Expression Analysis Highlights AXL as a Candidate Zika Virus Entry Receptor in Neural Stem Cells

Graphical Abstract

Highlights

- Single-cell analysis reveals expression and specificity of candidate Zika receptors

- AXL shows strong expression in human radial glia, brain capillaries, and microglia

- Developing human retina progenitors also show high AXL expression

- AXL expression is conserved in rodents and human cerebral organoid model systems

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In Brief

The recent outbreak of Zika virus and the association with fetal abnormalities including microcephaly represents a global health emergency. Kriegstein and colleagues survey the expression of candidate Zika virus entry proteins to suggest that high AXL expression in neural stem cells may render this population selectively vulnerable to viral infection.
Expression Analysis Highlights AXL as a Candidate Zika Virus Entry Receptor in Neural Stem Cells

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SUMMARY

The recent outbreak of Zika virus (ZIKV) in Brazil has been linked to substantial increases in fetal abnormalities and microcephaly. However, information about the underlying molecular and cellular mechanisms connecting viral infection to these defects remains limited. In this study we have examined the expression of receptors implicated in cell entry of several enveloped viruses including ZIKV across diverse cell types in the developing brain. Using single-cell RNA-seq and immunohistochemistry, we found that the candidate viral entry receptor AXL is highly expressed by human radial glial cells, astrocytes, endothelial cells, and microglia in developing human cortex and by progenitor cells in developing retina. We also show that AXL expression in radial glia is conserved in developing mouse and ferret cortex and in human stem cell-derived cerebral organoids, highlighting multiple experimental systems that could be applied to study mechanisms of ZIKV infectivity and effects on brain development.

In February 2016, the World Health Organization declared the 2015 outbreak of the Zika virus (ZIKV) in Central and South America a global health emergency (Heymann et al., 2016) following a strong correlation between cases of ZIKV infection and a dramatic increase in microcephaly cases in Brazil (Oliveria Melo et al., 2016; Schuler-Faccini et al., 2016). Subsequent reports have now established the ability of ZIKV to cross the human fetal-placental barrier to infect the developing central nervous system (Calvet et al., 2016; Martines et al., 2016; Mlakar et al., 2016). The neurotropism and neurovirulence of ZIKV has been appreciated in model systems since the earliest description of the virus (Bell et al., 1971; Dick, 1952; Dick et al., 1952), but it has only recently been described in human neural stem and progenitor cells using in vitro systems (Tang et al., 2016; P.P. Garcéz, E.C. Loiola, R.M. da Costa, L.M. Higa, P. Trindade, R. Delvecchio, J.M. Nascimento, R. Brindeiro, A. Tanuri, and S.K. Rehen, 2016, PeerJ, preprint). Although pathology data is currently limited, the first imaging studies and cases with confirmed ZIKV infection in the prenatal brain showed devastating consequences, including severe microcephaly, lissencephaly, hydrocephaly, necrosis, periventricular and cortical calcification, diffuse astrogliosis, and activated microglia (Mlakar et al., 2016, Schuler-Faccini et al., 2016). The findings of massive cell death and necrosis reflect a far more destructive process than occurs in many genetic forms of microcephaly.

Primary microcephaly is thought to result from a depletion of the founder population of radial glia, the neural stem cells in developing brain, either through cell death or premature differentiation (Barkovich et al., 2012). Infrequent cases of neurodevelopmental brain malformations including microcephaly have been reported in association with viral infections, including cytomegalovirus (CMV), rubella virus, West Nile Virus, HIV, herpes simplex, and chikungunya (Ahifors et al., 1986; Gérardin et al., 2014; Lanari et al., 2012; Nakao and Chiba, 1970; O’Leary et al., 2006; Sinha et al., 1972; Teissier et al., 2014; von der Hagen et al., 2014). Of the few viruses known to cross the placental barrier, CMV infection causes similar neurodevelopmental brain abnormalities to those caused by ZIKV (Conboy et al., 1986; Fowler et al., 1992; Teissier et al., 2014). CMV neuroinvasiveness is mediated by a variety of entry factors, including integrins and EGFR, which are highly expressed by radial glia, a neural stem cell population. Higher expression of these entry proteins determines the initial susceptible cell population (Kawasaki et al., 2015).

Based on the role of neural stem cells in other forms of microcephaly, we hypothesized that human radial glia may selectively express proteins promoting ZIKV entry and infectivity during neurogenesis. In support of this hypothesis, two recent papers demonstrated the vulnerability of neural stem and progenitor cells to ZIKV using in vitro cultures derived from pluripotent stem cells (Tang et al., 2016; P.P. Garcéz, E.C. Loiola, R.M. da Costa, L.M. Higa, P. Trindade, R. Delvecchio, J.M. Nascimento, R. Brindeiro, A. Tanuri, and S.K. Rehen, 2016, PeerJ, preprint). Many surface proteins facilitate flavivirus entry into cells (Perea-Lecoin et al., 2014), but the precise mechanism remains largely unknown and additional factors may also contribute to infection. Several of these proteins are sufficient to support ZIKV entry into HEK293T cells that normally have low infectivity, including DC-SIGN (encoded by CD209), TIM1 (encoded by HAVCR1), TYRO3, and AXL. Furthermore, blocking or silencing
Figure 1. AXL Is Expressed in Human Radial Glia and Blood Vessels in the Developing Cortex at Mid-neurogenesis

(A) Heatmap showing expression levels of candidate flavivirus entry receptors in primary cells from developing human cortex. Genes directly implicated in ZIKV entry are indicated with dots. Cells are arranged based on inferred cell type identity (see Supplemental Experimental Procedures).

(B) Violin plots showing distribution of expression levels of AXL across single cells of each respective cell type.

(C) Overview of AXL expression in the developing human brain. Images show immunostaining of a section through human cortex at GW18. Radial glia marker SOX2 expression is enriched in the germinal zones, the ventricular zone (VZ), and the outer subventricular zone (OSVZ), while the expression of neuronal marker SATB2 is enriched in the cortical plate (CP). LV, lateral ventricle; MZ, marginal zone. AXL expression is enriched in the germinal zones and at the pial and ventricular edges. Right schematic highlights cell types that strongly express AXL receptor, including the radial glia and brain vasculature.

(D) Immunostaining of the pial edge of the developing cerebral cortex. AXL expression is found in the pial end-feet and pia-contacting radial fibers of the radial glia, visualized by VIM immunostaining. Examples of fibers with double-immunoreactivity for VIM and AXL are highlighted with arrows.

(legend continued on next page)
AXL reduces infectivity in cultured fibroblasts and alveolar epithelial cells by as much as 90% (Hamel et al., 2015). Understanding the expression patterns of putative flavivirus receptors could strengthen the possible link between ZIKV infection and microcephaly and support the discovery of a mechanism of ZIKV neurovirulence.

To identify cell populations that may be particularly vulnerable to ZIKV infection, we analyzed the expression of candidate genes mediating flavivirus entry across single cells from the developing human cerebral cortex (Figure 1A). We previously classified single cells from developing cortex as astrocytes, radial glia, intermediate progenitor cells, and immature excitatory and inhibitory neurons using patterns of genome-wide gene expression (Pollen et al., 2015). To survey additional cell types, we also analyzed cells from developing cortex that express markers of microglia and endothelial cells (Table S1). Importantly, while many candidate entry receptors and attachment factors have been described, other unknown factors may mediate ZIKV entry, and we also include a global table of gene expression across single cells (Table S2). Across cell types, we found that multiple putative flavivirus entry receptor genes, including AXL and heat shock protein genes, showed a strong pattern of enrichment in radial glia cells, astrocytes, endothelial cells, and microglia, suggesting that these cell types may be particularly vulnerable to ZIKV infection (Figures 1A and 1B).

AXL, known to mediate ZIKV and dengue virus entry in human skin cells (Hamel et al., 2015), showed particularly high expression in radial glia (78/96 radial glia displayed expression greater than 6 log2 normalized read counts). In contrast, other candidate genes known to permit ZIKV entry showed more limited expression at this threshold including TYRO3 (7/418 cells and 5/96 radial glia) and CD209 (DC-SIGN, 0/418 cells, Figure S1). Based on these observations, we further investigated the expression pattern of AXL protein in primary human tissue samples using immunohistochemistry. At mid-neurogenesis, AXL is expressed in a highly reproducible pattern throughout the cortex, with strong expression bordering the lateral ventricle and in the outer subventricular zone (OSVZ) (Figures 2C, 2D, and S1). Closer examination revealed that staining along the ventricle resulted from the expression pattern of AXL in developing ferret cortex and organoids, which resemble the VZ of primary human cortex, and in SOX2-expressing cells away from the lumen (Figure S2). The specific expression of AXL in radial glia-like and oRG-like cells in the organoids and limited expression in neurons is consistent with observations from single-cell mRNA-seq analysis of similarly derived cerebral organoids (Figures 2C and S2). Interestingly, human cerebral organoids also contain cells that resemble early choroid plexus cells (Sakaguchi et al., 2015), and these cells strongly express AXL (Figures 2C and S2), consistent with the expression pattern in embryonic mouse (Figure 2A).

Here we report that the candidate ZIKV receptor AXL is highly enriched in radial glia, the neural stem cells of the human fetal cerebral cortex, providing a hypothesis for why these cells are particularly vulnerable to ZIKV infection and providing a candidate mechanism for ZIKV-induced microcephaly. This finding supports recent suggestions that ZIKV preferentially targets in-vitro-derived progenitor cells rather than immature neurons (Tang et al., 2016). Furthermore, we show that AXL is expressed in the OSVZ (Figure S1). Immunostaining for AXL protein shows strong expression in SOX2-expressing cells at the outer edge of the neural retina (NR), and in addition, very strong staining in the ciliary marginal zone (CMZ). Patches of strong AXL staining are indicated by arrows. See also Figure S1.
by cortical astrocytes, blood microcapillaries, microglia, and progenitors in the neural retina and ciliary marginal zone. The latter finding could help explain how ZIKV causes ocular lesions (de Paula Freitas et al., 2016). The specificity of AXL expression in radial glia neural stem cells is also conserved in mouse and ferret cerebral cortex and in human PSC-derived cerebral organoids. We suggest that these diverse systems may support studies of ZIKV infectivity in radial glia and the downstream consequences that may mediate disease pathogenesis.

Transgenic mouse models of microcephaly mutations often show less severe phenotypes than human patients with the same mutation (Barkovich et al., 2012; Gruber et al., 2011; Lizarraga et al., 2010; Woods et al., 2005). Differences in brain development that include massively expanded OSVZ and increased diversity of cortical progenitors in the human cortex likely contribute to this difference. For example, the contribution of oRG cells to brain malformations such as microcephaly or lissencephaly is largely unknown, although this cell type becomes the predominant neural stem cell population in the developing primate and human cortex toward mid-gestation when OSVZ proliferation dramatically increases (Lukasiewicz et al., 2005; Hansen et al., 2010). Our results indicate that oRG cells express AXL at...
very high levels and are likely targets for ZIKV infectivity. Involvement of oRG cells, which have been linked to developmental and evolutionary cortical expansion (Hansen et al., 2010; Ostrem et al., 2014; Pollen et al., 2015), may make a significant contribution to the severe phenotype of ZIKV microcephaly and agria.

Signaling through AXL suppresses the innate immune response (Rotllin et al., 2007). In dengue virus infection, AXL not only supports virus entry, but its kinase domain also enhances virus infectivity following entry (Meertens et al., 2012). If ZIKV binds AXL during entry, it may similarly activate AXL signaling and suppress the innate immune response, enabling the virus to better establish an infection and prevent viral clearance (Miklar et al., 2016). These features suggest that a small-molecule inhibitor of AXL function may be protective against ZIKV infectivity. However, signaling through Axl normally supports neural stem cell survival, proliferation, and neurogenesis (Ji et al., 2014; Lemke and Bursten-Cohen, 2010), and Axl also maintains the blood-brain barrier, protecting against the neurotropism of other viruses (Miner et al., 2015). Interference with normal AXL has been shown to stimulate production of inflammatory cytokines, promote microglia activation, and eventually lead to the loss of neural stem cells (Ji et al., 2013). Therefore, while blocking AXL may protect against cellular infection or viral replication, perturbation of AXL function may also have multiple adverse consequences.

We propose a testable hypothesis: after breaching the placental-fetal barrier, ZIKV reaches the developing brain by hematogenous spread or via the cerebrospinal fluid (CSF) and invades radial glia cells as the first target population with highest AXL expression, either through their processes that often make contact with blood vessels, or via their apical end-feet that make direct contact with the CSF. By preferentially destroying radial glia cells, the founder cell population that generates all cortical neurons, ZIKV can produce severe microcephaly. Future studies will be needed to test this hypothesis and particularly whether AXL expression alone determines the cellular population with enhanced neurotropism for ZIKV in the developing human brain or whether other binding factors, including genes expressed at low levels, may be involved. In addition, further studies are urgently needed to determine (1) how the virus crosses the placenta to infect fetal brain and causes generalized growth restriction (Brasil et al., 2016) and (2) whether the virus infects adult human brain, as ZIKV has recently been detected in the CSF of adults (Carteaux et al., 2016; Mécheries et al., 2016). Finally, other flaviviruses that use similar entry receptors have not been strongly associated with fetal brain abnormalities, and future work must examine potential changes in recent strains of ZIKV. The current manuscript constitutes an initial step toward the understanding of how ZIKV might cause developmental brain malformations.

ACCESSION NUMBERS

The accession number for the single cell sequencing data reported in this paper is dbGaP: phs000989.v2.p1.

SUPPLEMENTAL INFORMATION

Supplemental Information for this article includes figures, two tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2016.03.012.

REFERENCES


