Missing link:

Animal models to study whether Zika causes birth defects

By Rachel Becker

When Patricia Pestana Garcez returned home to Brazil to start her new position as an assistant professor at the Federal University of Rio de Janeiro in June, she knew that she would study the neurobiology of brain malformations. But she hadn't yet decided whether to continue her research into microcephaly, a birth defect characterized by an unusually small head and caused by genetic mutation or maternal infection.

Over the next eight months, Brazil experienced an unprecedented surge in the number of babies born with microcephaly. The Brazilian Ministry of Health soon posited a link between this spike in birth defects and the rise of a mosquito-borne flavivirus called Zika that had recently entered the country. The two coinciding events, and the ensuing push for researchers to study a possible link, made Garcez's decision clear.

"We have something really important to research," she says. "It's an emergency."

Now, as public health officials scramble to decide what measures to implement to curb the spread of Zika—El Salvador has even asked women to postpone having children

until 2018—Garcez and other scientists have begun to accelerate their efforts to model the effects of the virus in cell lines and animals. They hope to figure out whether Zika can, in fact, cause neurological problems in these models, and if so, how.

Their work capitalizes on promising new techniques, such as the use of three-dimensional clusters of cells known as organoids that offer insights into brain development. "It's quite a beautiful technique," Garcez says. "It can really recapitulate at least the beginning of the cortical development."

Early origins

Zika virus was first discovered in the late 1940s by Scottish virologist George W.A. Dick and two of his colleagues formerly from the Rockefeller Foundation. While monitoring rhesus monkeys caged in the canopy of Uganda's Zika forest for signs of mosquito-borne disease, they sampled the blood of a febrile monkey and discovered a previously unknown virus, which they named after the region. The team later found the same virus in pulverized suspensions of

the *Aedes africanus* mosquito¹.

Since then, Zika virus has spread from Africa to Asia², transmitted by several species of *Aedes* mosquito and possibly also through sexual intercourse in humans³. Virus strains in the Asian lineage caused Zika outbreaks in Micronesia in 2007⁴ and in French Polynesia between 2013 and 2014⁵.

Despite those two recent outbreaks, Zika virus was not the first virus to spring to researchers' minds when, in December 2014, people in the northeastern states of Brazil started to come down with a mild febrile illness. Brazil is rife with flaviviruses, such as dengue, chikungunya and yellow fever—and in adults, Zika virus is asymptomatic about 80% of the time.

However, when it does manifest, affected individuals often develop a rash, joint pain, conjunctivitis and, occasionally, a fever that is typically much milder than the fever associated with a dengue infection.

But the combination of symptoms was unusual enough that Kleber Luz, an infectious disease specialist at the Federal University of Rio Grande do Norte in

Natal, Brazil, and Carlos Brito, a physician at the Federal University of Pernambuco, suspected that these people did not have dengue.

Luz sent serum samples from 21 of these patients to Claudia Nunes Duarte dos Santos, a virologist at the Carlos Chagas Institute—part of the Brazilian Ministry of Health's research branch, the Oswaldo Cruz Foundation (FIOCRUZ). Duarte dos Santos didn't find any of the usual suspects in these patients' sera. Instead, using sequencing techniques, she confirmed Zika virus in eight of the samples⁶. She and Luz told the Ministry of Health about their results immediately.

In parallel, a group led by virologists Gubio Campos and Silvia Sardi of the Federal University of Bahia in Salvador, Brazil, detected the presence of Zika RNA in seven of 24 people tested in the city of Camaçari, more than 650 miles south of Luz⁷. In May 2015, the Brazilian government announced that Zika virus was circulating in the country, adding it to a small list of regions in which the virus is actively transmitted. That list has now grown to 30 countries and territories worldwide.

Neurological problems emerge

The discovery of Zika in Brazil did not alarm clinicians initially. But Brito, who specializes in public health and infectious diseases, recalled that after the 2013 epidemic of Zika virus in French Polynesia, at least 72 people had experienced neurological symptoms, 40 of whom developed a condition called Guillain-Barré syndrome⁸. This condition occurs when the immune system attacks the myelin coating of peripheral nerves, causing weakness and paralysis that typically resolves with time. Although the virus was not confirmed in many of these individuals, the emergence of these neurological deficits so quickly after the outbreak raised suspicion.

In May 2015, Brito sent a message on the mobile app WhatsApp to neurologists around Brazil, alerting them to the historical link between Zika and neurological disease. WhatsApp, Brito says in an e-mail, is widely used in Brazil and enables the rapid communication among doctors that is critical for collecting information on cases in an outbreak investigation such as this one.

He learned of one group of patients being treated by Lucia Brito, head of neurology at the Hospital da Restauração in Recife, Brazil. Some of them had inflammation of the optic nerve; others of the brain and spinal cord—and many had Guillain-Barré syndrome.

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Newborn concern: A reported rise in cases of microcephaly has concerned health officials in Brazil. (Previous page: A container of larvae from the *Aedes aegypti* mosquito, a carrier of Zika virus.)

When Brito, along with FIOCRUZ public health investigator Ernesto Marques and Lucia Brito, tested cerebrospinal fluid and blood from 30 of the patients using RT-PCR, they detected Zika virus in seven samples. Carlos Brito says that they are currently investigating 70 more.

In October, Carlos Brito was called in to assess an unusually large number of microcephaly cases in the state of Pernambuco⁹. Since mid-2015, the Brazilian Ministry of Health has been investigating more than 4,700 reported cases of microcephaly. So far, 404 have been confirmed to have the condition or other central nervous system disorders, and 709 have been ruled out, as this article went to press.¹⁰ The Ministry of Health is still evaluating the remainder. After Brazilian scientists identified Zika virus genetic material in a stillborn infant with microcephaly, as well as in the amniotic fluid of mothers with affected babies¹¹, the country announced that there might be a link between Zika and microcephaly.

Cellular clues

The circumstantial evidence for a causal link between Zika infection in pregnant women and microcephaly in developing infants is piling up, but studying it presents a challenge. "We have no idea about the mechanism," Garcez says.

The virus might directly infect brain cells, or it could work via an indirect mechanism whereby viral toxins damage developing neural tissue, she says. Or, adds virologist Koen van Rompay of the California National

Primate Research Center, University of California Davis, maternal antibodies to the virus could be interfering with normal fetal development.

Recently, Duarte dos Santos identified Zika virus in placental macrophages—another, as yet unpublished, clue that it is possible for the virus to be transmitted to the fetus *in utero*. She has begun to expose mouse neural cultures to the virus to see which cells it preferentially infects and how the infection might change protein production and gene expression.

Garcez, meanwhile, has teamed up with Stevens Rehen, a stem cell biologist at the D'Or Institute for Research and Education in Rio de Janeiro. Rehen's lab specializes in modeling human brain development by using induced pluripotent stem cells (iPS cells). Last year, his lab began to grow organoids—three-dimensional amalgamations of brain tissue that mimic some of the structural features of a brain. To create them, Rehen's lab reprograms human epithelial cells to become iPS cells, which are then fed a cocktail of nutrients and growth factors that steer them toward becoming neural progenitors and, eventually, cerebral tissue¹². Garcez plans to work with members of Rehen's lab to expose the organoids to Zika to investigate how the virus and its products affect the developing brain.

Modeling with mice

Albert Osterhaus, a virologist at the University of Veterinary Medicine Hannover in Germany, thinks that developing an animal model—in addition to *in vitro* and

post-mortem studies—is necessary to show that maternal infection with Zika virus can actually cause microcephaly. After that, scientists can start to use the model to probe the underlying mechanisms.

Osterhaus, who has written about the mechanisms by which neurotropic viruses can cause neuropathology¹³, speculates that one way the virus might infect the fetal brain is via the bloodstream. When a pregnant woman is infected with Zika virus during her first trimester, the virus could move into her bloodstream and cross into the placenta, enter the fetus's bloodstream and travel to its developing brain where it could interfere with normal growth. “But to be honest, we don't know that,” he says. His lab currently has plans to develop an animal model.

Several other researchers are also trying to model the infection in mice and in nonhuman primates, something that scientists attempted in the 1950s¹⁴ and 1970s. A series of experiments conducted in the early 1950s by George W.A. Dick—the scientist who originally discovered Zika—found that although young mice were susceptible to Zika infection when the researchers administered the virus via an intraperitoneal injection, the older mice were not. Mice of all ages were susceptible, however, when given the infection intracerebrally¹⁵. In 1971, T.M. Bell and colleagues infected newborn and young adult mice via an intracranial injection. They observed necrosis in neurons within the hippocampus, inflammation, enlarged

glia and active viral replication¹⁶.

Even if some researchers have been able to model Zika infection in mice, however, mice are not a perfect model system for microcephaly because their brain development differs fundamentally from that in humans. Arnold Kriegstein, director of the developmental and stem cell biology program at the University of California, San Francisco, points to a pool of founder cells called radial glia as one key difference¹⁷. Humans have more—and more types—of radial glia than do mice, and these in turn give rise to more neurons in the human brain than in the mouse brain. Gene mutations and viruses that could destroy these founder cell populations might therefore result in more severe microcephaly in humans than in mice, Kriegstein explains.

Garcez will try to model vertical transmission of Zika by using an intraperitoneal injection to deliver the virus to pregnant mice. She then plans to assess if brain development changes in the pups of infected mothers.

Meanwhile, Duarte dos Santos plans to inject several clinical isolates of the virus into mouse models to determine whether and, if so, how Zika virus causes birth defects. These models include transgenic AG129 mice, which have deficient anti-viral cytokine responses and thus are susceptible to dengue infection¹⁸. Ann Powers, chief of the alphavirus laboratory at the US Centers for Disease Control and Prevention, adds that scientists at her institution will also use

AG129 mice, in addition to outbred, wild-type mice.

At the California National Primate Research Center, Koen van Rompay has been studying HIV in nonhuman primate models since the early 1990s. With the emergence of Zika as an infectious disease of global concern, he is now expanding his focus to model the virus in rhesus macaques. Macaques, he explains, are seasonal breeders—which means that he will need to infect them soon to study how maternal infection affects the fetal brain. Otherwise, he will have to wait months until the next breeding season. Consequently, he is scrambling to pull together enough pilot funding for his research team to infect the breeding females in the next one or two months. Van Rompay hopes that if they are able to recapitulate infant microcephaly via maternal infection with Zika virus in the macaques, they will then be able to ask questions about the mechanisms and test the safety and efficacy of potential vaccines and interventions.

“All of us here are volunteering our time for this, but we feel that this is the reason that we became scientists,” he says. “When there is some acute disease, or some acute emergency—this is why we got our training.”

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Fearful Flavivirus: A molecular model of the Zika virus.