.2 μm ± 0.8, 5.1 μm ± 0.8, 5.1 μm ± 0.8, 5.1 μm ± 0.8, and 5.01 μm ± 0.8 respectively. In response to PE, the vessel diameters significantly decreased at 3 minutes post treatment and remained constricted. We hypothesized that incomplete ablation might account for remaining contractile activity,
and therefore assessed the ablation efficiency of cerebral pericytes or vSMCs at 4 dpf. We note partial ablation of both lineages (Figure 14I’- N’’). As the concentration of Mtz used for the ablation was the maximal tolerated concentration at this developmental stage without inducing morphological defects, the observation of reduced contractile ability is consistent with pericytes playing an active role in vascular constriction at 4 dpf.

Similarly, cerebral vessels normally covered by vSMCs continue to constrict to vasoconstrictors in embryos with incompletely ablated vSMCs at 4 dpf. This constriction was weaker and not maintained. Cerebral vessels with diameters of ≥9.5 µm, normally covered with vSMCs, constrict in response to NA in the ablated state (P=0.001; Figure 15D and N-Q) and remain constricted for the duration of treatment. The average vessel diameter of these vSMC ablated vessels at 0, 1, 2, 3 and 4 minutes were 11.6 µm ± 1.4, 11.0 µm ± 1.8, 10.3 µm ± 1.7, 9.7 µm ± 1.9, and 9.5 µm ± 2.1 respectively. These ≥9.5 µm in diameter vessels also constrict in response to PE when vSMCs are ablated (P=0.070; Figure 15E R-U). The average vSMC-covered vessel diameters at 0, 1, 2, 3, 4 and 5 minutes were 11.7 µm ± 2.1, 11.3 µm ± 2.1, 11.3 µm ± 2.0, 11.3 µm ± 2.1, 11.2 µm ± 2.01 and 11.2 µm ± 2.0 respectively. Significant constriction only occurred between 0 and 4 minutes but interestingly, these vessels did not remain constricted. Relative to embryos with non-ablated vSMCs (Figure 13I) constriction in embryos with ablated vSMCs is later and not maintained (Figure 15E). This reduction in contractile ability is consistent with active contraction by vSMCs at 4 dpf.

When mural cells are ablated at 5 dpf and tested at 6 dpf, we see a complete reduction of contractile activity as neither pericyte nor vSMC covered vessels constrict (Figure 16).
Cerebral vessels ≤6.5 μm in diameter no longer respond to either NA or PE (Figure 16B-C). The average vessel diameter at 0, 1, 2, 3 and 4 minutes with NA treatment were 4.5 μm ± 0.9, 4.4 μm ± 0.9, 4.4 μm ± 0.9, 4.5 μm ± 1.0 and 4.5 μm ± 1.0 respectively. As for PE treatment, the average vessel diameters at 0, 1, 2, 3, 4 and 5 minutes were 4.8 μm ± 0.8, 4.9 μm ± 0.7, 4.9 μm ± 0.8, 4.9 μm ± 0.8, 4.9 μm ± 0.8 and 4.8 μm ± 0.8 respectively. Similarly, ≥9.5 μm diameter vessels with ablated vSMCs do not constrict to either NA or PE at 6 dpf (Figure 16D-E). The average vessel diameters at 0, 1, 2, 3 and 4 minutes of treatment with NA were 13.5 μm ± 2.9, 13.5 μm ± 2.7, 13.3 μm ± 3.0, 13.2 μm ± 3.0 and 13.1 μm ± 3.1 respectively. After treatment with PE, the average vessel diameters at 0, 1, 2, 3, 4 and 5 minutes were 12.5 μm ± 2.1, 12.4 μm ± 2.5, 12.4 μm ± 2.7, 12.1 μm ± 2.3, 12.2 μm ± 2.3 and 12.1 μm ± 2.4 respectively. Our 5-6 dpf genetic ablation data suggest that vSMCs actively contract at 6 dpf to constrict vessel diameter.

Additionally, we used another method to determine whether pericytes and vSMCs actively regulate vascular tone by comparing basal vessel diameter in the presence and absence of each mural cell. Vascular tone is the active regulation of a constant basal vessel diameter that is less than maximal dilation. At 4 dpf, vessels with ablated pericytes are significantly enlarged (P=0.016; Figure 16F). The average vessel diameter with pericytes present was 5.2 μm ± 0.9, while without pericytes, it was 5.5 μm ± 0.7. At 6 dpf, as expected, the average vessel diameter in the presence and absence of pericytes remained unchanged, 4.9 μm ± 0.8 and 4.9 μm ± 0.9 (Figure 16G), which is in agreement with our pharmacological data suggesting that pericytes regulate vessel diameter at 4 dpf but not 6 dpf.
vSMCs regulate tone in a complementary manner. At 4 dpf, there is no difference in cerebral vessel diameter in the presence and absence of vSMCs (Figure 16H). The average vessel diameter with and without vSMCs was 11.8 µm ± 1.8 and 11.7 µm ± 1.8 respectively. However, at 6 dpf, the average vessel diameter with vSMC coverage was 11.6 µm ± 1.6, while with ablated vSMCs it was 13.0 µm ± 2.5 (P=0.001, Figure 16l).

Taken together, we provide evidence that at 4 dpf, pericytes are a key regulator of cerebral vessel diameter, but at 6 dpf, vSMCs become the primary mediators of vascular tone.
Figure 14: Mural cell ablation using lineage-restricted nitroreductase expression.

Brightfield (A-H) and confocal (A’-H’) images of embryos at 4 dpf after mural cell ablation at 3 dpf (B, B’, F, F’), or at 6 dpf after ablation at 5 dpf (D, D’, H, H’). Circles in A’, C’, E’, and G’ highlight wildtype mural-endothelial cell association. I-N’’) Control experiment to demonstrate mosaicism in pericyte and vSMC transgenic lines. I) Overlap between pericyte transgenic lines: Tg(pdgfrβ:GFP) and Tg(pdgfrβ:Gal4;UAS:NTRmCherry) at 4 dpf. J-J’’) Inset of vessels in I. The circles in J represent the overlap of expression between the two transgenic lines while those in J’’ show two cells that were not marked by Tg(pdgfrβ:Gal4;UAS:NTRmCherry). K-K’’) Inset of vessels in I. L) Overlap between the two vSMC transgenic lines Tg(acta2:Gal4;UAS:NTRmCherry) and Tg(acta2:GFP) at 4 dpf is incomplete. M-N’’) Insets highlighting mosaicism in the marking of vSMCs by the two transgenic lines. The circles shows regions where Tg(acta2:GFP) and Tg(acta2:Gal4;UAS:NTRmCherry) overlap.

Scale bars represent 200 µm in A-H and 20 µm in A’-H’ and I-N’’. A, P, V and D refer to anterior, posterior, ventral and dorsal.
Ablated Pericytes

NA

PE

Ablated vSMCs

NA

PE

\(\alpha\) adrenergic agonists

4 days post fertilization (4 dpf)

<table>
<thead>
<tr>
<th>A</th>
<th>B</th>
<th>C</th>
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<tr>
<td>0</td>
<td>NA</td>
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<td>1</td>
<td>2</td>
<td>3 Mtz Treatment</td>
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\[ \text{Ablated Pericytes} \]

\[ \text{Ablated vSMCs} \]

\[ \text{NA} \]

\[ \text{PE} \]

\[ \text{F} \]

\[ \text{G} \]

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Figure 15: Cerebral vessels with ablated mural cells from 3-4 dpf show delayed constriction.

A) Experimental timeline. The zebrafish were treated with 50 μM Mtz from 3-4 dpf, treated with the vasoconstrictors at 4 dpf and then imaged. Vessels with ablated pericytes and diameters of ≤6.5 μm constrict to both NA (B, P=0.001; n=18 measurements from 4 embryos) and PE (C, P=0.0003; n=46 measurements from 10 embryos). Similarly, vessels with ablated vSMCs and with diameters of ≥9.5 μm also constrict to NA (D, P=0.001; n=9 measurements from 7 embryos) and PE (E, P=0.070; n=14 measurements from 10 embryos). F-M) Example of constriction to NA and PE in a ≤6.5 μm diameter vessel. N-U) Example of constriction to NA and PE in a ≥9.5 μm diameter vessel. Scale bars represent 10 μm for F-H, J-L, N-P, and R-T. Significance was determined by repeated measures one-way ANOVA with P≤ 0.05 = * and P≤ 0.005 = **.
Figure 16: Lack of cerebral vessel constriction in the absence of mural cells at 6 dpf.

A) Experimental timeline. The embryos were treated with Mtz from 5-6 dpf before treatment with vasoconstrictors at 6 dpf. Vessels with ablated pericytes and diameters of ≤6.5 μm did not constrict to either NA (B, P=0.065; n=50 measurements from 12 embryos) or PE (C, P=0.123; n=45 measurements from 6 embryos). Vessels with ablated vSMCs and diameters of ≥9.5 μm also did not constrict to either NA (D, P=0.233; n=12 measurements from 10 embryos) or PE (E, P=0.314; n=10 measurements from 9 embryos) after vSMC ablation. F) Furthermore, vessels with ablated pericytes have an enlarged diameter at 4 dpf (P=0.016; n=168 and 97 for the non-ablated and ablated groups). G) However, there is no difference in vessel diameter in the presence and absence of pericytes at 6 dpf (P=0.953; n=193 and 141 for the non-ablated and ablated groups). H) There is no difference in vessel diameter in the presence and absence of vSMCs at 4 dpf (P=0.738; n=68 and 23 for the non-ablated and ablated groups). I) However, vessels with ablated vSMCs have an enlarged diameter at 6 dpf (P=0.001; n=92 and 22 for the non-ablated and ablated groups).

Significance was determined by repeated measures one-way ANOVA for B-E and unpaired two-tailed t-tests for F-I with P≤ 0.05 = * and P≤ 0.005 = **.
3.5 Vessel dilation is mediated by pericytes and vSMCs

NO is a key molecule mediating vascular mural cell relaxation (Figures 6 and 7). To test the ability of developing mural cells to relax, we exposed zebrafish to NO donors at different stages of development. Dose response curves were carried out for each agent (Figure 17) and changes to heart rate were assessed and showed no effect (Figure 11C). A lack of n appears to be a confounding factor in the significance observed with the dose-response curves. We selected our NO donor concentrations based on the dose response curves and published concentrations in the zebrafish.

At 4 dpf, pericyte-covered vessels dilate in response to SNP (P=0.005; Figure 18A and E-I). The average pericyte-covered vessel diameter before and after SNP treatment was 5.1 µm ± 1.0 and 5.4 µm ± 1.1 respectively (P=0.010; 33/53 vessels; Figure 18B). To determine if this dilation is due to the active relaxation of pericytes, we genetically ablated pericytes with Mtz and then exposed the zebrafish to SNP. After ablation, vessels with diameters ≤6.5 µm constricted instead of dilating (P=0.003; Figure 18C), from 5.7 µm ± 0.5 to 5.4 µm ± 0.5, to SNP (P=0.001; 25/33 vessels; Figure 18D). The loss of dilation in the absence of pericytes supports the active role of pericytes in vascular dilation at 4 dpf. When we assessed the ability of pericytes to relax in response to SNP at 6 dpf, there was no response (Figure 19C). The average pericyte-covered vessel diameter before and after SNP was 5.0 ± 0.8 and 4.9 ± 0.9 respectively.

We also assessed the relaxation of vSMCs to SNP at 4 dpf, however surprisingly, there was no significant dilation (Figure 19A). The average vSMC-covered vessel diameter
before and after SNP treatment was 11.8 µm ± 2.0 and 11.8 µm ± 3.0 (Figure 19B). We noted a binary response from vSMC-covered vessels. Of 42 measurements, 16 dilated, one did not change in diameter while 25 constricted (Figure 19B). This suggests that at 4 dpf, the ability of vSMCs to relax may still be maturing. At 6 dpf, these vessels also did not respond to SNP (Figure 19D). The average vessel diameter before and after SNP treatment was 11.1 ± 1.3 and 10.8 ± 1.6 respectively.

We reasoned that if a vessel is close to maximal dilation, it may not be able to show a large dilation in response to NO donors. Thus, we tested whether vessels are able to dilate from a pre-constricted state. We contracted vSMCs using 10 µM of PE and then exposed them to 100 µM of NO donor SNAP (Figure 18J). Similar to our previous results at 4 dpf, vSMCs contracted but were unable to relax (P=0.0001; Figure 18K). The vSMC-covered vessels constricted to PE within the first minute and remained constricted for the duration of PE exposure (2 minute and 3-minute, P≤0.003 and P≤0.008 accordingly). The average vessel diameters at 0, 1, 2 and 3 minutes were 12.3 µm ± 1.8, 11.0 µm ± 1.7, 11.3 µm ± 1.1, and 11.2 µm ± 1.2 respectively. Some vessels appear to dilate to SNAP within first minute of treatment (4th minute), however, as time progresses, they constrict again and remain constricted for the duration of the experiment. The vessel diameters at 4, 5, 6 and 7 minutes were 11.9 µm ± 1.2, 11.5 µm ± 1.0, 11.0 µm ± 1.0 and 10.6 µm ± 1.4 respectively. Thus, we conclude that at 4 dpf, vSMC-covered vessels only constrict but do not dilate.
In contrast, when vSMCs are more mature at 6 dpf, they can relax from a pre-contracted state ($P=0.0002$; Figure 18L). In response to PE, vSMC covered vessels constrict ($P=0.001$ between 0 and 1 minutes, $P=0.002$ between 0 and 2 minutes and $P=0.010$ between 0 and 3 minutes). The average vessel diameter at 0, 1, 2, and 3 minutes were 11.1 µm ± 0.8, 10.1 µm ± 1.0, 10.2 µm ± 0.9 and 10.2 µm ± 1.0 respectively. When SNAP was added, these vessels then actively dilate (4th minute). There was significant dilation from experimental minute 1 (10.1 µm ± 1.0) to minute 4 (10.7 µm ± 1.1; $P=0.035$). Thus by 6 dpf, vSMCs have developed active vasomotor responses that permit both contraction and relaxation.
Figure 17: Dose response curves for NO donors at 6 dpf.

Unexpectedly, cerebral vessels either constrict or do not respond to most NO donors at 6 dpf. There was no change in vessel diameter in response to 1 µM SNP (A, P=0.065; n=3 measurements from 3 embryos) but constriction to 100 µM (B, P=0.004, n=8 measurements from 3 embryos), 500 µM (C, P=0.059; n=3 measurements from 3 embryos) and 1 mM (D, P=0.025; n=3 measurements from 3 embryos). E-H) NONOate did not significantly change vessel diameter at 1 nM (E, P=0.221; n=4 measurements from 4 embryos), 1 µM (F, P=0.017; n=5 measurements from 3 embryos), 5 µM (G, P=0.139; n=3 measurements from 3 embryos) or 100 µM (H, P=0.562; n=4 measurements from 4 embryos). I-L) SNAP also did not significantly change vessel diameter at 5 µM (I, P=0.180; n=5 measurements from 4 embryos), 10 µM (J, P=0.038; n=4 measurements from 4 embryos), 50 µM (K, P=0.179; n=3 measurements from 3 embryos) or 100 µM (L, P=0.439; n=4 measurements from 4 embryos).

For experiments, 1 mM SNP was selected based on previous SNP use in zebrafish, and 100 µM of SNAP and 5 µM of NONOate were used. Significance was determined by a one-way ANOVA with P≤ 0.05 = *.
Pericyte-covered vessels - SNP at 4 dpf

Ablated Pericytes

Unintact Pericytes

Normalized Vessel Diameter (µm)

Normalized Vessel Diameter (µm)

Time (min)

Time (min)

E F G H I

Pericyte endothelium

PE

SNAP

Time (min)

0 1 2 3 4 5 6 7

Ctrl

vSMC-covered vessels

4 dpf

6 dpf

Normalized Vessel Diameter (µm)

Normalized Vessel Diameter (µm)

Time (min)

Time (min)
Figure 18: Pericycle-covered vessels dilate at 4 dpf while vSMC-covered vessels dilate at 6 dpf but only from a pre-constricted state.

A) Pericycle-covered vessels dilate to SNP at 4 dpf (P=0.005; n=53 measurements from 15 embryos). B) Specifically, 33/53 pericycle-covered vessels dilate (P=0.010; n=53 measurements from 15 embryos). C) When pericytes are ablated, vessels with diameters of ≤6.5 μm constrict in response to SNP at 4 dpf (P=0.003; n=33 measurements from 10 embryos), on average the vessel diameters decreased from 5.65 μm to 5.43 μm (P=0.001, D). E-I) An example of pericycle-covered vessel dilation to SNP at 4 dpf. J) Experimental timeline for the pre-constriction followed by dilation of vSMC-covered vessels. Embryos were imaged prior to drug addition (0 min), followed by 3 minutes of exposure to PE (10 μM) and then to 4 minutes of exposure to SNAP (100 μM). Embryos were imaged every minute. K) At 4 dpf, vSMC-covered vessels do not dilate to SNAP from a pre-constricted state (P=0.0001; n=15 measurements from 11 embryos). L) At 6 dpf, vSMC-covered vessels dilate from a pre-constricted state (P=0.0002; n=15 measurements from 13 embryos). The shading in K and L highlight the different drug exposure and the corresponding changes in vSMC-covered vessel diameter according to the experimental timeline (J).

Scale bars represent 10 μm for E-I and significance was determined by paired two-tailed t-tests with P≤ 0.05 = *, P≤ 0.005 = ** and P≤ 0.0005 = ***.
Figure 19: The variable response of mural cells to NO donor SNP at 4 and 6 dpf.

A) vSMC-covered vessels do not dilate to SNP at 4 dpf (P=0.992; n=42 measurements from 23 embryos). B) vSMCs respond in a binary manner to SNP; 17/42 vessels dilate while 25 constrict (P=0.992; n=42 measurements from 23 embryos). C-D) At 6 dpf, neither pericyte (C, P=0.438; n=79 paired measurements from) nor vSMC-covered vessels (D, P=0.240; n=25 measurements from) dilate in response to SNP. Significance was determined by paired two-tailed t-tests with P≤ 0.05 = *, P≤ 0.005 = ** and P≤ 0.0005 = ***.
3.6 Pericytes relax via the EP$_4$ receptor to cause vessel dilation

Astrocyte end feet release prostaglandins (including prostaglandin E$_2$ (PGE$_2$), the ligand of EP$_4$ receptor) to evoke vasomotor responses in pericytes (Macvicar & Newman, 2015). In the mouse, pericytes are shown to relax via EP$_4$ receptors by increasing cAMP production (Hall et al., 2014). Blocking the EP$_4$ receptor using the antagonist AH23848 is predicted to compromise pericyte ability to relax. This is because PGE$_2$ induced pericyte relaxation is an independent and parallel pathway to NO induced relaxation (Hamilton et al., 2010). Both pathways convergence on increasing PKG and MLCP activity (Hamilton et al., 2010). We predict that once NO donor SNAP is added following AH23848, enhanced pericyte relaxation and pericyte-vessel vessel dilation is to be observed. Thus, in using AH23848 we can test the role of EP$_4$ receptors in pericyte relaxation. In response to AH23848 at 4 dpf, pericyte-covered vessels constrict within the first minute and remain constricted (P=0.002; Figure 20B and D-H). The average vessel diameters at 0, 1, 2, 3 and 4 minutes of exposure to AH23848 were 5.4 µm ± 0.7, 5.3 µm ± 0.7, 5.3 µm ± 0.7, 5.2 µm ± 0.8, and 5.1 µm ± 0.8 respectively. After SNAP addition, these vessels remained constricted. The average vessel diameter at 5, 6, 7 and 8 minutes were 5.1 µm ± 0.9, 5.2 µm ± 0.8, 5.2 µm ± 0.8 and 5.3 µm ± 0.8 respectively. Thus at 4 dpf, blocking the EP$_4$ receptor/pathway constricts pericyte-covered vessels and compromises the ability of pericytes to relax to NO donor SNAP.

Similar to our previous data at 6 dpf, we found AH23848 to have no effect on pericyte-covered vessels (P=0.55; Figure 20C). As expected, these vessels do not dilate to SNAP after treatment with AH23848. At 6 dpf, the average vessel diameters at 0, 1, 2, 3, 4, 5,
6, 7 and 8 minutes were 4.4 µm ± 0.6, 4.5 µm ± 0.6, 4.4 µm ± 0.6, 4.4 µm ± 0.6, 4.4 µm ± 0.6, 4.5 µm ± 0.7, 4.4 µm ± 0.6, 4.4 µm ± 0.6 and 4.4 µm ± 0.6 respectively.

It was important to determine the spatial relationship between pericytes and astrocytes at 4 dpf since both cell types are part of the neurovascular unit. We observed direct contact between pericytes and astrocytes in the 4 dpf zebrafish brain (Figure 20E-I), suggesting that astrocytic regulation of pericyte behavior via prostaglandins would be possible at this stage. Taken together, our pharmacological data shows regulation of pericytes via prostaglandin receptor and physical proximity between pericytes and astrocytes. These results are consistent with pericytes responding to prostaglandin signals at this stage to regulate vessel diameter.
A

Time (min)

0 1 2 3 4 5 6 7 8

Ctrl

AH23848

SNAP

Pericyte-covered vessels - 4 dpf

Normalized Vessel Diameter (μm)

0.50 0.75 1.00 1.25

B

C

Time (min)

0 1 2 3 4 5 6 7 8

Pericyte-covered vessels - 6 dpf

D

E

0 min

F

1 min

G

Merge

H

Inset

Dorsal view

A

P

Eye

Eye

Pericytes Astrocytes - 4 dpf

E

F

G

H

I
Figure 20: Pericytes relax via EP_4 receptor signaling to cause vessel dilation.

A) Experimental timeline. The embryos were imaged prior to any drug treatment to obtain baseline vessel diameter (0 min). AH23848 was then added and embryos were imaged every minute for four minutes. SNAP was added after 4 minutes, and the fish were imaged every minute for the next four minutes. B) At 4 dpf, pericyte-covered vessels constrict in response to AH23848 and remain constricted after SNAP treatment (P=0.002; n=27 measurements from 9 embryos). C) At 6 dpf, the pericyte-covered vessels do not respond to either vasoactive agent (P=0.55; n=33 measurements from 15 embryos). The shading in B and C reflect the change in pericyte-covered vessel diameter in accordance with the experimental timeline. D-H) An example of pericyte-covered vessel constriction in response to AH23848 at 4 dpf. E) Spatial relationship between pericytes and astrocytes (marked with GFAP:GFP) at 4 dpf. A and P refer to anterior and posterior. F-I) Insets of D. White circles in F-I highlight close contact between pericytes and astrocytes. Scale bars represent 50 µm in E and 10 µm in F-I. Significance was determined by one-way ANOVA with P≤ 0.05 = *.
CHAPTER FOUR: DISCUSSION
4.1 Vasoactive regulation in early zebrafish development

The advantages of optical clarity and availability of transgenic lines for pericyte and vSMC lineages in zebrafish allow us to explore the dynamic role of mural cells in maintaining vascular tone during embryonic development. We have done so for the first time using intravital imaging. No studies have been conducted previously during embryonic development, as development of vascular myogenesis occurs in utero in mammals and cannot be live imaged. The roles of pericytes and vSMCs in regulating vessel diameter are conflicting in the literature for several reasons; unlike in zebrafish, markers such as \textit{pdgfrβ} can label both mural cell types in mouse (Armulik et al., 2011; Nakayama et al., 2013), and mural cells have also been identified based on morphology, which can be unreliable (Attwell et al., 2016). Pericytes also have a variety of morphologies (Hartmann et al., 2015); some pericytes express contractile proteins and some do not. These factors make it difficult to clearly separate mural cell contribution to vascular tone regulation, potentially leading to contradictory conclusions regarding vasomotor regulation (Fernandez-Klett et al., 2010; Hill et al., 2015).

Our results support a switch in mural cell types that regulate cerebral vessel diameters at different stages of development. As the cerebral vasculature develops, mural cell numbers increase from 4 dpf to 6 dpf. At 4 dpf, pericytes actively constrict and dilate cerebral vessels to maintain vascular tone. At this stage, vSMCs have the capacity to contract in response to vasoconstricting agents. However, their response is slow, and there is heterogeneity in responses to these stimuli. At 6 dpf, vSMCs are the only mural cells we detect that regulate vascular tone in our assays using pharmacological stimuli
(Figure 21).

**Figure 21: Summary Figure - Developmental shift in mural cell ability to regulate cerebral vascular tone.**

A) At 4 dpf, both pericyte and vSMC-covered vessels constrict while only the pericyte-covered vessels dilate. B) At 6 dpf, both the pericyte and vSMC populations have increased in number. Pericyte-covered vessels no longer respond to vasoactive agents while vSMC-covered vessels constrict but can only dilate from a pre-constricted state. C) There is no vasomotor response when mural cells are ablated at 5 dpf and exposed to vasoactive agents at 6 dpf.
We find it intriguing that at 6 dpf, while vSMCs can contract, they can only relax from a pre-contracted state, suggesting that largest brain vessels in the zebrafish embryo are maintained in a dilated state during normal development. Interestingly, as hemodynamic load increases in the zebrafish trunk between 24-48 hpf, the diameter of the DA and posterior cardinal vein (PCV) increases (Sugden et al., 2017). However, by 72 hpf, ECs align themselves by changing shape to decrease vessel diameter (Sugden et al., 2017). These time points align with pericyte and vSMC development and recruitment to the vessels. Once recruited to the vessels, these mural cells then regulate changes in vessel diameter by actively contracting or relaxing.

4.2 Pericytes actively contract and relax to regulate cerebral vessel diameters

We provide several lines of evidence to show pericytes actively regulating cerebral vessel diameters. Firstly, our results show that at 4 dpf, pericytes are responsive to vasoactive agents, including vasoconstrictors (NA, PE and AH23848) and the vasodilator SNP. Secondly, genetic ablation of pericytes reduces vasoconstriction and removes vasodilation. Thirdly, the basal diameter of vessels with ablated pericytes is larger than wild type pericyte-covered vessels suggesting that pericytes contribute to the regulation of vessel diameter at this stage.

We observed a high, but not complete level of ablation of both pericytes and vSMCs at 4 dpf, and reduced, but not complete loss of cell activity. Incomplete ablation may be due to insufficient Mtz (although we used the highest concentration possible at this stage without inducing morphological defects). Another possibility is that mural cells are
undergoing rapid differentiation at this time and there may be a pool of mural cells that have vasomotor activity, but do not express enough of the marker to undergo ablation. A third possibility is that there is mosaic expression of the transgenes and not every mural cell is labelled (and therefore not targeted by Mtz ablation). Although there was a large overlap between our pericyte transgenics, there were a number of cells that were not marked by both lines. Transgenic lines for vSMCs are also relatively mosaic at 4 dpf. Despite these limitations, our evidence strongly suggests that at 4 dpf, pericytes are actively regulating vessel diameter.

4.3 Mechanism of pericyte contraction and relaxation

Intracellular calcium concentration dictates whether pericytes will contract or relax, at least in the mouse (Sakagami et al., 1999; Sakagami et al., 2001; Sugiyama et al., 2004). Pericytes can contract in response to endothelial-derived vasoactive agents (Matsugi et al., 1997) and they do contain microfilaments resembling actin and myosin containing fibers (Ho, 2004; Lebeux & Willemot, 1978). However, they do not express the same isoforms of actin and myosin as present in vSMCs. Also, it is possible that they express contractile gene such as acta but at an undetectable level with current markers and methods. In cultured pericytes, activation of endothelin-1 (ET-1) receptors in pericytes increases calcium, resulting in the alignment of F-actin and intermediate filaments and contraction (Dehouck et al., 1997). We used NA and PE to assess the contractile properties of pericytes during development because catecholamines such as NA have been shown to modulate tone in cultured pericytes (Markhotina et al., 2007), and in cerebral slices (Hall et al., 2014; Peppiatt et al., 2006).
Our understanding of pericyte relaxation is limited. Similar to vSMCs, pericytes relax by activation of potassium channels (Burnette & White, 2006; Cao et al., 2006; Jackson, 2005; Li & Puro, 2001; Quignard et al., 2003; Von Beckerath et al., 2000; Wiederholt et al., 1995). Activation of potassium channels by vasodilators such as NO reduces the activity of VDCC and ClCa in pericytes (Hamilton et al., 2010; Sakagami et al., 2001). The EP4 receptor contributes to pericyte relaxation in parallel to NO (Burnette & White, 2006; Dodge et al., 1991; Hamilton et al., 2010), which is needed to stop the production of vasoconstricting agent 20-HETE (Hall et al., 2014). When we blocked the EP4 receptor and then exposed the zebrafish to NO donor SNAP, the pericyte-covered vessels constricted to the EP4 antagonist and did not dilate in response to NO. This constriction and lack of dilation by the pericyte-covered vessels at this early stage highlights the importance of the prostaglandin pathway in pericyte relaxation in vivo.

4.4 A temporal shift in cell types regulating vascular tone

The relationship between neural activity and pericyte activity is not well understood. Although pericytes can locally modulate capillary diameters resulting in local changes in RBC flow, this activity is unrelated to neurovascular coupling according to one study (Fernandez-Klett et al., 2010). In another study, whisker pad stimulation results in robust calcium transients in pericytes, corresponding to changes in vessel diameter and blood flow, and pericytes rather than vSMCs were shown to initiate this process (Hall et al., 2014). However, a third study showed that stimulation of neuronal function via whisker pad stimulation results in robust calcium transients only in vSMCs, and subsequent
vSMC-covered vessel dilation (Hill et al., 2015). Hill et al. found delayed dilation in vessels without smooth muscle coverage, specifically after peak dilation in vessels with SMC coverage. Thus, this study suggested the dilation in non-vSMC covered vessels is a passive response to the upstream increase in blood flow (Hill et al., 2015).

Understanding when neurovascular coupling develops in an organism is crucial to elucidating the role of cerebral vascular mural cells in regulating vascular tone. When does neurovascular coupling develop in fish? Ulrich et al. determined that up until 3 dpf, the development of neuronal structures is not affected by the presence or absence of vessels in the zebrafish hindbrain (Ulrich et al., 2011). Furthermore, while there is increased neuronal activity at 6 dpf in the optic tectum in response to visual stimulus in fish larvae, it does not correlate to increased RBC speed until 8 dpf, a time when embryos exhibit neurovascular coupling in the optic tectum (Chhabria et al., 2018). These time windows correlate with the two windows of mural cell activity in our data. At 4 dpf, while neurovascular activity and coupling is immature, pericytes are present in large numbers in cerebral vessels and regulate cerebral vessel diameter and hence, blood flow. This is further supported by our results that show physical contact between astrocytes and pericytes at 4 dpf, both components of the neurovascular unit. This suggests that at 6 dpf, pericytes have additional controls from other cell types. Since pericytes are part of the neurovascular unit, perhaps neurons and astrocytes could be controlling pericyte activity by inhibiting rather than stimulating pericyte activity in response to exogenous vasoactive agents. Once neuronal activity has increased, the coupling of neuronal activity and changes in blood flow matures.
Our findings in developing zebrafish embryos reveal key similarities and differences to data generated primarily in the adult mouse brain model. We show that both mural cell types are involved in regulating cerebral vessel diameters but at different stages of development. Pericytes, which share a developmental origin of neural crest with vSMCs in the forebrain and hindbrain (Ando et al., 2016; Cavanaugh et al., 2015; Wang et al., 2011; Wang et al., 2014; Whitesell et al., 2014), regulate cerebral vessel diameter and local changes in CBF while vSMCs are differentiating at 4 dpf. It is important to remember that although the vessels covered by pericytes are the smaller capillaries, RBCs have a diameter of ~5 µm in the zebrafish and these cells have the ability to deform while squeezing through constricted blood vessel regions (Noguchi & Gompper, 2005; Pawlik et al., 1981; Skalak & Branemark, 1969). Thus, a small change in diameter on the smallest vessels might substantially decrease resistance and increase blood flow. Later, once vSMCs have matured and significant neuronal activity occurs, vSMCs become the primary mediators of vascular tone.
CHAPTER FIVE: FUTURE DIRECTIONS
5.1 Neurovascular signalling

Although gap junctions exist between pericytes, ECs and vSMCs (Borysova et al., 2013; Cuevas et al., 1984), there are no data in zebrafish to elucidate the mechanism by which neuronal activity causes changes in vessel diameter and blood flow. Do pericytes coordinate neurovascular coupling by signalling to upstream vSMC-covered vessels to respond to neural activity, or do molecular signals from neurons directly affect vSMC activity? Another possibility is that both pericyte signal to upstream vSMCs and direct signals from neurons reach vSMCs. In the mouse, one study showed that sensory-evoked dilation occurs in the first order pericyte-covered capillaries prior to the zero-order penetrating vSMC-covered arterioles (Hall et al., 2014), suggesting that signaling is transmitted from pericytes to the SMC-covered precapillary arterioles and to upstream arterioles. However, these results are disputed and these questions have yet to be answered. Clearly both pericytes and vSMCs are capable of responding to exogenous vasoactive agents, but the sources and developmental ontogeny of endogenous vasoactive signals remain unknown. Future studies involving transgenic zebrafish expressing mural cell markers in combination with fluorescent reporters for vasoactive compounds such as NO can help us to better understand intercellular signalling between mural cells during neurovascular coupling.

5.2 Understanding the variability in mural cell response to vasoactive agents

Our results support the current literature on the variability in mural cell response to vasoactive agents. We show that not all pericytes or vSMCs respond as expected to vasoactive agents in early development. Such variability in pericyte response has also
been observed in the adult mouse. Only a population of pericytes contract in response to the vasoconstrictor U46619 in a cranial window model, with half of the capillaries constricting and the other half dilating (Fernandez-Klett et al., 2010).

Why this variability in pericyte response exists has yet to be fully understood. One explanation relies on the heterogeneity in the expression of contractile machinery in pericytes, such as the expression of smooth muscle actin (αSMA). For instance only a subset of pericytes have been observed to express αSMA in the brain (Bandopadhyay et al., 2001), spinal cord (Roufail et al., 1995), retina (Nehls, 2004), and pancreatic islets (Almaça et al., 2018). A second explanation could be that only regions of the vessel in contact with the pericyte cell body constrict or dilate and not those regions in contact with processes. This possibility will depend on where the contractile machinery is location within a pericytes. Another explanation is that while mural cells in one region of the vasculature contract and constrict cerebral vessels, mural cells in the neighbouring regions must relax to dilate vessels and accommodate for the change in pressure and subsequent blood flow within the vascular network. This second possibility does not explain why some mural cells do not respond at all to vasoactive agents, as seen with our results.

While these explanations are reasonable, our findings provide further insights as to why this variability exists. Specific to our work, the timing onset of mural cell maturation seems to be essential in mural cell response to vasoactive agents. vSMCs differentiate and mature later than do pericytes and the number of both mural cell types increase from 4 to
6 dpf, indicating that these cells are still developing and maturing at these stages of development. Thus, depending on their maturity, mural cells will respond differently to these vasoactive agents. As for the variability observed in the adult stage, it could be that not all mural cells are receiving the same vasoactive signals. Due to their anatomical location as part of the neurovascular unit, pericytes are in closer proximity to astrocytes and neurons than are vSMCs. Depending on whether neuronal signals reach vSMCs directly or via coordination from pericytes, not all vSMCs may receive the same vasoactive signals. Thus, not all vSMCs may respond as expected to vasoactive agents. Going forward, studies involving local activation of neural or mural cell activity via optogenetic experiments coupled with genetically encoded calcium indicators can help unravel the interactions between mural cell subtypes and neurovascular coupling during development.

5.3 Cerebral small vessel disease (CSVD) and the role of mural cells

Understanding the role of mural cells in vascular tone regulating has great implications on human health and disease. CSVD is used to describe any pathological condition affecting the small vessels of the brain and compromising blood flow (Joutel and Faraci, 2014; Østergaard et al., 2016; Pantoni, 2010). CSVD results in further detrimental consequences such as ischemic stroke (Østergaard et al., 2016; Pantoni, 2010), a condition when cerebral vessels are blocked by a blood clot. In fact, CSVD causes 25-30% of ischemic strokes (Chauhan et al., 2016; Joutel and Faraci, 2014; Wardlaw et al., 2013), which is hypothesized to be the mechanism by which CSVD causes cerebral damage (Wardlaw et al., 2013).
Our lab took part in a genome wide association study (GWAS) that involved more >80000 patients to identify genes associated with stroke. Genetic variants upstream of the forkhead box F2 (FoxF2) gene, a transcription factor, were associated with an increased risk of all-stroke (Chauhan et al., 2016). In human patients, segmental deletions involving the Foxf2 gene exhibit extensive white matter hyperintensities, a feature of CSVD and ischemic stroke visible with magnetic resonance imaging (MRI; Chauhan et al., 2016, Visscher and Veldink, 2016; Wardlaw et al., 2013). At the cellular level, Foxf2 is expressed in both mural cell types and is involved in their development and differentiation (Chauhan et al., 2016). Future experiments such as Foxf2 knockout experiments in mural cells and the subsequent effect on mural cell activity can help us to better understand the role of Foxf2 in mural cell development and differentiation. Elucidating the dynamic shift in mural cell activity when it comes to regulating vascular tone during development will offer insights into other avenues required for understanding the role of mural cells in CSVD.
CHAPTER SIX: REFERENCES


the developing arterial system. Development, 141(7), 1544–1552.
development in the embryonic zebrafish hindbrain. *Developmental Biology, 357*(1), 134–151.


Williams, K., Alvarez, X., and Lackner, A. A. (2001). Central nervous system perivascular cells are immunoregulatory cells that connect the CNS with the peripheral immune system. *Glia, 36*(2), 156–164.


CHAPTER SEVEN: APPENDICES
7.1 Vehicle control (E3) experiments

Graph 1: Pericyte-covered vessels constrict to vehicle control at 4 dpf.

Pericyte-covered vessels constrict in response to the vehicle control (P=0.012; n=6 measurements from 2 embryos). There was significant constriction at 3 (P=0.040) and 5 minutes (P=0.008). The average vessel diameter at 0, 1, 2, 3, 4 and 5 minutes were 5.2 μm ± 1.3, 5.4 μm ± 1.3, 5.0 μm ± 1.3, 4.9 μm ± 1.3, 5.0 μm ± 1.3, and 4.9 μm ± 1.2.

The average pericyte-covered vessel diameters were compared between the vehicle control and vasoconstricting treated groups. There was significant response to both NA (P<0.0001) and PE (P=0.0582). Vessel diameter at each minute were also compared (Sidak’s multiple comparison test) and significance is summarized in the table below with ns = non-significant:

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Graph 2: Pericyte-covered vessels do not respond to the vehicle control at 6 dpf.

There was no change in pericyte-covered vessel diameter in response to vehicle control at 6 dpf (P=0.898; n=24 paired measurements from 5 embryos). The average vessel diameter at 0 and 10 minutes were 4.9 μm ± 0.8 and 5.0 μm ± 0.9.

The average pericyte-covered vessel diameters were compared between the vehicle control and the SNP treated group. There was no significant difference (P=0.890). Next, vessel diameter at each minute were compared (Sidak’s multiple comparison test) and the results are summarized in the table below with ns = non-significant:

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Cerebral vessels with diameter of $\leq 6.5 \, \mu m$ constrict to the vehicle control at 4 dpf ($P=0.013$; n=23 measurements from 5 embryos). The average vessel diameter at 0, 1, 2, 3, 4, and 5 minutes were 5.2 $\mu m \pm 0.8$, 5.2 $\mu m \pm 0.9$, 5.1 $\mu m \pm 0.8$, 5.0 $\mu m \pm 0.8$ and 5.0 $\mu m \pm 0.8$.

The average pericyte-covered vessel diameters were compared between the vehicle control and vasoconstricting treated groups. There was significant response to both NA ($P=0.003$) and PE ($P=0.020$). Next, average vessel diameter at each minute were compared (Sidak’s multiple comparison test) and the significance is summarized in the table below with ns = non-significant:

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Graph 4: Cerebral vessels with ablated vSMCs from 3-4 dpf constrict to vehicle control.

Cerebral vessels with diameters ≥9.5 μm constrict to the vehicle control at 4 dpf (P=0.011; n=4 measurements from 2 embryos). The average vessel diameter at 0, 1, 2, 3, 4, and 5 minutes were 10.1 μm ± 1.0, 8.1 μm ± 1.2, 8.6 μm ± 1.1, 9.0 μm ± 0.7, 9.2 μm ± 0.7, and 9.0 μm ± 0.5.

The average pericyte-covered vessel diameters were compared between the vehicle control and vasoconstricting treated groups. There was significant response to both NA (P<0.0001) and PE (P=0.002). Next, vessel diameter at each minute were compared (Sidak’s multiple comparison test) and the significance is summarized in the table below with ns = non-significant and s = significant:

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Graph 5: Cerebral vessels with ablated pericytes from 5-6 dpf do not respond to vehicle control.

Cerebral vessels with diameter of ≤6.5 μm do not respond to the vehicle control at 6 dpf (P=0.327; n=57 measurements from 9 embryos). The average vessel diameter at 0, 1, 2, 3, 4 and 5 minutes were 4.9 μm ± 1.0, 4.8 μm ± 1.1, 4.8 μm ± 1.1, 4.7 μm ± 1.1, 4.7 μm ± 1.1 and 4.8 μm ± 1.1.

Average vessel diameters from the vehicle control group was then compared to average vessel diameters in the vasoconstricting treatment groups. There was no significant difference in response to either NA (P=0.771) and PE (P=0.509). Next, vessel diameter at each minute was compared (Sidak’s multiple comparison test) and the results are summarized in the table below with ns = non-significant:

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Graph 6: vSMC-covered vessels constrict to phenylephrine and remain constricted after the addition of vehicle control at 4 dpf.

The embryos were exposed to phenylephrine after baseline (0 minute) for the duration of the experimental period. The vehicle control was then added after minute 3 and kept on until the end of the experimental period. The vSMC-covered vessels constrict to PE (P=0.073; n=6 measurements from 5 embryos). From Tukey’s multiple comparison test, there was significant constriction at 4 minute (P=0.021) and 5 minutes (P=0.040). The average vessel diameters at 0, 1, 2, 3, 4, 5, 6, and 7 minutes were 11.2 µm ± 0.8, 10.1 µm ± 0.9, 10.5 µm ± 1.0, 10.3 µm ± 1.1, 10.2 µm ± 0.9, 10.1 µm ± 0.9, 10.3 µm ± 0.6, and 10.6 µm ± 0.8.

Average vessel diameters from 4-7 minutes in the vehicle control group were then compared to average vessel diameter in the SNAP treated group. There was significant difference in vessel diameter between the two groups (P=0.018). Next, vessel diameter at each minute was compared (Sidak’s multiple comparison test) and the results are summarized in the table below with ns = non-significant:

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Graph 7: vSMC-covered vessels do not respond to phenylephrine or the vehicle control at 6 dpf.

The embryos were exposed to phenylephrine after baseline (0 minute) for the duration of the experimental period. The vehicle control was then added after minute 3 and kept on until the end of the experimental period. The vSMC-covered vessels do not respond to either agent ($P=0.155$; $n=5$ measurements from 5 embryos). The average vessel diameters at 0, 1, 2, 3, 4, 5, 6, and 7 minutes were 10.8 $\mu$m $\pm$ 0.6, 10.4 $\mu$m $\pm$ 0.9, 10.5 $\mu$m $\pm$ 0.7, 10.5 $\mu$m $\pm$ 0.9, 10.2 $\mu$m $\pm$ 0.8, 10.0 $\mu$m $\pm$ 0.7, 10.1 $\mu$m $\pm$ 0.6, and 10.2 $\mu$m $\pm$ 0.9.

Average vessel diameters from 4-7 minutes in the vehicle control group were then compared to average vessel diameter in the SNAP treated group. There was no significant difference in vessel diameter between the two groups ($P=0.911$). Next, vessel diameter at each minute was compared (Sidak’s multiple comparison test) and the results are summarized in the table below with ns = non-significant:

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Graph 8: Pericyte-covered vessels do not respond to DMSO vehicle control at 4 dpf.

DMSO did not result in pericyte-covered vessel constriction or dilation (P=0.421; n=17 measurements from 6 embryos). The average vessel diameters at 0, 1, 2, 3, 4, 5, 6, 7, and 8 minutes were 5.4 μm ± 0.6, 5.3 μm ± 0.6, 5.3 μm ± 0.5, 5.2 μm ± 0.5, 5.2 μm ± 0.5, 5.2 μm ± 0.5, 5.2 μm ± 0.5, and 5.2 μm ± 0.5.

Average vessel diameters from 1-4 minutes in the vehicle control group were then compared to average vessel diameter in the AH23848 treated group. There was no significant difference in vessel diameter (P=0.152). Next, vessel diameter at each minute was compared (Sidak’s multiple comparison test) and the results are summarized in the table below with ns = non-significant:

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Graph 9: Pericyte-covered vessels do not respond to DMSO vehicle control at 6 dpf.

DMSO did not result in pericyte-covered vessel constriction or dilation (P=0.567; n=16 measurements from 3 embryos). The average vessel diameters at 0, 1, 2, 3, 4, 5, 6, 7, and 8 minutes were 4.7 µm ± 0.5, 4.7 µm ± 0.6, 4.6 µm ± 0.7, 4.5 µm ± 0.7, 4.4 µm ± 0.7, 4.5 µm ± 0.4, 4.5 µm ± 0.5, 4.6 µm ± 0.5 and 4.5 µm ± 0.5.

Average vessel diameters from 1-4 minutes in the vehicle control group were then compared to average vessel diameter in the AH23848 treated group. There was no significant difference in vessel diameter (P=0.230). Next, vessel diameter at each minute was compared (Sidak’s multiple comparison test) and the results are summarized in the table below with ns = non-significant:

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