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Journal

Cell Stem Cell, 18(5)

ISSN

1934-5909

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Publication Date

2016-05-01

DOI

10.1016/j.stem.2016.03.012

Peer reviewed

HHS Public Access

Author manuscript

Cell Stem Cell. Author manuscript; available in PMC 2017 May 05.

Published in final edited form as:

Cell Stem Cell. 2016 May 5; 18(5): 591–596. doi:10.1016/j.stem.2016.03.012.

Expression Analysis Highlights AXL as a Candidate Zika Virus Entry Receptor in Human 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 (Oliveira 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.,

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Accession Numbers

Single cell sequencing data has been deposited on dbGaP accession number: phs000989.v2.p1.

Author contributions

T.J.N., and E.D.L, performed expression studies. T.J.N., A.A.P., and C.S.E., performed bioinformatic analysis. E.D.L. and M.B. generated cerebral organoids. T.J.N, A.A.P., and A.R.K. conceived of the project and wrote the manuscript with input from all authors

1971; Dick, 1952; Dick et al., 1952), but has only recently been described in human neural stem and progenitor cells using *in vitro* systems (Tang et al., 2016; Garcez et al., 2016, *PeerJ Preprints*). 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 (Ahlfors et al., 1986; Gerardin 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 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. 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; Garcez et al., 2016, *PeerJ Preprints*). Many surface proteins facilitate flavivirus entry into cells (Perera-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 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 (Figure 1 A-B).

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 OSVZ (Figures 2 C-D and S1). Closer examination revealed that staining along the ventricle resulted from specific localization of AXL to radial glia apical end-feet (Figures 2 D and S1). AXL was also detected at the pial end-feet of radial glia near the meninges (Figure 2 B). In recent years a second population of radial glial cells, known as outer radial glia (oRG), has been identified in the OSVZ of the developing human brain (Fietz et al., 2010; Hansen et al., 2010). We observed high levels of AXL in the cell bodies of oRG cells, accounting for the pattern of AXL labeling in the OSVZ (Figures 2C and S1). In addition, pronounced AXL immunostaining outlined brain capillaries (Figures 2 A and S1), consistent with AXL expression observed in endothelial cells by single cell analysis. We further examined AXL expression from stages of early neurogenesis (GW13.5) to term. We found that AXL expression persisted in radial glia throughout the period of neurogenesis, and in capillaries and astrocytes to term, but remained largely absent from SATB2-expressing neurons, even at later developmental stages (Figure S1).

A recent report of 29 infants with presumed ZIKV microcephaly reported that 10 (34.5%) had severe ocular abnormalities. The ocular lesions consisted of focal pigment mottling and chorioretinal atrophy particularly severe in the macula (de Paula Freitas et al., 2016). Therefore, we examined AXL expression in developing human retina. We dissected two human neural retina samples at GW10 and GW12 and captured single cells for mRNA sequencing. *AXL* was highly expressed in cells that had a stem cell gene signature (Figures 1G and S1). To confirm this finding, we immunostained tissue sections of developing retina. AXL was expressed along the outer margin of the neural retina, where it was co-expressed with SOX2, a marker of neural stem cells. In addition, AXL was highly enriched in cells of the ciliary marginal zone, adjacent to the neural retina (Figure 1G).

We next investigated the possible conservation of AXL expression across model systems that could be used to study the mechanism ZIKV infection and pathogenicity. Public repositories of *in situ* hybridization data indicate AXL expression in mouse radial glia (Figure 2 A). In addition, previous studies of mouse cortex reported enriched Axl expression in the apical end-feet of radial glia cells (Wang et al., 2011). We examined the expression pattern of AXL in developing ferret cortex, and found that, similar to human cortex, AXL is expressed in the end-feet of radial glia cells at the ventricular edge and in oRG cells in the OSVZ (Figure 2

B). Finally, we generated human iPS cell-derived cerebral organoids, and observed AXL expression along the lumen of neuroepithelial-like rosette regions in the 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 2 C 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 2 C and S2), consistent with the expression pattern in embryonic mouse (Figure 2 A).

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 by cortical astrocytes, blood microcapillaries, microglia, and by 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 pluripotent stem cell-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 fetal cortex towards mid-gestation when OSVZ proliferation dramatically increases (Lukaszewicz et al., 2005). 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.

Signaling through AXL suppresses the innate immune response (Rothlin 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 (Mlakar 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 Burstyn-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 to 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 that after breaching of 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 may 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 how the virus crosses the placenta to infect fetal brain as well as to cause generalized growth restriction (Brasil et al., 2016), and to determine whether the virus infects adult human brain, as ZIKV has recently been detected in the cerebrospinal fluid of adults (Carteaux et al., 2016; Mecharles 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 towards the understanding of how ZIKV might cause developmental brain malformations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgement

We are grateful to Hanna Retallack, Shaohui Wang, Anne Leyrat, Joe Shuga, Aaron Diaz, Mercedes Paredes, Joseph LoTurco, Melanie Bedolli, Lillian Adame, Joe DeRisi, and Jeremy Reiter for helpful comments, suggestions, and technical help. We thank the staff at the San Francisco General Hospital for providing access to primary tissue samples. A.A.P. is supported by a Damon Runyon Cancer Research Foundation postdoctoral fellowship (DRG-2166-13). This research was supported by NIH awards U01 MH105989, R37 NS35710, and R01NS075998 to A.R.K. and CIRM award GCIR-06673-A, and by gifts from Helen Ford and Bernard Osher. The authors also wish to thank Marc and Lynne Benioff for their financial support for these studies.

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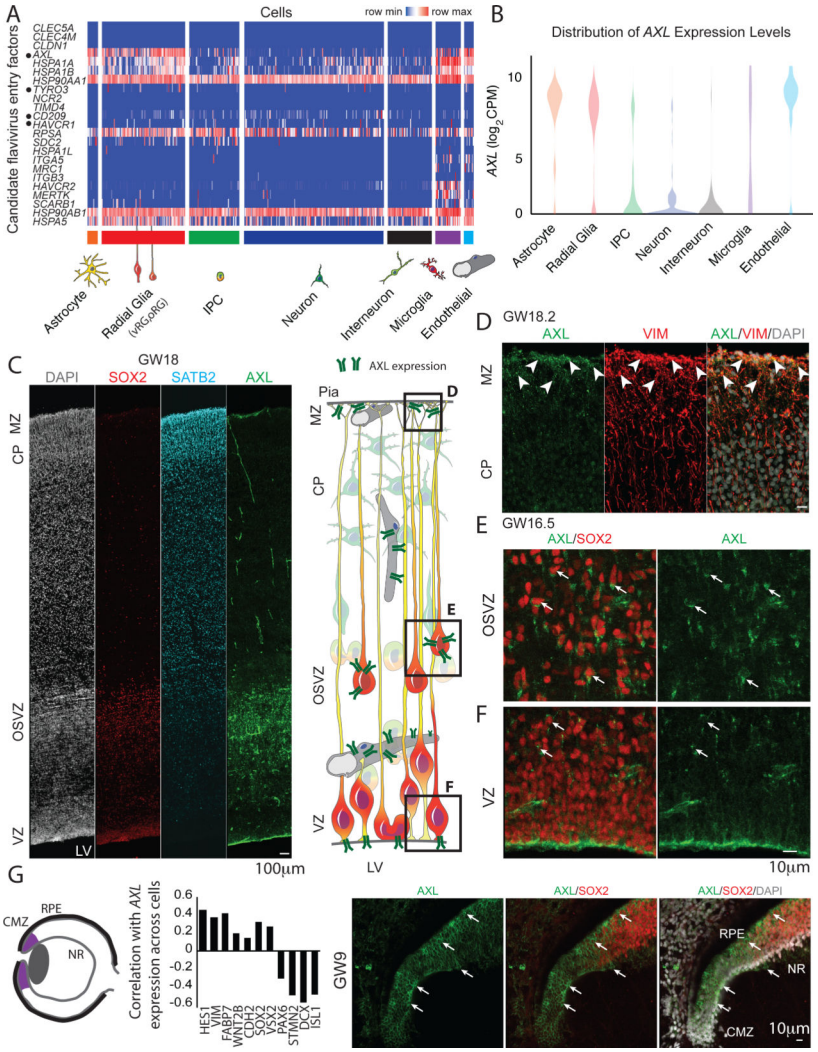

Figure 1. AXL is expressed in human radial glia and blood vessels in the developing cortex at mid-neurogenesis

(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 Supplementary 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. (E) AXL

immunostaining in the OSVZ, where AXL is expressed in the oRG cells, visualized by SOX2 nuclear immunoreactivity (arrows). (F) Strong AXL immunostaining in the VZ can be detected at the edge of the lateral ventricle. Examples of abventricular radial glia with strong AXL expression are highlighted with arrows. (G) Bar chart shows that *AXL* expression is highly correlated with a stem cell signature and anti-correlated with a neuronal signature across single cells collected from GW10 and GW12 primary human neural retina (See also 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.

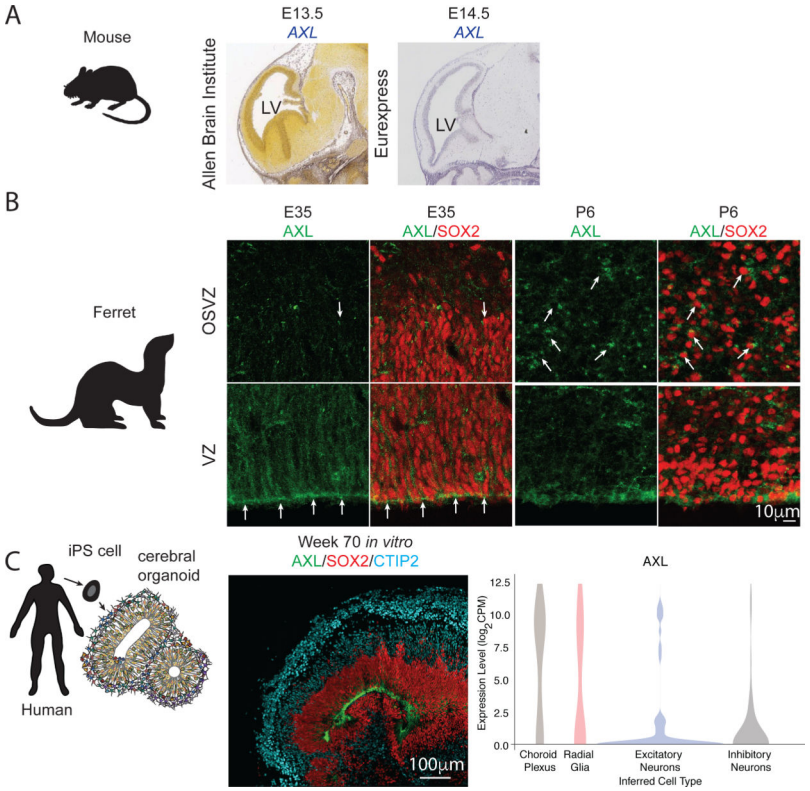

Figure 2. Radial glia expression of AXL is conserved in animal models of cortical development and recapitulated in pluripotent stem cell-derived neural progenitors

(A) Expression of *AXL* mRNA in the ventricular zone of the developing mouse cerebrum, suggesting that the radial glia expression of AXL is conserved in mouse. (B) Immunostaining of ferret cerebral cortex tissue sections at two neurogenic stages of development reveals conserved expression of AXL at the ventricular edge and in oRG cells (arrows). (C) Immunohistochemistry shows that AXL expression is enriched in radial glia-like cells around the lumen of the ventricular zone-like region of the organoid. Violin plots of single cell gene expression reveals high expression of *AXL* in choroid plexus-like and radial glia-like cells, but lower expression in neuronal populations. See also Figure S2.