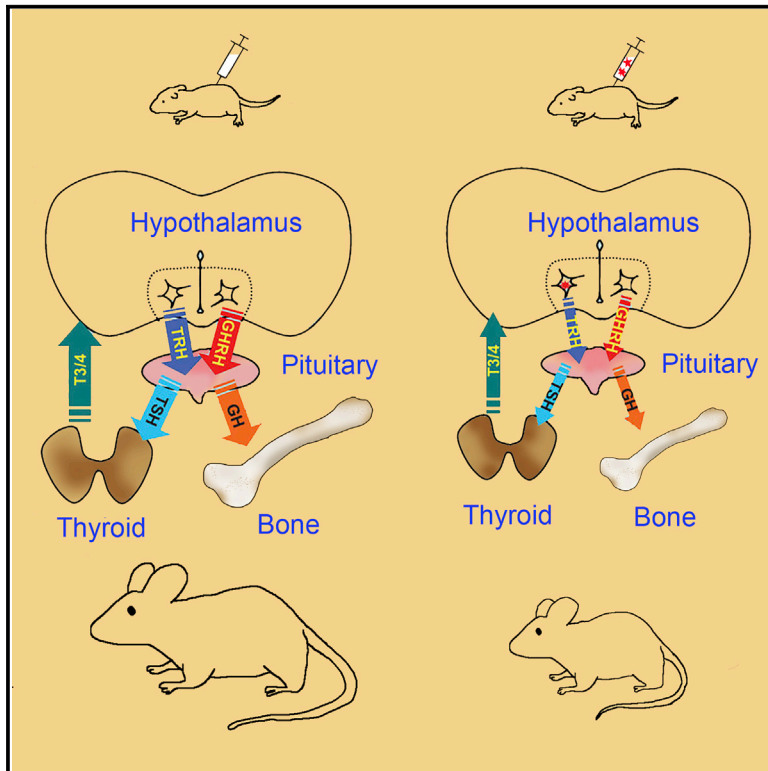


Cell Reports

Zika Virus Infection in Hypothalamus Causes Hormone Deficiencies and Leads to Irreversible Growth Delay and Memory Impairment in Mice

Graphical Abstract



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In Brief

Wu et al. find that ZIKV infection in mouse pups causes hypothalamic damage and dysfunction of the hypothalamic-pituitary axis, featuring multi-hormone deficiencies and resulting in irreversible growth delay and memory impairment during adulthood. Early administration of the relevant hormones could partially improve the long-term sequelae in the neuroendocrine system.

Highlights

- ZIKV infection in mouse pups causes irreversible growth delay and memory impairment
- Hormones from hypothalamus, pituitary, and thyroid are reduced during ZIKV infection
- Hypothalamic cells producing thyrotropin-releasing hormone can be infected by ZIKV
- Early administration of growth hormone improves growth delay caused by ZIKV infection



Zika Virus Infection in Hypothalamus Causes Hormone Deficiencies and Leads to Irreversible Growth Delay and Memory Impairment in Mice

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SUMMARY

Zika virus (ZIKV) can cause microcephaly in the fetus. However, its effects on body growth and the development of children with postnatal ZIKV infection are largely unknown. To examine this, we intraperitoneally challenged mouse pups with ZIKV. Infection causes an irreversible growth delay and deficits in spatial learning and memory, with growth-relevant hormones significantly reduced during infection. These effects are associated with ZIKV RNA expression in the hypothalamus, blood, and brain but not in the pituitary and thyroid. Infection is also associated with hypothalamic inflammation, and ZIKV antigen is detectable in neuroendocrine cells producing thyrotropin-releasing hormone. Moreover, early administration of growth hormone could significantly improve growth delay. Our results demonstrate that ZIKV can infect the hypothalamus, causing multi-hormone deficiencies and delayed growth and development in a mouse model. Therefore, prospective multidisciplinary follow-up of ZIKV-infected children may be necessary to understand potential effects of this virus on childhood development.

INTRODUCTION

Zika virus (ZIKV), a member of the vector-borne flaviviruses, is transmitted mainly by mosquito bites and causes Zika virus disease (ZVD), which has usually been a mild and self-limited illness in the half century since its discovery in 1947. However, a ZIKV outbreak in 2015 and 2016 affected millions of people in more than 70 countries around the world, and ZVD was characterized by severe complications of neurological disorders (Lessler et al., 2016; Miner and Diamond, 2017). As has been well documented, in adults, ZIKV infection may lead to Guillain-Barré syndrome, which is caused by an attack against the peripheral nervous system, and occasionally meningoencephalitis. In pregnant women, ZIKV congenitally infects the neuron progenitor cells, disrupts the development of the brain, and causes fetus microcephaly, as well as a wide spectrum of neurological manifestations that

go beyond microcephaly (Mlakar et al., 2016; Saad et al., 2018). In animal models, the brain is one of the organs containing a higher viral load (Li et al., 2016b; Wu et al., 2017), indicating notable neurotropism of ZIKV and apparent capabilities to pass through the blood-brain barrier.

In addition to regulating mentation and sensorimotor processes, the brain regulates neuroendocrine activity. The hypothalamic-pituitary axis in the brain is a key center in regulating the neuroendocrine system and plays an important role in body growth and development by secreting various hormones (Clarke, 2015; Plant, 2015). Under the control of relevant releasing or inhibiting hormones produced by the hypothalamic nuclei, the anterior pituitary secretes growth hormone (GH), thyroid-stimulating hormone (TSH), and some other hormones to stimulate body growth and development and to regulate the secretion of downstream endocrine organs, such as the thyroid to secrete thyroxine. Consistent with its function, the endothelial cells in the hypothalamus are unique in being fenestrated and thus highly permeable to blood-borne materials, including virus (Langlet et al., 2013). Diseases in the hypothalamus and pituitary in childhood, such as tumor, trauma, or infection, may result in hypopituitarism, which consists of deficiencies of these hormones individually or in combination and causes long-term adverse effects on growth, pubertal development, bone health, fertility, and quality of life (Beatrice et al., 2013). Because ZIKV has distinct neurotropism and the ability to cross the blood-brain barrier, its effects on the neuroendocrine system, especially on the hypothalamic-pituitary axis, need to be investigated.

Because the hypothalamic-pituitary axis is important for growth and development, infants and children are more susceptible to its dysfunction. However, in contrast to adults and fetuses, postnatal ZIKV infection in infants and children has been neglected. Very recently, a few published reports have described infants and children with postnatally acquired ZIKV infection in endemic areas (Li et al., 2017). According to the descriptions, perinatal and breastfeeding-related transmissions were considered potential routes for infant ZIKV infection. The clinical course is mild, with similar symptoms to adults, such as fever, rash, headache, arthralgia, and conjunctivitis in most children, and neurological complications and death are rarely reported (Goodman et al., 2016). However, a seroprevalence investigation after a ZIKV outbreak in French Polynesia in 2014 and 2015 showed that schoolchildren had a higher seroprevalence rate



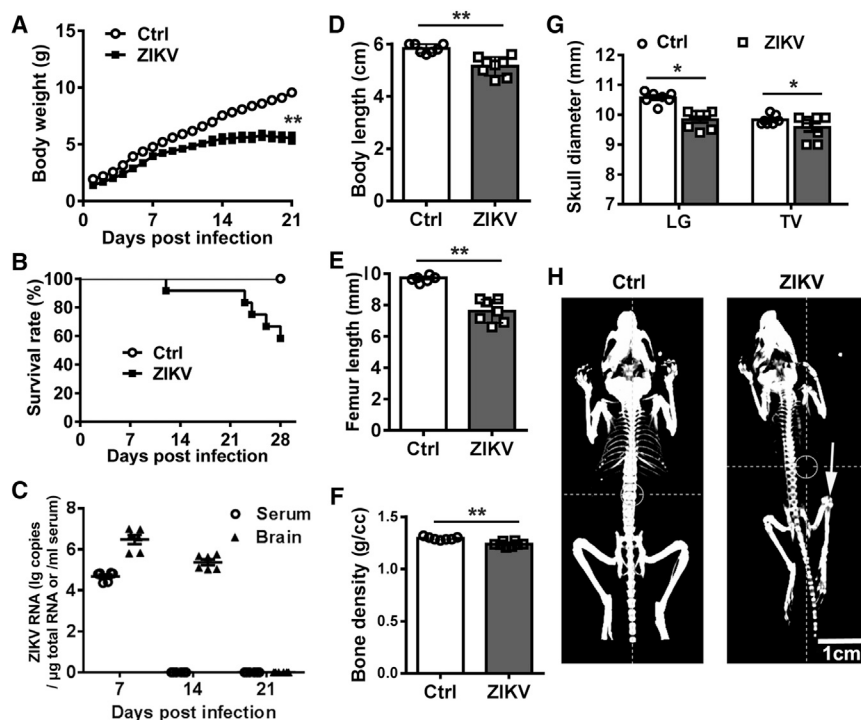


Figure 1. Effects of ZIKV Infection on Growth and Development of Mouse Pups

1-day-old BALB/c mouse pups were injected intraperitoneally (i.p.) with 10^5 pfu of ZIKV resuspended in 0.9% NaCl solution (ZIKV) or an equal volume of 0.9% NaCl as a control (Ctrl). (A and B) Body weight changes (A) and survival (B) were recorded daily for 28 days after infection ($n = 12$ for each group).

(C) The copy number of ZIKV RNA in serum and brain was determined by qRT-PCR at 7, 14, and 21 days post-infection (dpi) ($n = 6$ for each time point).

(D–G) Body length (D), femur length (E), (F) femur bone density (F), and longitudinal (LG) and transverse (TV) skull diameters (G) were measured using micro-computed tomography (microCT) scan at 21 dpi ($n = 8$ for each group).

(H) Representative microCT images. Arrows indicate heterogeneity of the bone density. Scale bar, 1 cm.

Data are presented as mean $\pm$ SEM. Unless otherwise stated, all data hereafter were analyzed using Student's *t* test. Body weight changes were analyzed using repeated-measures ANOVA. Femur length and LG and TV skull diameters were analyzed using the Mann-Whitney U test. * $p < 0.05$ versus Ctrl, ** $p < 0.01$ versus Ctrl.

and lower proportion of asymptomatic infection and that some patients presented with neurological manifestations in the first years of life (Aubry et al., 2017). Very notably, there is a report describing a 10-month-old boy presenting with acute ZIKV infection associated with a stroke (Landais et al., 2017). All of the aforementioned information indicates that children may be at high risk for ZIKV infection (Aubry et al., 2017).

Considering the ability of ZIKV to cause CNS lesions and disrupt the development of the fetus, its effects on the growth and development of children urgently require investigation. Long-term population-based cohort follow-up studies in children are therefore necessary but currently lacking and may take years to perform (Hotez, 2016).

To address these questions, mouse pups were used in the present study as an animal model to investigate the long-term effects of ZIKV infection on growth and development. Our data provide helpful clues for understanding the clinical impact of ZIKV infection in children and suggest that long-term population-based cohort follow-up studies in ZIKV-infected infants and children are urgently needed.

RESULTS

ZIKV Infection Resulted in Growth Delay

To study the effects of ZIKV infection on the growth and development of mice, pups of immunocompetent mice were intraperitoneally (i.p.) inoculated with 10^5 plaque formation units (pfu) of ZIKV (ZIKV group) resuspended in physiological saline (0.9% NaCl solution) or an equal volume of physiological saline (control group) 1 day after birth. Beginning 7 days post-infection (dpi), body weight gain in the ZIKV group was much slower than in

the control group (Figure 1A; $p < 0.01$). Except for slow growth, no apparent symptoms, such as paralysis or disability, were observed. Most deaths of infected mice occurred after 21 dpi, and the survival rate was approximately 40% to 60%, depending on the experiments (Figure 1B). From 21 to 28 dpi, the weights of survivors in the ZIKV group were significantly lower than control (data not shown). By qRT-PCR, ZIKV RNA was detected in the serum of infected mice at 7 dpi with a load of approximately 10^4 – 10^5 copies per microgram total RNA or per milliliter serum and disappeared at 14 dpi (Figure 1C). ZIKV RNA showed a longer duration in brain than in blood; it was detected at 7 and 14 dpi and disappeared at 21 dpi (Figure 1C). Anti-ZIKV antibody was detected in the sera of all surviving mice, confirming that the mouse pup model could be used to observe the effect on growth after ZIKV infection in pups.

Micro-computed tomography (microCT) analysis was performed at 21 dpi, at which time an obvious difference in body weight change was observed between the ZIKV and control groups. The microCT images showed that body length, femur length, bone density, and longitudinal and transverse diameters of the skull in ZIKV-infected mice were all less than in control mice (Figures 1D–1F, $p < 0.01$; Figure 1G, $p < 0.05$). Major organs were collected at 14 and 21 dpi. The weights of organs, including heart, lung, liver, spleen, and kidney, from ZIKV-infected mice were less than those from control mice, and the difference was more obvious at 21 dpi, but the viscera index (viscera weight/body weight) remained almost unchanged (Figures S1A–S1F). Combined with the decreased body weight, these results suggested that ZIKV infection caused a growth delay in mice.

To explore whether the growth delay was associated with nutrition intake and absorption, changes in intestinal morphology

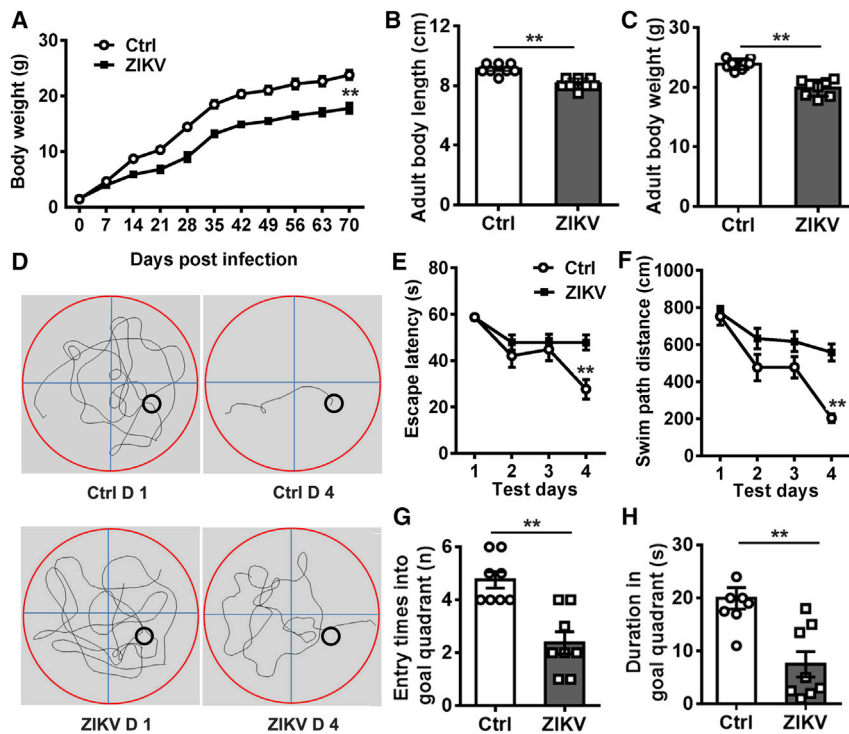


Figure 2. Effects of ZIKV Infection on Body Growth and Memory Development of Adult Mice

(A) Body weight changes were determined every week during the following 70 dpi after ZIKV infection ($n = 12$ for each group).

(B and C) Body length (B) and body weight (C) of female mice were measured at 120 dpi ($n = 8$ for each group). The spatial reference memory and learning ability of ZIKV-infected (ZIKV) and control (Ctrl) female mice were investigated using the Morris water maze at 120 dpi. In brief, mice were placed in a large circular water pool and required to find a visible or invisible platform that would allow them to escape the water during 4 days of the test. (D) A representative swim path on the first (D1) and the fourth (D4) test days.

(E and F) Escape latency (E) and the distance (F) to reach the platform were measured every day.

(G and H) At test day D5, when the platform was removed, (G) the number of entries and (H) the time spent in the goal quadrant where the platform was originally located were then recorded.

Data are presented as mean $\pm$ SEM ($n = 8$ for each group). Body weight, escape latency, and distance to reach the platform were analyzed using repeated-measures ANOVA. ** $p < 0.01$ versus Ctrl.

and function during ZIKV infection were examined. However, neither ZIKV RNA nor histopathological changes was found in the small intestine at 14, 21, and 28 dpi after infection (Figure S2A). Moreover, ZIKV group mice showed normal serum levels of albumin and prealbumin during infection (Figures S2B and S2C), indicating that the growth delay was not due to malnutrition and dysfunction of the intestine.

ZIKV Infection Led to Impairment of Memory and Learning in Adulthood

The effects of ZIKV infection on adulthood were characterized after 28 dpi. As shown in Figure 2, body weight in the ZIKV group was persistently lower than in the control group from 28 to 70 dpi (Figure 2A; $p < 0.01$). Even up to 120 dpi, adult body length and adult body weight in the ZIKV group were still lower than in the control group (Figures 2B and 2C; $p < 0.01$). However, the increasing body weight rate after 28 dpi was similar to control, as revealed by the two parallel curves (Figure 2A), implying that body growth was restored to a considerable level after ZIKV was eliminated from the brain. Nevertheless, the deficit of growth caused during the infection was not compensated.

The Morris water maze was used to analyze the effect of ZIKV infection on the memory development and cognitive functions of adult mice (D'Hooge and De Deyn, 2001). As shown in Figure 2D, in comparison with the control group, mice from the ZIKV group failed to shorten escape latency (Figure 2E) and distance (Figure 2F) to reach the platform during the 4 day training at approximately 120 dpi, showing impaired learning ability after ZIKV infection. On day 5, when the platform was removed, mice from the ZIKV group entered the target quadrant, where the plat-

form was originally located, fewer times (Figure 2G), and they stayed within it for less time (Figure 2H). All the aforementioned parameters revealed a significant difference between the two groups ($p < 0.01$). As revealed by swimming speed, mice in the ZIKV group had no impaired physical mobility (Figure S3); these results thus showed a defect in memory in ZIKV-infected mice. Taken together, the behavioral assay supported the defect in learning ability and memory in ZIKV-infected mice.

GH Deficiency and Pituitary Changes in ZIKV-Infected Mice

The pituitary plays an important role in body growth by secreting GH and TSH, and the latter in turn stimulates the thyroid to synthesize and secrete thyroxine. GH and TSH were thus measured using ELISA at 1, 7, 14, 21, and 28 dpi. Mice in the control and ZIKV groups showed similar change tendencies in serum hormone concentrations: GH gradually decreased within 21 days after birth and slightly increased at 28 days (Figure 3A). TSH displayed a reverse tendency with GH. It gradually increased within 21 days after birth and was maintained at a relatively high level at 28 days (Figure 3B). ZIKV infection did not change the dynamics of GH and TSH, but it significantly reduced GH and TSH at 7 and 14 dpi (Figures 3A and 3B; $p < 0.05$ for both). At 28 dpi, when ZIKV had been eliminated from serum and brain, GH and TSH recovered to the normal control level.

To test the mechanism underlying the reduction of these hormones, the pituitary was collected at different time points post-infection and subjected to qRT-PCR and morphological assessments. ZIKV RNA was not detected in the pituitary at any time point. After birth, the size of the pituitary gradually increased in

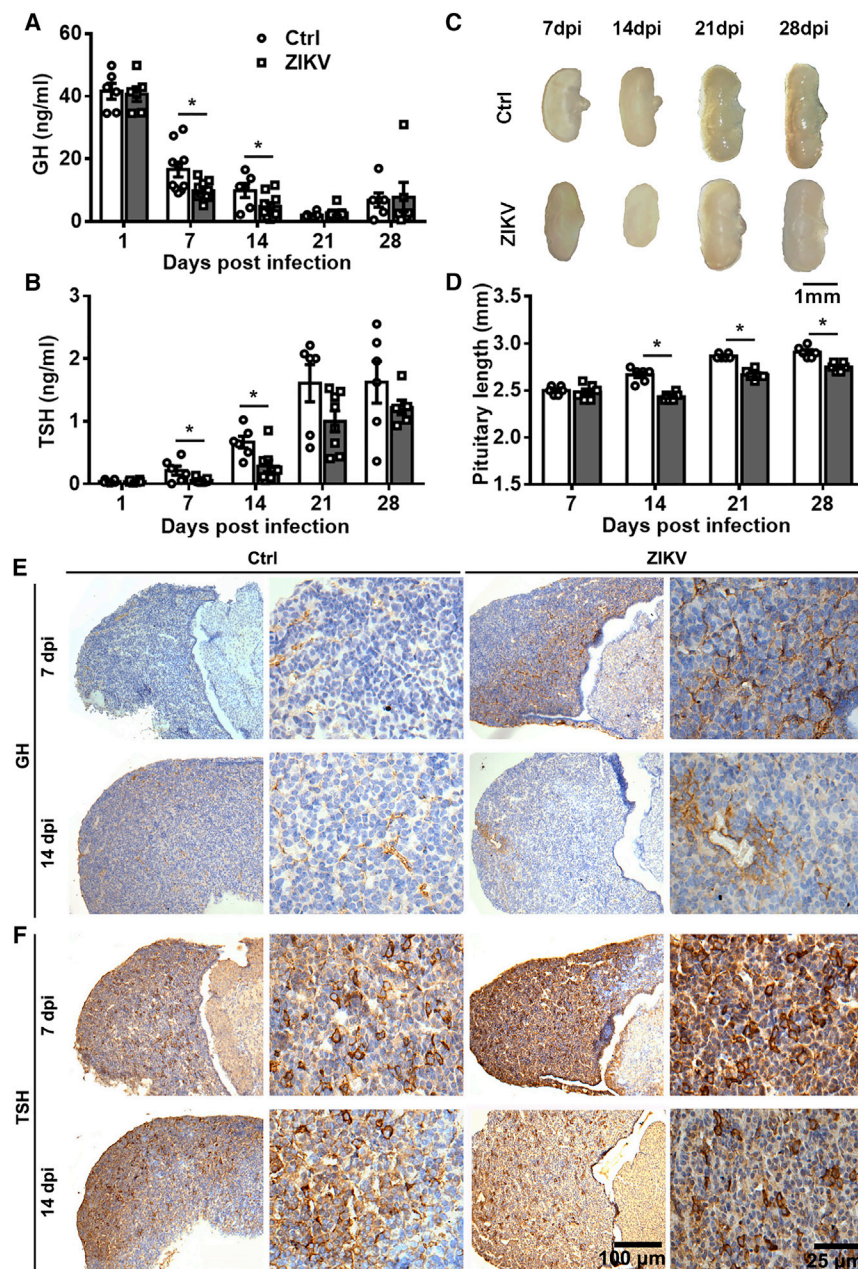


Figure 3. Changes in Hormone Production and Organ Size of the Pituitary after ZIKV Infection in Mice

Sera and pituitaries were collected from ZIKV-infected (ZIKV) or control (Ctrl) mice at 1, 7, 14, 21, and 28 dpi.

(A and B) Serum levels of (A) growth hormone (GH) and (B) TSH were measured using ELISA ($n = 8$ for each time point).

(C and D) Representative images of pituitary (C) and average length of pituitary (D) at 7, 14, 21, and 28 dpi ($n = 4$ for each time point). Scale bar, 1 mm. (E and F) Paraffin sections of pituitary at 7 and 14 dpi were subjected to immunohistochemistry (IHC). (E) Anti-GH and (F) anti-TSH antibodies were used as the primary antibodies. Scale bar, 100 or 25 μm .

Data are presented as mean $\pm$ SEM. * $p < 0.05$ versus Ctrl.

Thyroxine Deficiency and Histopathological Changes in the Thyroid in ZIKV-Infected Mice

Because ZIKV-infected mice showed impairment of learning and memory as well as reduced TSH, changes in thyroxine and the histopathology of the thyroid were characterized. Serum levels of T3 and T4, the two forms of thyroxine, were significantly lower in ZIKV-infected mice at 7, 14, and 21 dpi compared with control (Figures 4A and 4B; $p < 0.05$ except for T4 at 21 dpi at $p < 0.01$), showing similar dynamics as TSH. At 28 dpi, when ZIKV had been eliminated from serum and brain, T3 and T4 partially recovered to the control level. Using qRT-PCR and immunostaining, no ZIKV RNA or antigen was detected in thyroid at any time point. On the basis of H&E staining, the number of thyroid follicles appeared to remain unchanged, but the diameters of most follicles were obviously smaller than the control. Follicular cell hyperplasia and colloid reduction were often observed in the ZIKV group at 14 dpi (Figure 4C).

both groups, but it was always smaller in the ZIKV group than the control group (Figures 3C and 3D; $p < 0.05$). However, no obvious morphological changes were observed in pituitary using H&E staining (Figure S4A). By immunohistochemistry (IHC) staining, the GH-producing and TSH-producing cells were observed in pituitaries from the control and ZIKV groups. Compared with the control, GH-producing and TSH-producing cells had stronger staining signals in the ZIKV group at 7 and 14 dpi (Figures 3E and 3F). In combination with the lower levels of GH and TSH in serum, these results might indicate that the secretion of these two hormones was possibly disrupted in ZIKV-infected mice.

However, morphological changes in the thyroid were recovered at 21 dpi (Figure 4C), suggesting that the function of the thyroid was not fully developed in the absence of TSH. In these experiments, the concurrency of ZIKV infection in brain, growth delay, and the decreases in GH, TSH, and thyroxine suggested that ZIKV infection affected the neuroendocrine system and led to the growth delay.

Growth Delay Was Partially Rescued by Hormone Therapy

Furthermore, to confirm that the decreases in GH and thyroxine caused by ZIKV infection were closely linked to the growth delay

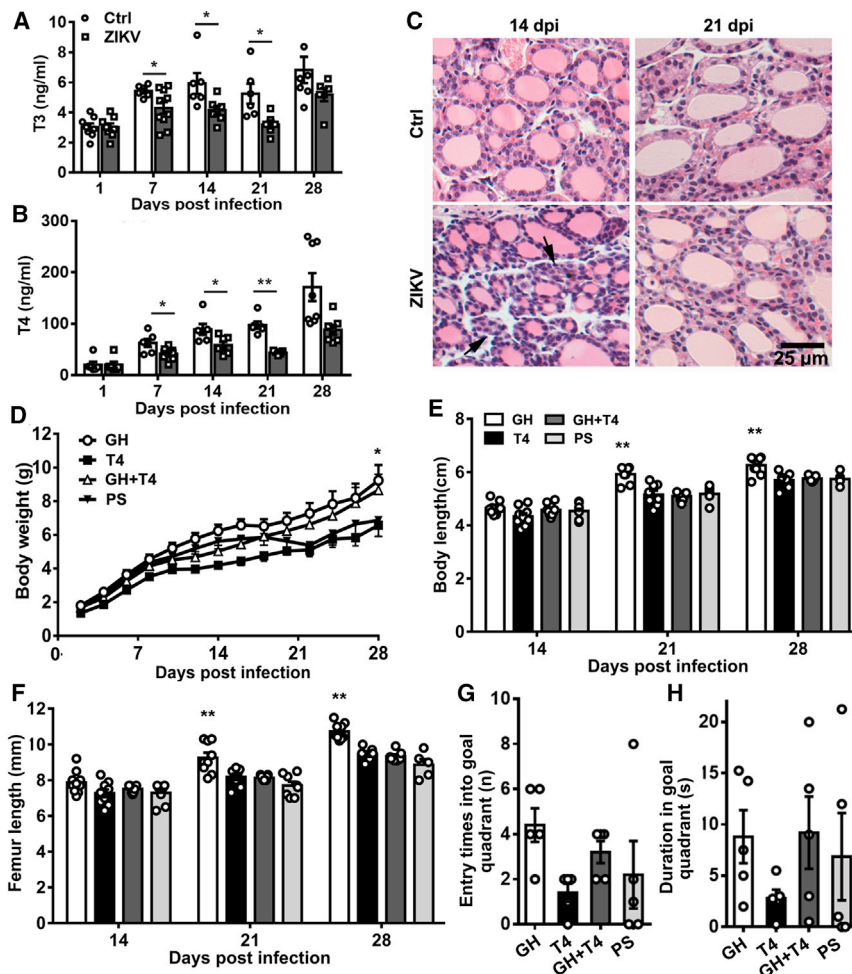


Figure 4. Hormone Production and Morphological Changes in the Thyroid as Well as Efficacy of Hormone Therapy

One-day-old BALB/c mouse pups were i.p. injected with 10^5 pfu of ZIKV resuspended in 0.9% NaCl solution.

(A–C) Sera were collected from ZIKV-infected (ZIKV) or control (Ctrl) mice at 1, 7, 14, 21, and 28 dpi. Serum levels of (A) T3 and (B) T4 were measured using ELISA ($n = 8$ for each time point). (C) Paraffin sections of thyroid at 14 and 21 dpi were analyzed using H&E staining. Arrows indicate follicular cell hyperplasia. Scale bar, 25 μ m.

(D–H) One-day-old BALB/c mouse pups were i.p. injected with 10^5 pfu of ZIKV resuspended in 0.9% NaCl solution, followed immediately by i.p. administration of recombinant human GH (6 μ g/g) (GH), T4 (0.1 μ g/g) (T4), GH (6 μ g/g) plus T4 (0.1 μ g/g) (GH+T4), or 0.9% saline (physiological saline [PS]) once a day. (D) Body weights were monitored daily. MicroCT was performed at 14, 21, and 28 dpi. (E) Body length and (F) femur length were measured using microCT at 14, 21, and 28 dpi. The Morris water maze was performed at 42 dpi when infected mice had grown to adulthood. (G) The number of entries and (H) the time spent (duration) in the goal quadrant where the platform was originally located were then recorded.

Data are presented as mean $\pm$ SEM. Number of entries was analyzed using the Mann-Whitney U test. * $p < 0.05$ versus PS, ** $p < 0.01$ versus PS.

and memory impairment, physiological doses of GH and/or T4 were administered following the infection of ZIKV to evaluate their therapeutic effects on the disorder. Treatment with GH clearly improved the body growth delay, as indicated by markedly increased body weight, body length, femur length, and skull diameters (Figures 4D, 4F, S4B, and S4C). Compared with the physiological saline group, all aforementioned parameters showed a similar change pattern in the GH treatment group: no obvious difference at 14 dpi and partial but significant improvement at 21 and 28 dpi ($p < 0.05$ for body weight, $p < 0.01$ for body length and femur length) (Figures 4E, 4F, S4B, and S4C). However, treatment with GH plus T4 or T4 alone showed no obvious improvement on all aforementioned parameters (Figures 4E, 4F, S4B, and S4C), indicating that the deficiency of GH rather than T4 was the main reason for the growth delay in ZIKV-infected mice.

In contrast to growth delay, memory impairment was not restored by any hormone therapy. GH treatment showed an improved trend, but no significant differences were found on entry times and duration in the goal quadrant from the Morris water maze test compared with the PS group (Figures 4G and 4H). The fact that T4 showed no therapeutic effect on body growth and

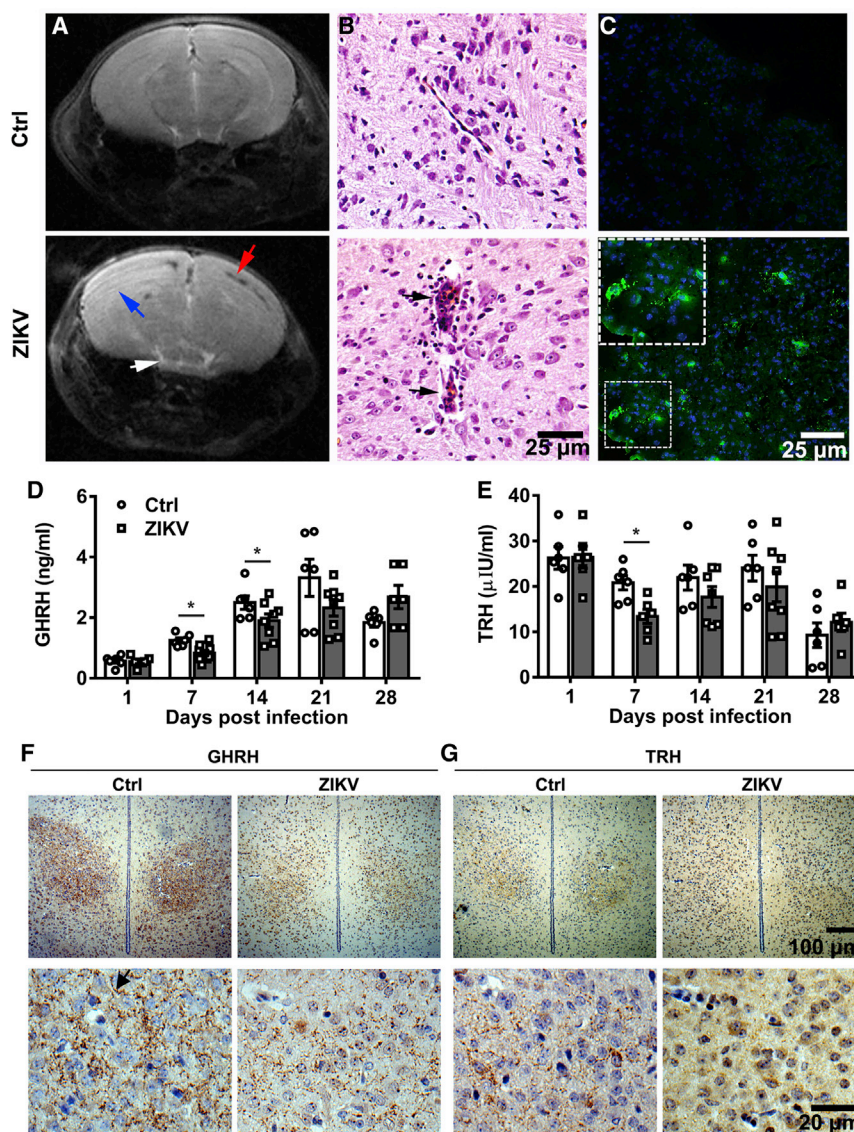
physiological saline group ($p < 0.05$ or $p < 0.01$; Figure S4D). Taken together, these results might suggest that the deficiency of GH was the main contributor to the growth delay in ZIKV-infected mice.

ZIKV Infection in the Hypothalamus Interfered with Its Regulation of the Pituitary

Despite the dysfunction of the pituitary and thyroid as well as the partially effective therapeutic effect of GH, neither destruction of the normal structure nor infiltration of inflammatory cells was observed, and no ZIKV was detected in these two organs in ZIKV-infected mice. Taken together, these results implied that the morphological and functional changes in the pituitary and thyroid were likely attributed to the dysfunction of their upstream hypothalamus.

MRI was used to analyze the pathological changes in mouse brain in the ZIKV group. As shown in Figure 5A, T1 MRI revealed an obviously high-density signal in the right cerebral cortex and subcortex as well as a strip-shaped low signal in the left cerebral subcortex, indicating edema, inflammation, and necrosis. Very notably, the hypothalamic area showed a loss of normal imaging, which was replaced by a signal with a heterogeneous density,

memory impairment was unexpected (Figures 4D–4H, S4B, and S4C), suggesting that it was not the key factor for ZIKV effects on mouse pups. Additionally, all treatments improved bone density with similar levels in comparison with the phys-



indicating inflammation and bleeding. The hypothalamus was further subjected to H&E staining. Infiltration of inflammatory cells and congestion were observed in the hypothalamus of ZIKV-infected mice at 14 dpi (Figure 5B), validating the observations obtained using MRI. By qRT-PCR, ZIKV RNA was detected in the hypothalamus at 7 and 14 dpi and disappeared at 21 dpi (Figure S5A), and ZIKV antigen was also detected therein (Figure 5C), further supporting ZIKV infection in the hypothalamus.

Neuroendocrine cells in the hypothalamus synthesize and secrete GH-releasing hormone (GHRH), thyrotropin-releasing hormone (TRH), and GH release-inhibiting hormone (GHIH) to stimulate or to inhibit the secretion of pituitary hormones such as GH and TSH. As measured using ELISA, GHRH showed an increasing tendency within the 3 weeks after birth in the control group (Figure 5D). ZIKV infection did not change the tendency but significantly reduced its level at 7 and 14 dpi (Figure 5D;

$p < 0.05$). TRH maintained stable levels within 3 weeks after birth (Figure 5E) in the control group, while it was reduced to the lowest level at 7 dpi ($p < 0.05$), followed by gradual recovery in the 2 weeks after ZIKV infection (Figure 5E). Unlike GHRH and TRH, the serum level of GHIH gradually increased after birth in both groups and was significantly reduced at 28 dpi after ZIKV infection (Figure S5B; $p < 0.05$).

Cross-sections of the hypothalamus were used to analyze the distribution of GHRH-producing and TRH-producing cells by IHC staining. In the control group, GHRH-positive and TRH-positive cells were distributed along the third ventricle in the neurosecretory zone of the arcuate nucleus, which was the main nucleus for GHRH and one of the nuclei for TRH (Nurh-dayat et al., 1999) (Figures 5F and 5G, top row). Many immunoreactive beaded fibers were observed in close vicinity to these cells (Figures 5F and 5G, bottom row), which were likely the fibers that transported hormones from the hypothalamus to the pituitary (Clarke, 2015). In the ZIKV group at 14 dpi, the stain signal for both GHRH and TRH weakened, and the beaded fiber also decreased (Figures 5F and 5G). At 28 dpi, the stain signal for GHRH and TRH as well as the beaded fiber in the ZIKV group recovered to a similar level as the control (Figures S5C and S5D). These results thus provided further evidence supporting that ZIKV infection in the hypothalamus disrupted the production of regulatory hormones and consequently caused the dysfunction of the downstream pituitary and thyroid.

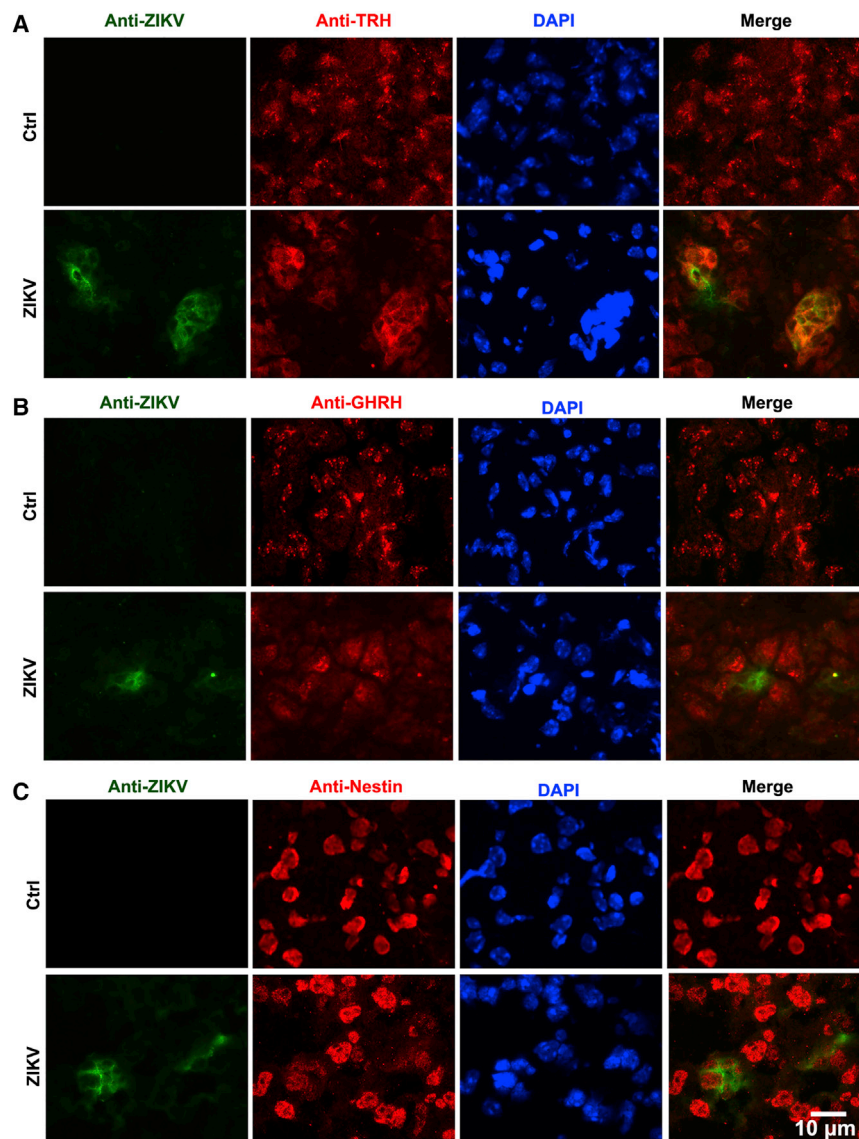


Figure 6. Co-localization of ZIKV Antigen with TRH, GHRH, and Nestin in the Hypothalamus

Frozen sections of brains were collected from ZIKV-infected (ZIKV) or control (Ctrl) mice at 14 dpi and subjected to co-immunofluorescence staining with anti-ZIKV antibody and (A) anti-TRH antibody, or (B) anti-GHRH antibody or (C) anti-Nestin antibody. Scale bar, 10 μ m.

was no obvious increase in apoptosis in the hypothalamus in the ZIKV group at 14 dpi, as revealed by either TUNEL staining or IHC staining (Figures S6A and S6B). Taken together, our results demonstrated that either ZIKV infection itself or the subsequent inflammatory environment affected the neuroendocrine cells in the hypothalamus, reduced their hormone secretion, and finally led to dysfunction of the pituitary and thyroid.

ZIKV Infection Resulted in Overexpression of GHRH and GH in the Long Term

The long-term effects of ZIKV infection were characterized at 210 dpi. Body weight, body length, femur length, and skull longitudinal and transverse diameters in the ZIKV group remained significantly lower than control, with unchanged bone density (Figures 7A–7D, S7A, and S7B). Seven kinds of hormones, as mentioned above, were measured using ELISA. Among them, GH produced by the pituitary and GHRH produced by the hypothalamus were much more abundant in the ZIKV group than in the control group (Figures 7E and 7F; $p < 0.01$ for GH, $p < 0.05$ for GHRH), suggesting an

TRH-Producing Cells in the Hypothalamus Were Susceptible to ZIKV Infection

To further investigate the characteristics of ZIKV infection in the hypothalamus, co-immunofluorescence staining was performed using anti-GHRH, anti-TRH, and anti-ZIKV antibodies. As shown in Figure 6, a cluster of cells co-stained with anti-ZIKV and anti-TRH was observed in the hypothalamus, indicating that TRH-producing cells were susceptible to ZIKV infection (Figure 6A). In contrast, co-localization of anti-ZIKV and anti-GHRH was not detected, but ZIKV-positive cells were localized very close to GHRH-positive cells (Figure 6B). In addition, the co-localization of anti-ZIKV and anti-Nestin was observed, suggesting that the progenitor cells of neuroendocrine cells were also infected (Figure 6C). Intriguingly, there was a dramatic reduction of immunoreactive beaded fibers for both anti-GHRH and anti-TRH in the ZIKV group, regardless of whether the cells were susceptible to ZIKV infection (Figures 6A and 6B). However, there

overproduction of these two hormones in the long term after ZIKV infection. Thyroxine (T3 and T4) and TRH as well as GHIH showed similar levels in both the ZIKV and control groups (Figures S7C–S7F). TSH, however, as a regulatory hormone of T3 and T4 secreted by the pituitary gland, was significantly elevated in the ZIKV group compared with the control group (Figure S7G; $p < 0.05$). H&E staining revealed that the neurosecretory zone in the arcuate nucleus seemed to expand in the ZIKV group with the appearance of more cellular nuclei (Figure 7G). Using IHC staining, many more anti-GHRH-positive rather than anti-TRH cells were observed in the hypothalamus in the ZIKV group (Figures 7H and 7I, top). Moreover, clear immunoreactive beaded fibers were observed around the GHRH-positive cells (Figure 7H, bottom), suggesting that the overexpression of GHRH might be the main cause of the high level of GH in the ZIKV group. Interestingly, the TRH-producing cells in the ZIKV group did not increase but were scattered throughout a larger region,

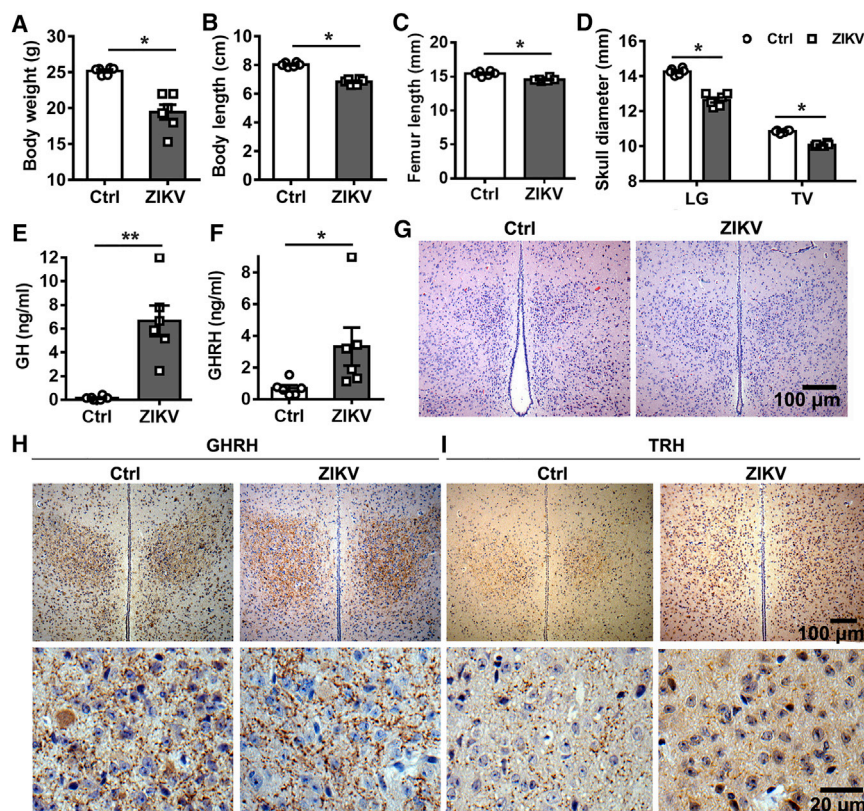


Figure 7. Long-Term Effects of ZIKV on Growth and Development

(A–D) Body weight (A), body length (B), femur length (C), and longitudinal (LG) and transverse (TV) skull diameters (D) of ZIKV-infected (ZIKV) or control (Ctrl) mice were measured using microCT at 210 dpi.

(E and F) Serum levels of (E) GH and (F) GHRH were measured using ELISA ($n = 8$ for each group).

(G–I) Paraffin sections of hypothalamus were subjected to (G) HE staining and IHC with (H) anti-GHRH antibody or (I) anti-TRH antibody. Scale bar, 100 or 20 μm as indicated.

Data are presented as mean $\pm$ SEM. Serum levels of GH and GHRH were analyzed using the Mann-Whitney U test. * $p < 0.05$ versus Ctrl, ** $p < 0.01$ versus Ctrl.

unlike the concentrated pattern in the control (Figure 7I). This observation was consistent with the unchanged TRH serum level at this time point. Additionally, there were no obvious differences in organ size of the thyroid or pituitary between the groups in gross anatomy (Figure S7H). Because no change in apoptosis was detected between the ZIKV and control groups either during acute infection (Figure S6) or at 210 dpi (data not shown), the mechanism underlying the proliferation of GHRH-producing cell remains to be addressed in the future. Taken together, ZIKV could infect the TRH-producing neuroendocrine cells in the hypothalamus, cause multi-hormone deficiencies, and finally delay body growth and memory development in mouse pups.

DISCUSSION

Recent research on ZIKV infection has made great progress in animal models, pathogenesis, and vaccines, especially the damage caused by ZIKV infection to the nervous system (Govero et al., 2016; Ma et al., 2016; Miner and Diamond, 2017; Shan et al., 2017; Sheng et al., 2017; Wang et al., 2017). However, reports are rare regarding the effects ZIKV has on the neuroendocrine system and the consequent long-term effects on body development and growth, especially in infants and children who acquire ZIKV infection postnatally. In this study, using mouse pups, we demonstrated that ZIKV could infect the hypothalamus, cause hypopituitarism, and finally result in growth delay and impairment of learning and memory.

We focused on the thyroid, pituitary, and hypothalamus because growth delay and memory impairment were the most obvious manifestations of the infection. Among the seven hormones measured, six were significantly reduced in ZIKV-infected mice, except for GHIH, indicating that all three hormone-producing organs were affected after ZIKV infection. Moreover, there was no virus detection and no obvious histopathological changes in the thyroid and pituitary, indicating that they

might not take primary responsibility for the growth delay and memory impairment induced by ZIKV infection. Thus, we focused further on the hypothalamus, an upstream organ that regulates the function of the pituitary and thyroid. As expected, ZIKV was detected in the hypothalamus, wherein histopathological changes were also observed. The hormones GHRH and TRH secreted by the hypothalamus were also reduced after ZIKV infection. The hypothalamus is a central neuroendocrine organ and plays a crucial role in regulating hormone release as well as body growth and development (Clarke, 2015). Therefore, the decreased pituitary size and follicular cell hyperplasia, as well as the reductions of T3, T4, and GH, were considered to be closely associated with the deficiency of upstream regulating hormones.

Next, we focused on target cells for ZIKV in the hypothalamus. As revealed by co-immunostaining, ZIKV antigen co-localized with anti-TRH-positive cells, which were distributed in a cluster pattern, indicating that TRH-producing cells, at least those in the hypothalamus, were susceptible to ZIKV infection. Co-localization of ZIKV antigen and Nestin was also observed, in agreement with previous reports and suggesting that neuronal precursor cells are susceptible to ZIKV (Cugola et al., 2016; Li et al., 2016a; Tang et al., 2016). Cells adjacent to GHRH-producing cells were stained with anti-ZIKV antibody. On the basis of this result, it was difficult to judge whether GHRH-producing cells were resistant to ZIKV infection or the expression of GHRH was inhibited in ZIKV-infected cells and thus precluded staining. Further studies are needed to clarify this issue.

The mechanisms underlying the change in hormones in the acute infection phase and long term after ZIKV infection are an interesting question. During acute infection, the expression of TRH and GHRH generally decreased with the reduction of beaded fibers of TRH and GHRH, potentially because of the direct effect of ZIKV on cells in the hypothalamus. Moreover, inflammatory cell infiltration and congestion were observed in the hypothalamus, implying that the local inflammatory environment induced by ZIKV infection was likely another reason for the change in hormone expression. In the long term, the serum level of GHRH significantly increased, together with an equivalent increase in the GH level, its downstream hormone. In contrast, most hormones related to the hypothalamus-pituitary-thyroid axis showed little change, except for an increase in TSH in the ZIKV group. Because no histopathological change was found in the pituitary, we speculate that the increased levels of GH and TSH might have been due to the overexpression of GHRH, which is essential for the expansion of the somatotrope lineage during pituitary development (Frohman and Kineman, 2002) and can potentiate the TSH-releasing activity of TRH (Looij et al., 1995). We cannot completely explain the cause of these differences on the basis of our present results. However, the histopathological changes in the hypothalamus in the long term after ZIKV infection may provide some explanation. The neurosecretory zone in the arcuate nucleus expanded and contained mainly GHRH-producing cells. In contrast, the TRH-producing cells showed a dispersed distribution in a larger region in the ZIKV group, unlike the concentrated pattern observed in the control. These results suggested a hyperplasia of GHRH-producing cells, which might be attributed to the following possibilities: some proliferating factors released during ZIKV infection (Sheikh and Mohamed, 2015) and the potential cross-talk between TRH-producing and GHRH-producing cells by an unknown mechanism. Additionally, no increase in apoptosis was detected in ZIKV-infected hypothalamus. Thus, the homeostasis of GHRH-producing cells was no longer maintained and finally led to over-proliferation and overexpression of relative hormones. For TRH-producing cells, viral direct damage might counteract the role of these proliferating factors and thus avoid over-proliferation. The feedback among thyroxine, TSH, and TRH may be another mechanism of over-proliferation avoidance (Joseph-Bravo et al., 2015).

The dysfunction of the hypothalamic-pituitary axis can be caused by diverse etiologies. Recently, infectious diseases are increasingly also being recognized as important factors. Infection by Hanta virus, which usually causes hemorrhagic fever, has been found to be complicated by a late hypopituitarism (Pekic et al., 2005). The manifestation appeared 1–2 years after the viral infection and could be improved after replacement therapy. Regarding flavivirus, no adverse effects on the hypothalamus and pituitary have been documented, but West Nile virus (WNV) infection has been associated with the onset of depression (Murray et al., 2007). Intriguingly, the GH level in adults is important for both mental and emotional well-being and for maintaining a high energy level. Adults with GH deficiency often have higher rates of depression (Prodan et al., 2012). Therefore, as a neurotropic virus, the long-term effect of ZIKV on the hypothalamic-pituitary axis is required to be further investigated.

GH secreted by the pituitary and thyroxine secreted by the thyroid are the most essential hormones through which the hypothalamus and pituitary regulate the growth and development of children, particularly the increase in height during childhood. Growth failure and short stature of children are the major manifestations of GH deficiency. Although malnutrition induced by insufficient food intake and adsorption during diseases including viral infection is also able to cause growth delay, the pattern is different from that induced by GH deficiency. Growth delay caused by malnutrition is usually accompanied by an increase of GH followed by accelerated growth for compensation when the malnutrition is corrected, which is thus called compensatory growth or catch-up growth (Griffin, 2015). In contrast, the growth delay observed in ZIKV-infected mouse pups was accompanied by a reduction of GH and growth deficit during the acute infection period, and it was not compensated after the virus was eliminated. Notably, treatment with GH or GH plus T4 could partially improve the growth delay and memory impairment in this study. Moreover, neither ZIKV RNA nor histopathological changes were observed in the small intestine in ZIKV-infected mice, and the infected showed normal a nourishment state. Taken together, the growth delay was considered to be caused by ZIKV-induced hormone deficiency but not malnutrition during acute infection.

In addition to GH, thyroxine secreted by the thyroid plays an important role in the growth and development of an organism by regulating metabolism, and its deficiency in infants may lead to intellectual impairment. In our experiments, T3 and T4 deficiency persisted up to 2 weeks (from 7 to 21 dpi) in mouse pups after ZIKV infection. Moreover, memory impairment was validated on the basis of the results of the Morris water maze, in which the surviving adult mice following ZIKV infection showed little progress in learning and memory ability after training. Cognitive sequela occurs in survivors following infection by various flaviviruses. Key factors for innate immunity, including complement and interleukin-1, mediate the damage to neurons and thus play important roles in the cognitive sequelae caused by WNV (Garber et al., 2018; Vasek et al., 2016). Recently, TNF- α has been demonstrated to mediate neurological abnormalities after ZIKV infection (Nem de Oliveira Souza et al., 2018). Therefore, two key factors were considered to be involved in the memory impairment in this study. First, injury to neurons and damage to the cerebral cortex by ZIKV infection observed in our study were considered main reasons for memory impairment. Secondary T3 and T4 deficiency might also contribute to the memory impairment. However, treatment with T4 alone could not improve the learning and memory ability of the infected mice, suggesting that ZIKV-induced neuronal damage and dysregulation were the dominant causal factors of the cognitive dysfunction. Thus, the limited therapeutic effect of T4 was reasonable in the case of neuron injury by ZIKV infection.

Additionally, bone density was also changed in the ZIKV-infected mouse pups. However, different from body growth and memory development, bone density was rescued by all hormone therapies, indicating that both GH and T4 played an important role in bone density formation. Moreover, a recent study reported that the low bone density was likely associated with systemic inflammation (Nem de Oliveira Souza et al., 2018). Therefore, both systemic inflammation and hormone deficiency were

considered contributors to the heterogeneity of bone density observed in the ZIKV-infected mice.

Our results were obtained using mice, with a lifespan much shorter than humans and development and growth that might be affected to a greater extent than in humans. In comparison with human infants, rodents' neurodevelopment is immature at birth. It is thought that the 12- to 13-day-old rat pup has a cerebral cortex comparable with that of a full-term newborn human baby (Dutta and Sengupta, 2016; Romijn et al., 1991). The infection of 1-day-old mouse pups used in our experiment may be similar to that later in pregnancy. Thus, these results cannot simply be reflective of human infants. However, the multi-hormone deficiencies caused by ZIKV in mice do imply the need to monitor the growth and development of infants and children who acquire postnatal ZIKV infection. Congenital infection later in pregnancy was possibly better to be included. Unfortunately, long-term population-based cohort follow-up studies in children are currently lacking and may take years to perform. Until recently, non-congenital ZIKV infection in children has been reported. Most children who suffer from ZIKV infection are asymptomatic or affected by a mild and self-limited illness without death or complicated disease (Li et al., 2017). However, the long-term outcomes of the infection in terms of children's health are unknown. It should be noted that whether the pups died from or survived ZIKV infection, they all suffered from growth delay, indicating that the occurrence of growth delay was not correlated with the severity of infection. A similar concern has recently been raised in children with asymptomatic ZIKV infection (Saad et al., 2018). Thus, regardless of the severity of symptoms, infants and children who acquire postnatal ZIKV infection should be monitored for development and growth.

Growth delay and memory impairment are long-term adverse effects that influence not only growth and puberty but also the ability to function normally within adult society. Since the recent outbreak of ZIKV in 2015, manifestations resulting from deficiencies of GH and thyroxine, if such manifestations exist, are supposed to be gradually emerging. Our results thus indicate that appropriate prospective multidisciplinary follow-up of children infected with ZIKV are necessary, not only to understand the natural history of this new agent but also to provide better development and quality of life for these children and their families.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and can be found with this article online at <https://doi.org/10.1016/j.celrep.2018.10.025>.

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AUTHOR CONTRIBUTIONS

Y.-H.W. and X.-Y.C. performed the animal experiments and most other experiments. W.Y. and D.-Y.F. maintained the cells and virus. Y.-H.W., X.-Y.C., D.L., P.-G.W., and J.A. designed the experiments and analyzed the results. Y.-H.W., P.-G.W., and J.A. prepared and revised the manuscript. J.A. organized the collaboration and directed the project.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse anti-GHRH polyclonal antibody	Cloud-Clone	Cat#MAA438Hu22
Mouse anti-TRH polyclonal antibody	Cloud-Clone	Cat#MAA839Hu22
Mouse anti-Nestin polyclonal antibody	Novus Biological	Cat#MAB1259; RRID:AB_2251304
Goat anti-mouse IgG(Alexa Fluor 488)	Life Technologies	Cat#A-11029; RRID:AB_2534088
Donkey anti-rabbit IgG (Alexa Fluor R 594)	Life Technologies	Cat#R-37119; RRID:AB_2556547
Rabbit anti-GHRH polyclonal antibody	Cloud-Clone	Cat#PAA438Mu01
Rabbit anti-TRH polyclonal antibody	Cloud-Clone	Cat#PAA839Mu01
Rabbit anti-GH polyclonal antibody	Cloud-Clone	Cat#PAA044Mu01
Rabbit anti-TSH polyclonal antibody	Bioss	Cat#bs-0460R; RRID:AB_10856531
Secondary HRP-conjugated goat anti-rabbit antibody	Zhongshan Golden Bridge Bio Co.	Cat#PV-9001
Mouse anti-ZIKV polyclonal antibody	Sheng et al., 2017	N/A
Bacterial and Virus Strains		
ZIKV (strain CAS-ZK01, Asian lineage)	Ma et al., 2016. provided by Dr. George Fu Gao	N/A
Chemicals, Peptides, and Recombinant Proteins		
GH	Saizeng, China	Cat#S20050025
Euthyrox(Levothyroxine Sodium Tablets, T4)	Merck, Germany	Cat#7464776116
Optimal cutting temperature compound(OCT)	SAKURA	Cat#4583
Critical Commercial Assays		
Rat/Mouse GH ELISA Kit	Millipore	Cat#EZRMGH-45K
Mouse GHRH ELISA Kits	Cloud-Clone	Cat#CEA438Hu
Mouse TRH ELISA Kits	Cloud-Clone	Cat#CEA839Hu
Mouse GHIH ELISA Kits	Cloud-Clone	Cat#CEA592Mu
Mouse TSH ELISA Kits	Cloud-Clone	Cat#CEA463Mu
Mouse T3 ELISA Kits	Cloud-Clone	Cat#CEA453Ge
Mouse T4 ELISA Kits	Cloud-Clone	Cat#CEA452Ge
Mouse albumin ELISA Kits	Cusabio	Cat#CSB-E13878m
Mouse prealbumin ELISA Kits	Cusabio	Cat#CSB-EL025270MO
<i>In Situ</i> Cell Death Detection Kit	Roche	Cat#11684817910
Quant One Step qRT-PCR	Tiagen	Cat#FP303-01
Experimental Models: Cell Lines		
Aedes albopictus mosquito cell line (C6/36)	National Infrastructure of Cell Line Resource	Cat#3131C0001000400021
Vero cells	National Infrastructure of Cell Line Resource	Cat#3111C0001CCC000060
Experimental Models: Organisms/Strains		
Pregnant BALB/c mice	Academy of Military Medical Sciences	N/A
Software and Algorithms		
SPSS 17.0 software	IBM	https://www.ibm.com/account/reg/cn-zh/signup?formid=urx-19774
GraphPad.Prism.v6.01	GraphPad Prism	https://www.graphpad.com/scientific-software/prism/
Inveon research work place 1.5 software	Siemens Inveon	N/A
Video tracking system	LIME LIGHT v3.0	N/A
7500 Real Time PCR System	Applied Biosystems	N/A
7.0T MR scanner	Bruker Pharmascan	N/A

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Jing An (anjing@ccmu.edu.cn).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mouse

Pregnant BALB/c mice from the Academy of Military Medical Sciences (Beijing, China) were bred and maintained under a specific pathogen-free animal facility at Capital Medical University.

Ethics statement

All animal experiments were approved by and conducted in accordance with the guidelines established by the Institutional Animal Care and the Animal Ethics Committees of Capital Medical University (Approval number: AEEI-2015-048).

Virus

ZIKV (strain CAS-ZK01, Asian lineage) was propagated in the *Aedes albopictus* mosquito cell line (C6/36) and the titers were determined by plaque assay on Vero cells under overlay medium containing 1.2% methylcellulose. Stocks were stored at -80°C until use.

METHOD DETAILS

Mouse experiments

One-day-old mouse pups were injected intraperitoneally (i.p.) with 10^5 pfu of ZIKV in 50 μL physiological saline (0.9% NaCl solution), or an equal volume of 0.9% NaCl solution as a control. The body weight and mortality of the mice were recorded for 70 days and at 120 and 210 dpi. Major samples from the viscera, including heart, lung, liver, spleen and kidney, were collected at 7, 14, 21, and 28 dpi. Pituitary, thyroid, and hypothalamus were collected at 7, 14, 21, 28 and 210 dpi for morphological studies and ZIKV detection.

MicroCT

X-ray microtomographic equipment, a Quantum FX Caliper microCT Imaging System (Siemens Medical Solutions, USA), was used to determine the bone growth and bone density of mouse pups at 21 dpi or 210 dpi. The X-ray source was set at 80 KV and 520 μA . Mice were scanned up to a field of view (FOV) of 48.64 mm $\times$ 72.96 mm and imaged with a 2D isotropic voxel size of 190 μm . Data analyses were performed using Inveon research work place 1.5 software (Siemens Inveon, Germany). Body length was measured from nose to anus, skull transverse diameter was the length between two ears, and skull longitudinal diameter was the length from the forehead to the foramen magnum. During the experiments, the animals were anesthetized with a mixture of isoflurane (0.75~1.5%) and oxygen (100%). Their breathing rate and body temperature were monitored. After scanning, the mice were observed for 1 h to ensure adequate anesthetic recovery.

Hormone therapy

One-day-old mouse pups were divided into 4 groups (10 mice for each group): GH, T4, GH+T4, and PS. Following the infection of ZIKV, the mice were i.p. injected with a dose of 6 $\mu\text{g/g}$ of recombinant human GH (Somatropin Injection, Saizeng, China) alone or 0.1 $\mu\text{g/g}$ of T4 (L-T4, Merck, Germany) alone or combined GH (6 $\mu\text{g/g}$) + T4 (0.1 $\mu\text{g/g}$) once per day as previously reported (Do et al., 2015; Gesing et al., 2014). The mice in the PS group were i.p. injected with 0.9% saline daily. All mice were monitored daily, and body weights were tested for 28 days. At 14, 21, and 28 dpi, body length, femur length, skull diameters and bone density were tested by MicroCT. The Morris water maze was used to analyze the effects of the hormone against ZIKV infection on the memory development at 42 dpi as the mice grew to adulthood.

Morris water maze testing

The Morris water maze test was used to analyze spatial reference memory and learning ability in ZIKV-infected mice. The water maze (150 cm in diameter) was filled with water and made opaque using black ink. A hidden 10-cm-diameter platform was positioned in the northwest quadrant. Mice collected at 42 dpi or 120 dpi were trained for 4 days (D1-D4) as follows: they were individually placed in the water at a starting point (E) and given 60 s to find the platform. They were then dried for approximately 10 min, followed by the next three trials starting at different points of S, W and N. On day 5, the platform was removed, the quadrant was defined as the goal quadrant and the mice were placed in the opposite quadrant (southeast quadrant). A 60 s probe trial (platform removed) was conducted to evaluate whether the mice localized the platform to the spatial position. The swimming track was recorded and measured

using a video tracking system (LIME LIGHT v3.0, Germany). Spatial reference memory was determined by measuring the escape latency and swim path distance every day.

ELISA

The concentrations of GHRH, TRH, GHIH, GH, TSH, T3 and T4 in mouse serum were quantified by ELISA according to the manufacturer's instructions. GH analyzing reagents were purchased from Millipore (Rat/Mouse Growth Hormone ELISA Kit, Millipore, USA). GHRH, TRH, GHIH, TSH, T3 and T4 reagents were purchased from Cloud-Clone (mouse GHRH, TRH, GHIH, TSH, T3 and T4 ELISA Kits, Cloud-Clone, USA). To reflect the nutritional status of infected mice, serum levels of albumin and prealbumin were also measured by ELISA (mouse albumin and prealbumin ELISA Kits, Cusabio, China). Briefly, 96-well plates were precoated with anti-GHRH, TRH, GHIH, GH, TSH, T3, T4, albumin or prealbumin monoclonal antibodies at 4°C overnight. Subsequently, 50 µL standard or sample (50 µL/well) was added, followed immediately by 50 µL Detection Reagent A. After incubation and washing, the prepared Detection Reagent B was added and incubated for 30 min. Finally, 90 µL Substrate Solution was added and incubated at 37°C for 10 min, followed by addition of 50 µL Stop Solution. The absorbance was measured at 450 nm in a Multiskan spectrum 1500 (Thermo, USA).

ZIKV mRNA quantification

Serum and major organs were harvested from ZIKV-infected or control mice at different time points as indicated. RNA was extracted from tissue lysates according to the manufacturer's protocol. Real-time qPCR analyses were performed as previously reported (Wu et al., 2017) with Quant One Step qRT-PCR (Tiangen, China) on a 7500 Real Time PCR System (Applied Biosystems, USA). Quantification of the copies of ZIKV mRNA was determined using the standard curve method. The ZIKV genome RNA transcribed *in vitro* was quantified and used as a standard template to establish the standard curve. ZIKV mRNA was expressed as the copy number per µg total RNA or per ml serum.

MRI

MRI of mice brain was performed with a 7.0T MR scanner (Bruker Pharmascan, Germany) using the RARE sequence: matrix = 256 × 256, repetition time (TR) = 3000 ms, echo time (TE) = 45 ms, field of view = 2.5 × 2.5 cm², flip angle = 180°, number of averages = 3, and acquisition time = 4 min 48 s. During the MRI experiments, the animals were anesthetized with a mixture of isoflurane (0.75~1.5%) and oxygen (100%). Their breathing rate and body temperature were monitored.

Immunofluorescence staining

The hypothalamus collected from ZIKV-infected or control mice was embedded immediately in optimal cutting temperature compound (OCT, SAKURA, USA) to prepare frozen sections (6 µm in thickness). The sections were incubated with mouse anti-ZIKV polyclonal antibody (PAb) (1:200), anti-GHRH (1:100, Cloud-Clone, China), anti-TRH (1:100, Cloud-Clone, China), or anti-Nestin (1:100, novus biological, USA) as primary antibody at 4°C overnight, and then incubated with goat anti-mouse IgG (1:400, Alexa Fluor 488, Life technologies, USA) or donkey anti-rabbit IgG (Alexa Fluor R 594, Life technologies, USA) at 37°C for 1 h. All images were captured with an Olympus microscope (IX71, Olympus, Japan).

Hematoxylin and eosin staining

The pituitary, hypothalamus, thyroid and small intestine collected from ZIKV-infected or control mice were fixed in 40% paraformaldehyde for 24 h. Next, paraffin embedding, section slicing (6 µm in thickness) and HE staining were performed according to standardized procedures.

Immunohistochemistry staining

To detect the expression of hormones in hypothalamus and pituitary, the tissue sections were incubated with rabbit polyclonal anti-GHRH (Cloud-Clone, China), anti-TRH (Cloud-Clone, China), and anti-GH (Cloud-Clone, China) or anti-TSH (Bioss, China) antibodies overnight. After washing, the slides were stained with a secondary HRP-conjugated goat anti-rabbit antibody (Zhongshan Golden Bridge Bio Co., Ltd., China) for 1 hour. The reaction was visualized by the addition of 3,30-diaminobenzidine (DAB) as a chromogen and then stopped by removing the DAB and washing with running water.

Detection of cell apoptosis

To detect apoptotic cells, hypothalamus paraffin sections (5 µm in thickness) were subjected to the transferase dUTP nick-end labeling (TUNEL) assay according to the manufacturer's instructions. In brief, sections were processed for TUNEL using an *In Situ* Cell Death Detection Kit (Roche, Germany). Each stained section was examined under a fluorescence microscope using a high power field (200 ×), and TUNEL-positive cells in the hypothalamus were evaluated. The cell apoptosis assay by IHC staining was detected by Beijing Gpbiotech (Beijing, China).

QUANTIFICATION AND STATISTICAL ANALYSIS

SPSS 17.0 software and GraphPad.Prism.v6.01 were used for statistical analyses. The normality of datasets was determined by Kolmogorov-Smirnov test and F test to compare variances. The data with normal distributions was analyzed using the repeated-measures analysis of variance or the Student's *t* test, while those with abnormal distributions or heterogeneity of variance were analyzed by the nonparametric Mann-Whitney U test. Data are presented as the mean $\pm$ standard error of the mean (SEM). $p < 0.05$ (*) and $p < 0.01$ (**) were considered statistically significant and highly significant, respectively.