

Zebrafish larvae exposed to ginkgotoxin exhibit seizure-like behavior that is relieved by pyridoxal-5'-phosphate, GABA and anti-epileptic drugs

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SUMMARY

The etiology of epilepsy is a very complicated, multifactorial process that is not completely understood. Therefore, the availability of epilepsy animal models induced by different mechanisms is crucial in advancing our knowledge and developing new therapeutic regimens for this disorder. Considering the advantages of zebrafish, we have developed a seizure model in zebrafish larvae using ginkgotoxin, a neurotoxin naturally occurring in *Ginkgo biloba* and hypothesized to inhibit the formation of the neurotransmitter γ -aminobutyric acid (GABA). We found that a 2-hour exposure to ginkgotoxin induced a seizure-like behavior in zebrafish larvae. This seizure-like swimming pattern was alleviated by the addition of either pyridoxal-5'-phosphate (PLP) or GABA and responded quickly to the anti-convulsing activity of gabapentin and phenytoin, two commonly prescribed anti-epileptic drugs (AEDs). Unexpectedly, the ginkgotoxin-induced PLP depletion in our experimental setting did not affect the homeostasis of folate-mediated one-carbon metabolism, another metabolic pathway playing a crucial role in neural function that also relies on the availability of PLP. This ginkgotoxin-induced seizure behavior was also relieved by primidone, which had been tested on a pentylenetetrazole-induced zebrafish seizure model but failed to rescue the seizure phenotype, highlighting the potential use and complementarity of this ginkgotoxin-induced seizure model for AED development. Structural and morphological characterization showed that a 2-hour ginkgotoxin exposure did not cause appreciable changes in larval morphology and tissues development. In conclusion, our data suggests that this ginkgotoxin-induced seizure in zebrafish larvae could serve as an *in vivo* model for epileptic seizure research and potential AED screening.

INTRODUCTION

Epilepsy is a common neurological disorder characterized by seizures. Approximately 50 million people worldwide are affected by epilepsy. Epilepsy is usually controlled, but not cured, with medication. However, 30-40% of epileptic patients are 'pharmacoresistant' to the currently available antiepileptic drugs (AEDs) (Loscher, 2011). This is probably due to the very complicated yet elusive mechanisms underlying epilepsy. Therefore, much remains to be learned about how epilepsy and epileptic seizures are induced and how they can be controlled. Meanwhile, new drugs targeting different etiological factors or mechanisms underlying different types of epileptic seizures are needed.

The lack of a proper animal model for *in vivo* studies and drug screening is a hindrance to research and drug development for

diseases. This is especially true for neuropathological disorders including epileptic seizures. Drug development for seizures is largely reliant on animal models, even at very early stages of compound screening. This is because the anticonvulsant effect of a compound can be observed more easily on live animals than using cultured cells in dishes. In the past few decades, rodent seizure models induced by a broad spectrum of mechanisms have contributed significantly to our knowledge on epilepsy and AED discovery. Recently, zebrafish, a vertebrate with complementary advantages to rodents, has been widely used in laboratories of both basic medical studies and clinical research. By combining the physiological complexity of animal studies and the throughput of *in vitro* screening, the zebrafish has proven to be a powerful tool for target identification, disease modeling and drug development (Kari et al., 2007). Currently, several pharmacological and genetic zebrafish models of epilepsy or behavioral seizures have been developed either by chemical induction (such as pentylenetetrazole and kainate) or genetic alteration (for a comprehensive review see Stewart et al., 2012). Considering the complexity of etiology underlying epilepsy, additional seizures models induced by different mechanisms would be useful.

Ginkgotoxin (4'-O-methylpyridoxine) is a neurotoxin naturally occurring in *Ginkgo biloba*. Various supplements from *Ginkgo biloba* are available over the counter and widely used in alleviating medical conditions including bronchial asthma, irritable bladder, depression, dizziness and tinnitus (Kajiyama et al., 2002; Kastner et al., 2007; Leistner and Drewke, 2010; Wada et al., 1985). However, use or accidental ingestion of *Ginkgo biloba* resulting in an overdose of ginkgotoxin is reported to cause poisoning, characterized by

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epileptic convulsions, vomiting, unconsciousness and irritability. These symptoms can be fatal if not promptly treated. The first documented case of ginkgotoxin poisoning appeared in 1881 and many other cases have been reported thereafter (Hasegawa et al., 2006; Miwa et al., 2001; Wada et al., 1985). In Japan, the *Ginkgo biloba* seeds, also called 'Gin-nan', are used as a food ingredient and their consumption has resulted in about 70 food poisoning episodes due to ginkgotoxin (Kajiyama et al., 2002; Leistner and Drewke, 2010). Patients with the symptoms of vomiting and convulsion after eating ginkgo nuts were also reported (Hasegawa et al., 2006; Kajiyama et al., 2002; Leistner and Drewke, 2010; Miwa et al., 2001). The urine and/or serum of these patients contained a large presence of ginkgotoxin.

Ginkgotoxin is structurally related to pyridoxine, pyridoxamine and pyridoxal, the three primary and inactive precursors of pyridoxal-5'-phosphate (PLP; the active form of vitamin B6). Ginkgotoxin has been shown to induce epileptic convulsions (Yagi et al., 1993). PLP is a cofactor for enzymes involved in numerous biochemical reactions, including several enzymes in the biosynthesis of neurotransmitters (Kastner et al., 2007). GABA is an inhibitory neurotransmitter and involved in many neurological functions. Altered GABA levels are believed to cause a number of pathological conditions including epilepsy and general motor disorders (Soghomonian and Martin, 1998). Glutamate decarboxylase (GAD), a PLP-requiring enzyme, catalyzes the rate-limiting step of GABA formation. Studies have shown that GABA levels, determined from tissue samples of rabbits fed with ginkgotoxin, were significantly reduced, suggesting that ginkgotoxin might be a GAD inhibitor (Nitsch and Okada, 1976). However, the activity of recombinant GAD was found not to be significantly affected by ginkgotoxin at physiological concentrations, indicating that the ginkgotoxin-induced decrease in GABA levels is unlikely to be caused by the direct binding and inhibition of GAD activity (Buss et al., 2001). In mammals, two enzymes are essential for producing the cofactor PLP in cells, i.e. pyridoxal kinase and pyridoxine 5'-phosphate oxidase (McCormick and Chen, 1999; Ting et al., 2000). Ginkgotoxin has been shown to be a substrate for pyridoxal kinase, which converts it to ginkgotoxin phosphate (Kastner et al., 2007). Low concentrations of ginkgotoxin phosphate inhibit pyridoxal kinase, but not pyridoxine 5'-phosphate oxidase (Kastner et al., 2007; Salamon et al., 2009). Therefore, the presence of ginkgotoxin is likely to diminish PLP formation, leading to decreased GABA and imbalance between inhibitory and excitatory neurotransmitters and, eventually, seizures.

Here, we explore the potential of establishing a zebrafish seizure model with ginkgotoxin. We hypothesize that, like in mammals, exposure to ginkgotoxin will induce seizure-like behavior in zebrafish larvae due to the lack of PLP and/or GABA.

RESULTS

Swimming behavior of ginkgotoxin-exposed zebrafish larvae

We found that zebrafish larvae exposed to ginkgotoxin exhibited a stereotypical behavioral change culminating in seizure-like convulsions in a time- and dose-dependent manner. To induce seizures, embryos at 5 dpf were moved to water containing ginkgotoxin of various concentrations (0.2-1 mM) and incubated for 2 hours before video recording and analysis. Control larvae at

5 dpf swim infrequently but robustly in small dart-like steps in all dimensions (Brustein et al., 2003). However, ginkgotoxin-treated larvae exhibit a hyperactive swimming pattern with short bursts of rapid jerking lasting up to a few seconds, propelling them through the water at high speed [supplementary material Movies 1 (control) and 2 (ginkgotoxin)]. This seizure-like behavior was dose-dependent as an obvious hyperactive swimming pattern was stably established after 1 hour of incubation when ginkgotoxin concentration reached 0.5 mM and higher (Fig. 1). It was also exposure time-dependent, because this seizure-like swimming pattern was observed in larvae incubated for 2 hours, with 0.2 mM ginkgotoxin (Fig. 1). These results support our hypothesis that ginkgotoxin can induce seizure-like behavior in zebrafish. It should be noted that we had also tested the seizure-induction activity of ginkgotoxin phosphate, the phosphorylate derivative of ginkgotoxin, which is thought to be more potent than ginkgotoxin for PL kinase inhibition. The results were similar to those observed with ginkgotoxin (data not shown). Therefore, only ginkgotoxin was used for the subsequent characterization.

To fine-tune the protocols for the most efficient induction and quantification of seizure-like behavior, larvae at three different developmental stages were incubated in 0.5 mM ginkgotoxin for the indicated durations and then video-recorded for larval swimming behavior, following the schedule depicted in Fig. 2. We could only manually video-record each larva separately due to the limitations of the instrument. Also, due to the fast development of

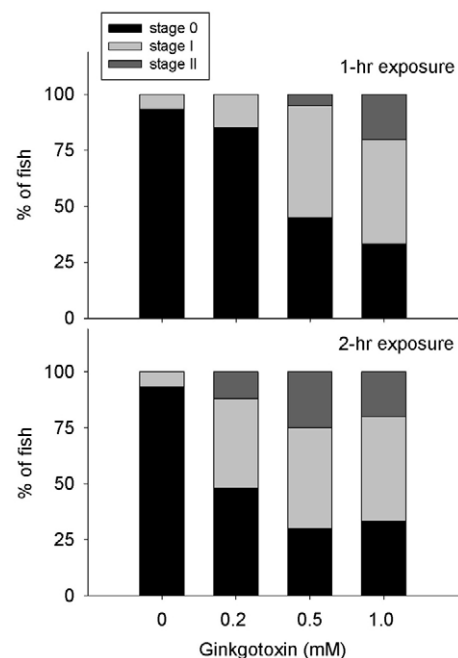


Fig. 1. Seizure-like behavior observed in zebrafish larvae exposed to ginkgotoxin. Zebrafish larvae at 5 dpf were incubated in water containing ginkgotoxin of various concentrations for 1 hour (upper) or 2 hours (lower) before observing under a microscope. Ginkgotoxin-exposed larvae were categorized into three stages based on the characteristics of their swimming behavior: stage 0, no or low swimming activity; stage I, mildly increased swimming activity; stage II, whole body convulsion and misshaped. There were a total of ten larvae in each group.

embryos, we recorded for only 1.5 minutes for each larva in order to maintain all the larvae of different groups in a comparable developmental stage during the time of data acquisition for all groups. Control larvae, which were incubated in fish water only, at 3 dpf had complete touch-evoked swimming responses but showed very little spontaneous swimming; whereas the larvae treated with ginkgotoxin for 2 hours (the 3-dpf/2-hr group; see Fig. 2 for description of the groups) exhibited a hyperactive swimming pattern similar to those observed in ginkgotoxin-treated 5-dpf larvae [supplementary material Movies 3 (control) and 4 (ginkgotoxin)]. The path tracking analysis showed that larvae of both the 3-dpf/2-hr and 6-hpf/66-hr groups had the most significant increase in larval distance displacement, approximately sevenfold in comparison to the control group (Fig. 3A,B). An approximately threefold increase was observed in 5-dpf larvae exposed to ginkgotoxin for 2 hours (Fig. 3B). These results showed that ginkgotoxin exposure induced seizure-like behavior in the embryos at all three stages examined. Also, the two strategies of 3-dpf/2-hr and 6-hpf/66-hr generated larvae with the most obvious seizure-like swimming behavior.

Biochemistry of ginkgotoxin-exposed zebrafish larvae

We found that the ginkgotoxin-induced seizure is most probably due to the lack of PLP and GABA. *In vitro* studies showed that human pyridoxal kinase was inhibited by ginkgotoxin, implying a possible decrease in intracellular PLP and GABA generation in organisms exposed to ginkgotoxin (Kastner et al., 2007; Salamon et al., 2009). To test this hypothesis, 0.5 mM of PLP or GABA was added to the fish water simultaneously with ginkgotoxin (Fig. 2, subgroup 2 of all groups). As expected, the hyperactive swimming pattern induced by ginkgotoxin was alleviated in the presence of

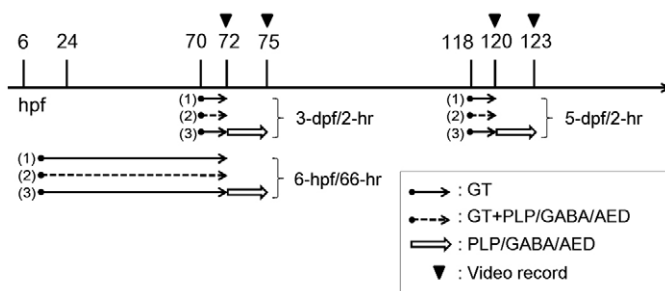


Fig. 2. Schedule for treatment with ginkgotoxin and anti-convulsing compounds and data acquisition. Embryos at three different stages were collected separately into three groups, namely 3-dpf/2-hr, 5-dpf/2-hr and 6-hpf/66-hr. The embryos in each group were divided into three subgroups (1, 2, 3) and treated with ginkgotoxin and AEDs following three different strategies. Subgroup 1 was treated with ginkgotoxin (GT) only for the duration indicated by solid arrow before video-recording for data acquisition. Subgroup 2 was treated simultaneously with both ginkgotoxin and rescuing compounds or AED for the duration indicated by dashed arrow before data acquisition. Subgroup 3 was treated with ginkgotoxin first for the duration indicated by the solid arrow. Then, the ginkgotoxin was replaced with rescuing compounds or AED and larvae were incubated for additional 3 hours before data acquisition. 3-dpf/2-hr refers to 3-dpf embryos incubated in ginkgotoxin for 2 hours. 5-dpf/2-hr refers to 5-dpf embryos incubated in ginkgotoxin for 2 hours. 6-hpf/66-hr refers to 6-hpf embryos incubated in ginkgotoxin for 66 hours.

either PLP or GABA (PLP/GABA)(Fig. 3B). The rescuing effect was most obvious in the larvae of the 6-hpf/66-hr group (embryos at 6 hpf were exposed simultaneously to both ginkgotoxin and PLP/GABA for 3 days). The rescuing effect was least effective in the larvae of the 5 dpf/2-hr group (ginkgotoxin and PLP/GABA were added at 5 dpf for 2 hours). It should be noted that similar rescuing effects were also observed when PLP/GABA was added after a stable pattern of seizure-like behavior was developed (Fig. 2, subgroup 3 of all groups; data not shown). Our data support the notion that ginkgotoxin-induced seizures are due to the impeded formation of PLP and GABA. These results also suggest that the ginkgotoxin-treated embryos could be used as a zebrafish model for vitamin B6 deficiency.

Pharmacology of ginkgotoxin-induced seizures in larval zebrafish

We found that the ginkgotoxin-induced seizure-like behavior in larval zebrafish also responded to the anti-convulsing activity of AEDs. Two commonly used AEDs, phenytoin and gabapentin, were added to fish water either simultaneously with ginkgotoxin (Fig. 2, subgroup 2) or after a stable pattern of hyperactive swimming had developed (Fig. 2, subgroup 3). This explored the potential of the seizure-like convulsions observed in ginkgotoxin-exposed larvae for candidate drug screening. We found that in comparison with the ginkgotoxin control group, simultaneously adding 1 mM of phenytoin or gabapentin with ginkgotoxin to 6-hpf embryos significantly reversed the seizure-like swimming behavior after incubating for 3 days (Fig. 2, 6-hpf/66-hr, subgroup 2; Fig. 3B). Anti-convulsing effects were also observed in the other two groups (3-dpf/2-hr and 5-dpf/2-hr) containing larvae at later stages and shorter incubation time (Fig. 2, 3-dpf/2-hr, subgroup 2 and 5-dpf/2-hr, subgroup 2; Fig. 3B). Similar anti-convulsing effects were observed when AEDs were added after a clear seizure-like behavior was established (Fig. 2, subgroup 3 of all groups; data not shown). Our data showed that the seizure-like behaviors observed in all the ginkgotoxin-treated embryos with these three seizure-induction strategies could be alleviated by adding AEDs, as expected. These results also suggest that of the three different strategies used and the two parameters measured, the strategies of 6-hpf/66-hr and 3-dpf/2-hr generated embryos with the highest sensitivity to AEDs, which is best reflected in the larval traveling distance.

Effects on PLP-requiring folate-mediated one-carbon metabolism

PLP is an essential cofactor involved in many important biological processes and metabolic pathways (Corken and Porter, 2011). Inhibition by ginkgotoxin of pyridoxal kinase might lower the cellular concentration of PLP to levels that will affect the conversion of many apo-B6 enzymes into their catalytically active holo-enzyme form. A crucial B6 enzyme in virtually all cells is serine hydroxymethyltransferase, in both its cytoplasmic and mitochondrial forms (Schirch and Szebenyi, 2005). These enzymes are crucial to folate-mediated one-carbon metabolism, which is responsible for the biosynthesis of nucleic acid and amino acids and the generation of the major intracellular methyl donor. This pathway is also crucial for the development and functions of neural tissues (Beaudin and Stover, 2009). Therefore, a possibility exists that the diminished PLP resulting from ginkgotoxin exposure interferes with embryonic folate one-carbon homeostasis. To test this hypothesis, we measured the embryonic folate contents of the control embryos and the

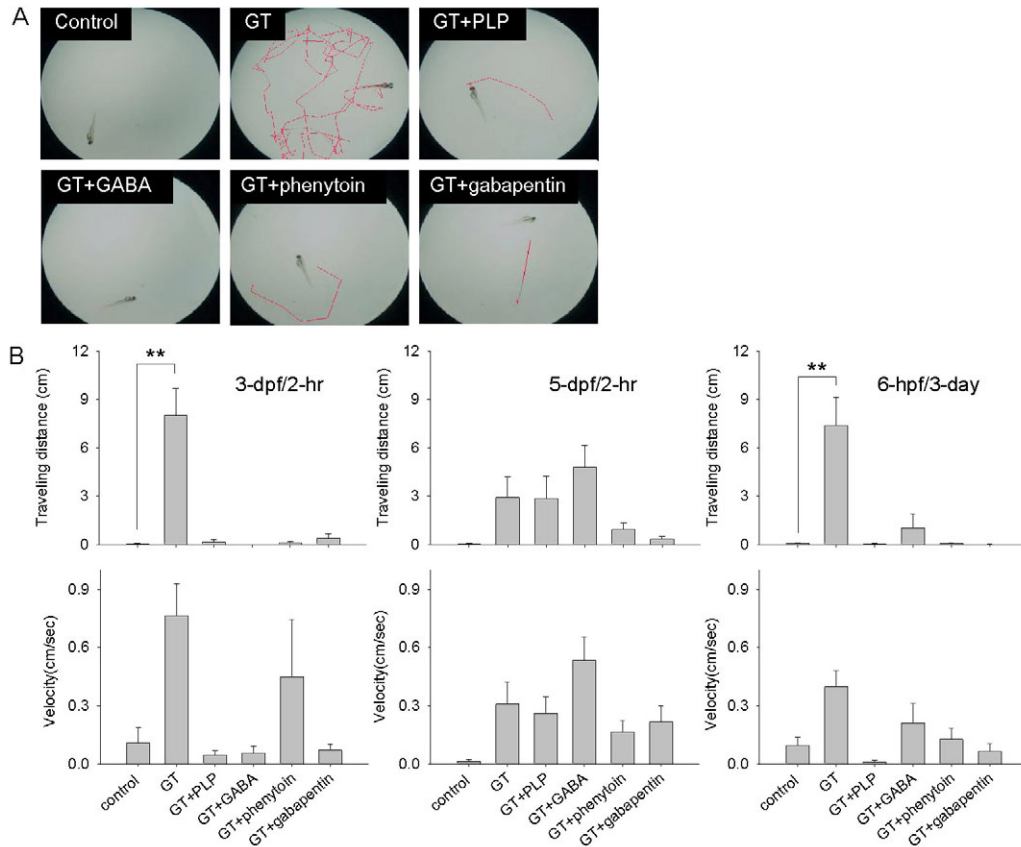


Fig. 3. Quantitative analysis of behavioral seizures induced by ginkgotoxin exposure and of response to AEDs. Embryos at indicated stages (3 dpf, 5 dpf or 6 hpf) were co-treated with both ginkgotoxin (GT) and anti-convulsing compounds simultaneously for the indicated time duration before video-recording (subgroup 2). (A) Sample locomotion tracking of 1.5 minutes are shown for individual larva at the end of treatment. (B) The traveling distance and velocity of larval displacement were analyzed. At least ten larvae were recorded for each group. Bars show mean + s.e.m. ****** $P < 0.0001$.

embryos exposed to ginkgotoxin (6-hpf/66-hr). Our data showed no significant difference between the control group and ginkgotoxin-treated group in either their total folate content or the folate derivatives (Fig. 4). It should be noted that the total folate content in embryos was lower than the sum of the three folate derivatives shown in the data. This could be due to the different methods we used for measuring the individual folate species and total folate (see Methods). The microbial assay for total folate measurement required overnight incubation of samples at 37°C, which probably partially destroyed 10-formyltetrahydrofolate and tetrahydrofolate (Fu et al., 1999). These results show that a 3-day exposure of 6-hpf embryos to 0.5 mM ginkgotoxin did not affect embryonic folate content and one-carbon homeostasis in our experimental setting, implying that the ginkgotoxin-induced seizures were not caused by any alteration in embryonic folate status.

Morphological characterization of ginkgotoxin-exposed zebrafish larva

GABA is essential for proper development of neural tissues (Bradford, 1995). To evaluate whether the ginkgotoxin-induced apparent PLP/GABA deficiency interferes with embryonic development, especially those locomotion-related tissues, embryos in the 6-hpf/66-hr group were subjected to morphological and

histochemical examination. The ginkgotoxin-treated larvae appeared to be morphologically normal, with their appearance and size similar to those of a control group (Fig. 5A-D). Hematoxylin and eosin (H&E) staining of cryosections in the head region revealed a slightly flatter forebrain in the ginkgotoxin-treated larvae, but the gray and white matters of brain were similar (Fig. 5E,F). The retinas of both the ginkgotoxin-treated and control groups also showed proper lamination and normally differentiated lenses. The transverse sections of the larval anterior trunk showed comparable characteristics for the myotomes and notochord in both the control and ginkgotoxin groups, even though their shapes seemed to vary (Fig. 5G,H).

In-situ hybridization using probes specific for tissues of interest was performed to closely examine embryonic structures and development at earlier stages. This was to determine whether ginkgotoxin caused any abnormality that could not be observed with histochemical characterization of 3-dpf larvae. Embryos at 6 hpf were incubated in water containing 0.5 mM ginkgotoxin for 18 hours and then harvested for analysis. In-situ hybridization revealed no obvious difference in the expression and distribution of both *myoD* (specific for somatic muscle) and *krox20* (specific for rhombomeres) between ginkgotoxin-treated embryos and the control group (Fig. 5I-L). Examining the embryos with both *vglut2.1*

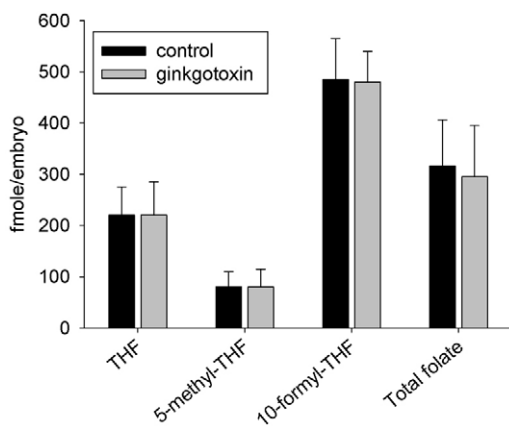


Fig. 4. PLP-requiring folate-mediated one-carbon pools affected by ginkgotxin exposure. Zebrafish embryos at 6 hpf were raised in fish water (control) or water containing 500 μ M ginkgotxin (GT) until 3 dpf and then harvested for folate content analysis. Total folate was measured with a microbial assay, whereas individual folate species were measured with HPLC. No significant differences were observed in folate content between the ginkgotxin-treated group (gray bar) and control group (black bar). Bars show mean + s.e.m. THF, tetrahydrofolate.

and *vglut2.2* probes also reveal no appreciable difference between untreated control and ginkgotxin-treated embryos, indicating that the development of glutamatergic neurons are not affected by exposure to ginkgotxin (Fig. 5M-P).

The development of the posterior lateral line (PLL), the sensory organ in aquatic organisms for detecting the movement and vibration of surrounding water, was also characterized in embryos at 3 dpf with DASPEI staining, which specifically labels the neuromasts deposited along the lateral line. This enabled evaluation of whether the change in swimming behavior was due to deformed PLL. We found that the position and the size of neuromasts along the PLL of ginkgotxin-treated larvae were comparable to those of control larvae (Fig. 5Q,R). These results indicate that the seizure-like behavior observed in ginkgotxin-treated larvae is not due to any abnormal development of larval muscle, rhombomeres or PLL.

However, in-situ hybridization probing with *pax2.1* revealed retarded spinal cord neurons. *Pax2.1* in zebrafish embryos has been shown to be transcribed first in the midbrain-hindbrain boundary region, then in the optic stalk, otic system, pronephros and nephric ducts, and lastly in specific interneurons of the hindbrain and spinal cord (Pfeffer et al., 1998). The signals corresponding to mid-hind brain and otic vesicle appeared to be similar in both control and ginkgotxin-treated groups (Fig. 6A,B). Nevertheless, the signals at optic stalk and spinal cord were significantly diminished in ginkgotxin-exposed embryos compared with the control group. Further examination was performed using *alx:GFP* transgenic fish, which express GFP specifically in excitatory interneurons under the control of the *alx* promoter (Kimura et al., 2006). These *alx* neurons are believed to be probable pre-motor interneurons that regulate motoneuron activity during escape and fast swimming. Confocal microscopy images of the notochord of the ginkgotxin-treated *alx:GFP* transgenic embryos showed a reduced number of interneurons compared with a control group (Fig. 6G,H). Fluorescent images of notochord of *alx:GFP* transgenic embryos

treated with ginkgotxin at 3 dpf for 2 hours (3-dpf/2-hr, subgroup 1) were also examined and the results showed no appreciable difference between the control and ginkgotxin-treated groups (data not shown). These results indicate that the effects of ginkgotxin are tissue-specific and dependent on the duration of exposure and the developmental stage at which exposure occurs. In addition, early and long-term exposure to ginkgotxin impedes the development of the spinal cord interneuron.

Previously, we showed that adding either PLP or GABA alleviates the ginkgotxin-induced seizures, even when the larvae have undergone a long-term exposure at a very early stage. As expected, the obstructed development of the optic stalk and spinal cord interneuron was also reversed when embryos were co-treated with PLP or GABA (Fig. 6C,D). The number of embryos with an obstructed spinal cord neuron decreased from 58% to 27% and 17% upon the addition of PLP or GABA, respectively. These results indicate that the ginkgotxin-induced neuropathological phenotypes, including both seizure-like behavior and obstructed neuronal development, are mostly due to the lack of PLP and GABA. Our data also shows that an obstructed spinal cord with interneurons of reduced number is sufficient to support a normal larval swimming pattern.

We noticed that obstructed development of the optic stalk, otic vesicle and spinal cord neuron was also observed in embryos exposed to GABA alone, without the presence of ginkgotxin (Fig. 6F). These results indicate that proper levels of GABA are crucial to the proper development of the spinal cord interneuron.

Primidone relieves seizures induced by ginkgotxin but not those induced by pentylenetetrazol

As described above, our results show that a ginkgotxin-induced seizure is mostly due to the reduced synthesis of PLP and GABA. This is different from the epileptogenic action of pentylenetetrazol (PTZ), a convulsant agent commonly used to induce seizures in experimental animals for epilepsy studies and drug discovery (Baraban et al., 2005). Therefore, there exists a possibility that the ginkgotxin-treated larvae will respond to AEDs differently to the PTZ-induced seizure model and be able to identify new drug candidates. To test this hypothesis, primidone, an anticonvulsant used to treat seizures that failed to reverse the PTZ-induced seizures in larval zebrafish, was tested on both PTZ and ginkgotxin models in parallel (Berghmans et al., 2007). To circumvent any possible ambiguity resulting from the obstructed interneuron (caused by long-term exposure to ginkgotxin), only the strategy of co-treating 3-dpf embryos with convulsing agents and 2 mM primidone for 2 hours was used for this test (3-dpf/2-hr). Folic acid (1 mM) was used in the place of primidone as a negative control. We found that primidone relieves the seizures induced by ginkgotxin but not by PTZ (Fig. 7). The percentage of ginkgotxin-exposed larvae with normal swimming behavior (stage 0) was increased from 28% to 80% in the presence of primidone. A more than tenfold decrease was observed in the traveling distance of larval displacement in the group co-treated with ginkgotxin and primidone, as compared with the ginkgotxin-treated control group. No significant change was observed in the PTZ-treated groups in either stage distribution or larval traveling distance. These results show that the ginkgotxin-induced seizure model responds differently to the PTZ model for certain AEDs.

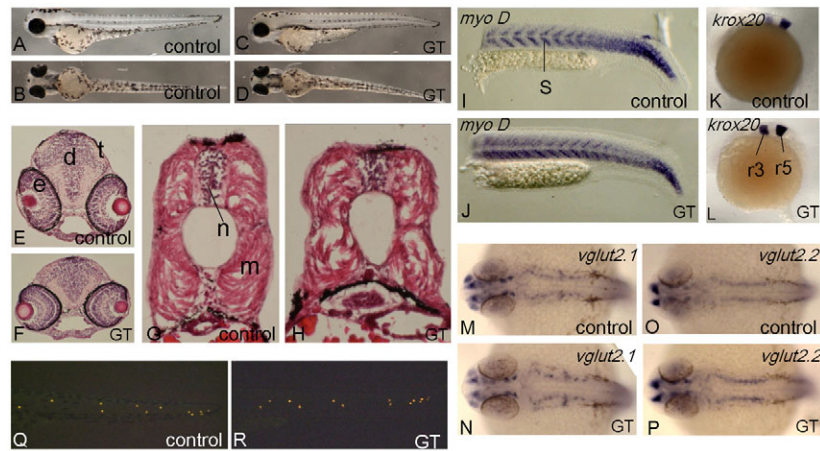


Fig. 5. Ginkgotoxin does not affect the development and appearance of zebrafish larvae. Zebrafish embryos were raised in fish water (control) or water containing 0.5 mM ginkgotoxin (GT) from 6 hpf to 72 hpf. (A-D) The lateral (A,C) and dorsal (B,D) views of embryos revealed no observable morphological differences in larval size and appearance between control and ginkgotoxin-treated groups. (E-H) The H&E-stained transverse cryosections of larval head regions and anterior trunk are oriented dorsal (top) to ventral (bottom) (E, F). Histochemical examination of the myotomes and notochord of embryos revealed no obvious difference between the control and ginkgotoxin-treated groups despite the variation in their shape (G,H). (I-P) The development of embryos were characterized at 24 hpf with in-situ hybridization using probes specific for somatic muscle (*myoD*, caudal lateral trunk views), rhombomere (*krox20*, lateral view with anterior to the left) and glutamatergic neurons (*vglut2.1* and *vglut2.2*, dorsal view). (Q,R) The posterior lateral line formation was characterized at 72 hpf by staining with DASPEI. m, myotome; n, notochord; e, eyes; d, diencephalon; t, tectum; s, somite; r3, rhombomere 3; r5, rhombomere 5.

DISCUSSION

Our results demonstrate a zebrafish model with seizure-like behavior that is induced by ginkgotoxin in zebrafish larvae. This ginkgotoxin-induced seizure-like behavior is rapidly induced and lasts longer than 3 hours. The seizure-like swimming pattern is reversed in the presence of PLP or GABA. Seizure-induction activity of ginkgotoxin has also been reported in other animal models. In South Africa, several deaths in cattle and sheep have been associated with ginkgotoxin poisoning because these animals feed on an *Albizia* species, which contains ginkgotoxin (Kajiyama et al., 2002). Ginkgotoxin was also shown to trigger seizures and even death at higher doses in animal models, including Guinea pigs and rats (Leistner and Drewke, 2010; Wada et al., 1985). The in vivo evidence reported in the current study supports the speculated

mechanism that the ginkgotoxin-induced seizure phenotype is mainly due to the depletion of PLP and GABA.

The ginkgotoxin-treated larvae respond quickly to the anti-convulsing activity of AEDs but are not identical to PTZ-exposed larvae, highlighting the potential use and complementarity of this model for AED development. We also showed that short-term ginkgotoxin exposure did not cause appreciable changes in larval morphology, tissue development or folate-mediated one-carbon homeostasis. Our data suggest that this ginkgotoxin-induced seizure in zebrafish larvae might serve as an in vivo model for epileptic seizure research and potential AED screening.

Our data also suggest that the strategy of incubating 3-dpf larvae in 0.5 mM ginkgotoxin for 2 hours (3-dpf/2-hr group in Fig. 2) is the most appropriate strategy among the three protocols

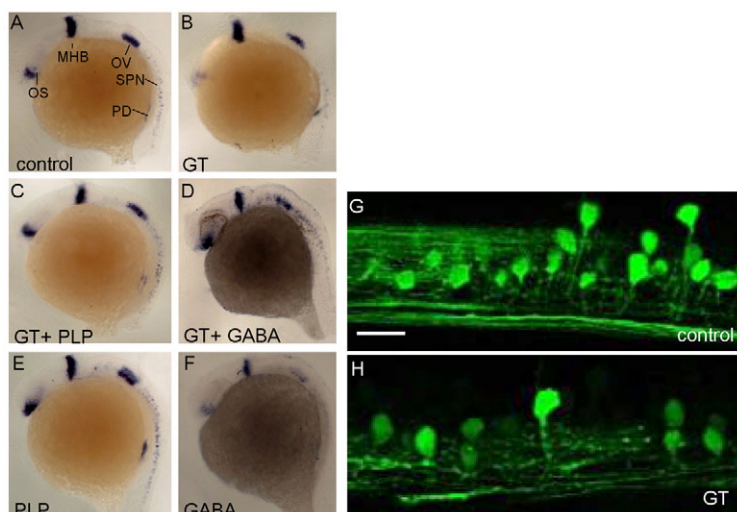


Fig. 6. Ginkgotoxin-induced malformation of optic stalk and spinal cord neuron was partially reversed by PLP or GABA supplementation.

Zebrafish embryos were raised in fish water (control) or water containing 500 μ M ginkgotoxin added at 6 hpf (GT). (A,B) The development of embryos were characterized at 24 hpf using in-situ hybridization with *pax2.1* probe (lateral view with anterior to the left). Development of otic stalk and spinal cord neuron were interfered with in the ginkgotoxin-treated group (B) and the effect was reversed in the presence of 0.5 mM PLP (C) or 0.5 mM GABA (D). Adding PLP alone did not cause observable changes in embryos (E), whereas adding GABA alone to the water impeded the development of *pax2.1*-expressing tissues, especially optic stalk, otic vesicle and spinal cord neuron (F). (G,H) Confocal microscopy images of the notochord in the trunk of Tg(*abx:GFP*) transgenic embryos at 3 dpf. OS, optic stalk; MHB, midbrain-hindbrain boundary; OV, otic vesicle; SPN, spinal cord neurons; PD, pronephric duct. Scale bar: 8 μ m.

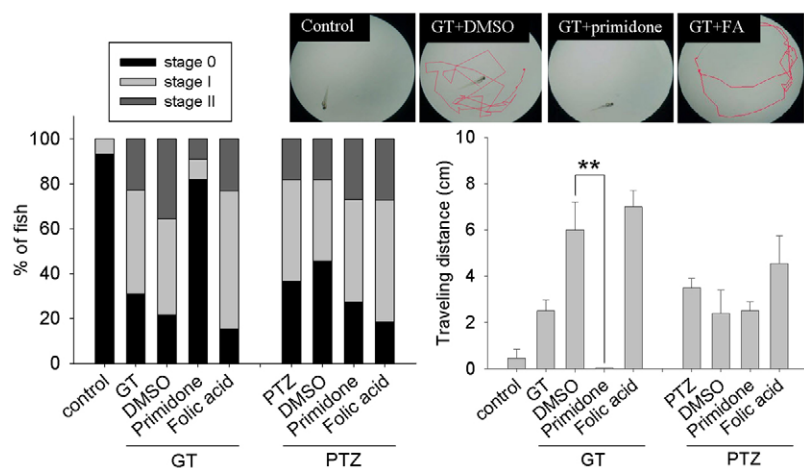


Fig. 7. Primidone relieved the larval seizure-like behavior induced by ginkgotoxin exposure but not by PTZ treatment.

Zebrafish larvae at 3 dpf were incubated in water containing convulsing agents with or without the presence of primidone for 2 hours before video recording and analyzed for the stage distribution (left) and total distance of larval displacement (right). The larval behavior was categorized into three stages on the basis of the characteristics of their swimming patterns: stage 0, no or low swimming activity; stage I, mildly increased swimming activity; stage II, whole body convulsion, erupting swimming and misshaped. There were at least ten larvae included for each group. Bars show mean + s.e.m. $**P < 0.0001$.

examined to induce larval seizures for drug screening. This is because this strategy induced the most obvious seizure-like behavior, which was also highly sensitive to AED activity (Figs 3, 7). In addition, unlike the early stage and long-term exposure, a 2-hour incubation does not cause any significant change in larval morphology and structure, circumventing any ambiguity for subsequent data interpretation (Figs 5, 6). As previously mentioned, we could record for only 1.5 minutes for each larva and ten larvae for each group every time due to the limitations of the instrument and the fast growth of larvae. Even so, we found that this was sufficient to distinguish the affected individuals from the control group, judging from larval traveling distance. This platform for compound screening is also time-efficient: it took less than 8 hours for us to finish the test for at least three compounds, starting from the induction of seizure by adding ginkgotoxin to the completion of analysis for larval traveling distance. As mentioned, the major limitation is the video recording of each individual larva. Therefore, we are convinced that a high-throughput monitoring system equipped with video recording devices will significantly improve the efficiency and bring the best use of this model for potential AED screening.

The larvae of the 6-hpf/66-hr group also sensitively responds to the anti-convulsing effects of rescuing compounds including PLP, GABA and AEDs, suggesting that the 6-hpf/66-hr strategy could also be used for drug screening. What is unexpected and interesting is that this reactivity was not attenuated by the obstructed interneuron resulting from the long-term exposure to ginkgotoxin in the 6-hpf/66-hr (subgroup 3) group, for which the rescuing compounds were added after both the seizure-like behavior and obstructed interneurons, supposedly, had been established. These data indicate that only a portion, but not all, of the developing neurons are required for the spinal cord to be functional. This observation is consistent with current knowledge on neuron maturation that programmed cell death is activated in cells receiving insufficient growth factors. This mechanism prevents neuronal overgrowth and helps maintain the proper number and networking of developed neurons (Freeman et al., 2004). In our case, the 'mission' of maintaining the proper number of developed spinal cord neurons was achieved by the toxicity resulting from early and prolonged exposure to ginkgotoxin. These data further strengthen our hypothesis that ginkgotoxin-induced seizure is

mainly caused by the lack of PLP and GABA, and not by other structural impairments. The observation that adding only GABA impedes spinal cord neuron development is in agreement with earlier reports that GABA regulates the proliferation of neural progenitor cells. This supports the notion that the appropriate level of GABA, or more likely the balance between inhibitory and excitatory neurotransmitters, is crucial to the development of neural tissues (Haydar et al., 2000).

We are convinced that the drug candidates identified by this ginkgotoxin-induced seizure model overlap with, but are not identical to, those identified using models in which seizure is induced by compounds interacting with neurotransmitter receptors. This is evidenced by our data showing that primidone failed to relieve larval epilepsy induced by PTZ; but does alleviate the seizure-like behavior induced by ginkgotoxin. The exact working mechanism of primidone is unclear but it is believed to work via interactions with voltage-gated sodium channels, which inhibit high-frequency repetitive firing of action potentials (Macdonald and Kelly, 1995). The effective differences could be attributed to different targeted mechanisms underlying these two models. Ginkgotoxin reduces PLP and GABA levels, whereas PTZ is a blocker of the chloride ionophore complex to the GABA_A receptor (Huang et al., 2001). Conversely, ginkgotoxin-induced seizures do not involve the binding to neurotransmitter receptors or ion channels, circumventing the concern for the competition between seizure-inducing agents and tested compounds.

Why does ginkgotoxin apparently decrease both PLP and GABA concentrations *in vivo*? Ginkgotoxin is a known inhibitor of pyridoxal kinase, which uses not only pyridoxal as a substrate but also pyridoxine and pyridoxamine (Fig. 8). A second route for synthesizing PLP is by the oxidation of pyridoxine 5'-phosphate by pyridoxine 5'-phosphate oxidase (Fig. 8). Past studies suggest that ginkgotoxin phosphate is not an effective inhibitor of this oxidase (Salamon et al., 2009). However, because pyridoxal kinase is the only known enzyme that phosphorylates pyridoxine to pyridoxine phosphate, this pathway to the formation of PLP would also be decreased by ginkgotoxin (Fig. 8). Therefore, a reduction in the *in vivo* concentration of PLP could be accounted for by the inhibition of only pyridoxal kinase by ginkgotoxin and ginkgotoxin phosphate. But why is the apparent concentration of GABA decreased?

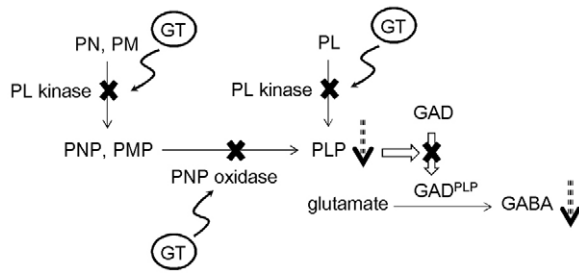


Fig. 8. Speculated mechanisms underlying ginkgotoxin-induced seizures.

Ginkgotoxin and ginkgotoxin phosphate inhibit pyridoxal kinase (PL kinase) for substrates pyridoxal, pyridoxamine and pyridoxine, resulting in decreased levels of PLP and pyridoxine-5'-phosphate (PNP). Inhibition of PL conversion to PLP directly decreases the amount of PLP formed by this pathway. The decrease in formation of PNP reduces PLP formation by pyridoxine phosphate oxidase. There is also some inhibition of pyridoxine phosphate oxidase by ginkgotoxin phosphate (Salamon et al., 2009). A lower *in vivo* PLP concentration reduces the conversion of apo-GAD to holo-GAD (GAD to GAD^{PLP}). Ginkgotoxin phosphate might also compete with PLP for binding to apo-GAD. Both mechanisms result in decreased intracellular GAD activity and GABA formation, leading to the occurrence of seizures. PN, pyridoxine; PM, pyridoxamine; PNP, pyridoxine-5'-phosphate; PMP, pyridoxamine-5'-phosphate; PL, pyridoxal; GT, ginkgotoxin.

Decrease in GABA could be the result of a decrease in the activity of GAD in its conversion of glutamate to GABA. One of the explanations for this putative decrease in GAD activity is that ginkgotoxin phosphate is an inhibitor in the conversion of apo-GAD to holo-GAD, thus reducing the amount of active enzyme. However, this seems unlikely because conversion of apo-B₆ enzymes to their active holo-form requires that the aldehyde of PLP forms an aldimine (Schiff-base) with an ε-amino group of an active site lysine residue. Ginkgotoxin does not contain this aldehyde group. Tight binding of PLP to apo-B₆ enzymes requires this aldehyde and the 5'-phosphate group. Other possible reasons for a loss of GAD activity is that it does not bind PLP as tightly or as rapidly as other competing apo-B₆ enzymes or that GAD has a rapid turnover rate in neural tissue, making it more sensitive to the level of PLP. How apo-B₆ enzymes compete *in vivo* for available PLP is an unexplored area of vitamin B₆ metabolism. However, studies have been reported that restriction of pyridoxine in MCF-7 cells leads to a significant drop in activity of cytosolic serine hydroxymethyltransferase (SHMT), a B₆ enzyme playing the central role in one-carbon metabolism, compared with mitochondrial SHMT (Perry et al., 2007). This suggests that PLP is not distributed equally among competing apo-B₆ enzymes. A modest decrease in PLP availability could result in low levels of certain neurotransmitters, with dire consequences on brain function and leading to neurological disorders.

Dozens of B₆ enzymes in the cell must be converted from their inactive apo-form to their catalytically active holo-form by the covalent attachment of PLP. A decrease in the *in vivo* concentration of PLP would be expected to cause a decrease in the activity of these B₆ enzymes (transaminase, decarboxylases, transferases, etc.) resulting in widespread disruption of cellular metabolism. Correlation between impaired folate status and numerous neurological disorders has been well documented (Beaudin and

Stover, 2007). A recent study reported that the presence of a C677T variant of 5,10-methylenetetrahydrofolate reductase was associated with increased seizure risk in epileptic pathogenesis, also implying a connection between the disturbance of folate-mediated one-carbon metabolism and the occurrence of epilepsy (Scher et al., 2011). However, despite the above-mentioned reports that restricted pyridoxine resulted in low SHMT activity in cultured cells, testing the effect of ginkgotoxin on SHMT in zebrafish larvae indicated that this important pathway is not affected, as judged by the distribution of one-carbon adducts of tetrahydrofolate (Fig. 4). Both the differences in the models used for testing (cultured human cells versus zebrafish larvae) and the parameters measured (individual enzyme activity versus distribution of folate derivatives) could account for the observed difference in SHMT activity between the current study and that of Perry et al. (Perry et al., 2007).

Another unanswered question is the potential change of GABA receptors in response to B₆ deficiency. An increased expression of GABA receptors would be expected in these ginkgotoxin-treated larvae. Besides serving as a drug-screening platform, these ginkgotoxin-exposed embryos might provide a useful *in vivo* tool for investigating the relationship between B₆ deficiency and the etiology of seizure and epilepsy.

To conclude, we show in the current study that a 2-hour exposure of 3-dpf zebrafish larvae to 0.5 mM ginkgotoxin induces a seizure-like behavior that is appropriate to serve as an *in vivo* model for seizure-related studies. Under this condition, no significant effect on larval structures and morphology is observed. This ginkgotoxin-induced seizure behavior is relieved by adding GABA or PLP into the embryo's water, confirming that this seizure-like behavior is caused by lack of the active form of vitamin B₆ and decreased GAD activity. The quick response of these ginkgotoxin-treated larvae to the anti-convulsing activity of AEDs makes this model suitable for potential AED screening. Moreover, the distinct response to primidone between ginkgotoxin- and PTZ-induced seizure models highlights the usefulness and complementarity of this ginkgotoxin-induced seizure model for AED development.

METHODS

Materials

The HPLC Aquasil C18 column and guard columns were purchased from ThermoFisher Scientific (Waltham, MA). The *Lactobacillus casei* for measuring total folate was obtained from Food Industry Research and Development Institute (Hsin-Chu, Taiwan). Ginkgotoxin and ginkgotoxin phosphate were synthesized in one of our laboratories (Martin Safo) at the Institute of Structural Biology and Drug Discovery, Virginia Commonwealth University, Richmond VA. The fully reduced monoglutamyl folates, except 10-CHO-THE, were generous gifts from Rudolf Moser (Merck Eprova, Switzerland). 10-CHO-THF was prepared by increasing the pH of 5,10-CH⁺-THF solution to 8.0 as previously described (Chang et al., 2010). The plasmids for preparing probes for *in-situ* hybridization were generous gift from Jen-Ning Tsai (School of Medical Laboratory and Biotechnology, Chung Shan Medical University, Taiwan). The plasmids for preparing *vglut2.1* and *vglut2.2* probes for *in-situ* hybridization were generous gift from Shin-ichi Higashijima (Laboratory of Developmental Gene Regulation, RIKEN Brain Science Institute and National Institutes of Natural Sciences, Japan). All other chemicals, including

anticonvulsant drugs and buffers were purchased from Sigma-Aldrich (Milwaukee, Wisconsin).

Animals

Zebrafish (*Danio rerio*, AB strain) were obtained from NTHU-NHRI Zebrafish Core Facility, Taiwan and bred and maintained at 28°C in a 10–14 hour light-dark diurnal cycle following the standard procedure (Westerfield, 2000). The Tg(*alx*:GFP) transgenic line was a generous gift from Hitoshi Okamoto and Shin-ichi Higashijima (Laboratory of Developmental Gene Regulation, RIKEN Brain Science Institute and National Institutes of Natural Sciences, Japan). Embryos were staged according to published procedures (Kimmel et al., 1995). All usage and experiments, conducted with the adult and larval zebrafish described in the current study, followed the Animal Use Protocol (IACUC Approval No. 99059) approved by the Institutional Animal Care and Use Committee, National Cheng Kung University, Tainan, Taiwan.

Drug treatment

Wild-type embryos were used for ginkgotoxin treatment and drug testing. A stock solution of ginkgotoxin (40 mM) was prepared in water and stored at –20°C. The stock solution was added to fish water, in which embryos of the desired stages were placed, to reach the desired concentrations ranging from 0.2 mM to 1 mM. Control embryos were raised in fish water without ginkgotoxin. For testing embryo response to anticonvulsants, pyridoxal-5'-phosphate and GABA were dissolved in water to make 40 mM stock solutions. Gabapentin, phenytoin and primidone were prepared in DMSO to make 100 mM stock solutions. The tested AED compounds were added to the fish water to reach the indicated working concentrations either simultaneously with ginkgotoxin or 3 hours before the embryos were collected for behavioral analysis and other examinations. The effectiveness of the AEDs was monitored by direct visual observation of the larval swimming pattern and quantified by the changes in rate and distance of larval displacement in the presence of the tested drug. At least ten larvae were used for each group.

Behavioral analysis

Zebrafish larva were placed individually in 200 µl fish water on a single concave microscope slide and observed under a dissecting microscope. The spontaneous swim episodes were video-recorded using a high-resolution Panasonic Digital video camera (DMC-FX55GT) for 1.5 minutes. For analysis of seizure stages, recording sessions were stored in a computer and later categorized by visual judgment. For quantitative analysis of locomotion, the recording sessions were analyzed for the total distance and velocity of larval displacement for each larva individually with the EthoVision XT8.0 locomotion tracking system (Noldus Information Technology, Leesburg, VA). Control groups were embryos of the same stages without any chemical treatment.

Characterization of tissue development

Histochemical analysis of the chemically treated larvae was performed following the protocols described in the Zebrafish book (Westerfield, 2000). In brief, anesthetized larvae were fixed in 4% paraformaldehyde, soaked in 30% sucrose and embedded in OCT. Sections of 8–10 nm were prepared on a Thermo Scientific Shandon

Cryostat 0620E (Waltham, MA). These sections were incubated in xylene to remove OCT and rinsed with PBS before H&E staining for pathological examination. Whole-mount in-situ hybridization was performed following the standard protocol described by Jowett and Thisse (Jowett, 2001; Thisse et al., 1993). Plasmids containing cDNA specific to rhombomere (*krox20*), somite (*myoD*), neural tissues (*pax2.1*) and glutamatergic neurons (*vglut2.1* and *vglut2.2*) were linearized with appropriate restriction enzymes. Digoxigenin-UTP-labeled antisense RNA probes were synthesized by in vitro transcription with DIG-RNA labeling kit (SP6/T7; Roche) and used for in-situ hybridization. Embryos were placed in glycerol and observed under a dissecting microscope and photographed. The confocal microscopy images of spinal cord interneuron of *alx*:GFP transgenic larvae were acquired on a Leica TCS SP2 microscope.

Folate measurement

The microbial folate assay was used for total folate measurement with a minor modification (Horne and Patterson, 1988). In brief, five embryos were homogenized in 0.1 ml of folate extraction buffer (10 mM potassium phosphate, pH 7.5 containing 0.1% 2-mercaptoethanol and 2% ascorbic acid) and flushed with nitrogen. Then 1 µl of homogenate was added to 0.3 ml of culture broth containing 20 µl of *Lactobacillus casei* liquid culture and incubated at 37°C overnight. The optical density of the overnight culture was measured next morning with a spectrophotometer and used to estimate folate concentration by interpolation with a standard curve constructed with known concentrations of folic acid. A modified reversed-phase HPLC method on an Aquasil C18 column with HPLC in combination with recombinant γ-glutamylhydrolase was used for assessment of individual folate derivatives as previously described (Kao et al., 2009). Approximately 50 larvae were collected, homogenized and sonicated in 0.25 ml of extraction buffer. The lysates were heated in boiling water for 5 minutes before centrifugation. The clear supernatant containing folylpolyglutamate folate was transferred to a clean tube and 1 µl of a purified recombinant γ-glutamyltetrahydrofolate hydrolase (4 µg/µl) was added to the supernatant for 5 minutes to convert folyl polyglutamates to their monoglutamate forms. The use of the purified γ-glutamylhydrofolate hydrolase greatly shortened the incubation time and improved reproducibility compared with previous methods that used impure hydrolase. For measurement of 10-formyltetrahydrofolate, recombinant zebrafish 10-formyltetrahydrofolate dehydrogenase (FDH) was added to the lysate to convert 10-formyltetrahydrofolate to tetrahydrofolate (Chang et al., 2010). Tubes were flushed with nitrogen gas before capping. After incubating at 37°C for 5 minutes, the tubes were boiled for 3 minutes to stop the enzymatic reaction and then immediately cooled on ice. After centrifugation and filtration to remove precipitated protein, 50 µl of the clear supernatant was injected into an Aquasil C18 column (150×4.6 mm, 3 µm; Thermo Electron Corporation, Waltham, MA) on an HPLC system (Agilent 1100) for folate detection. The potential folate peaks in extracts were identified by overlapping the retention times between the prospective peaks and folate standards. Each data point was the average of at least three determinations. The content of each folate derivative measured was quantified by interpolating the peak areas with the standard curves constructed with pure monoglutamyl folate standards of known concentrations. The amount of 10-

TRANSLATIONAL IMPACT

Clinical issue

Epilepsy is a neurological disorder affecting more than 50-million people worldwide. The development of so-called 'pharmacoresistant' seizures render many patients suffering from epileptic symptoms with no medical solution. Therefore, clarifying the mechanisms underlying epilepsy and the development of drug-resistant seizures is important. In addition, more effective drugs, possibly targeting different mechanisms, are urgently needed. Appropriate animal models of epileptic seizures are crucial for both mechanistic and drug development studies, because the anticonvulsant effect of a compound can be readily observed in live animals. Although several established rodent seizure models have advanced this field in the past few decades, a more time- and cost-effective animal model is needed to increase the efficiency of anti-epileptic drug (AED) development, and of basic and preclinical research.

Results

In this paper, the authors describe a model in which seizure-like behavior is induced by ginkgotoxin in zebrafish larvae. After testing different strategies, they report that treating larvae at 3 days post fertilization (dpf) with 0.5 mM ginkgotoxin for 2 hours results in robust seizure-like swimming behavior that is sensitive to AEDs, without showing signs of tissues damage. They also show that ginkgotoxin-induced seizures can be reversed by adding either the inhibitory neurotransmitter γ -aminobutyric acid (GABA) or pyridoxal-5'-phosphate (PLP; the active form of vitamin B6 and an enzyme required for GABA formation) simultaneously with ginkgotoxin, supporting the previously proposed mechanism that the seizure-like phenotype is due to an interference of GABA or PLP formation. In addition, ginkgotoxin-induced seizures were reversed by adding gabapentin and phenytoin, two commonly used AEDs. Finally, seizures could also be reversed by primidone, an AED that does not have this effect in another model of seizure in zebrafish larvae induced by pentylenetetrazol (PTZ).

Implications and future directions

The establishment of the ginkgotoxin-induced zebrafish seizure model provides an alternative in vivo system for AED development and seizure-related studies. These data demonstrate the use of the model for drug testing, and demonstrate that it might be sensitive to different AEDs than a previously reported model involving PTZ-induced seizures. The new model could be applied to high-throughput drug screening using a system equipped with video recording devices, as a means to identify candidate compounds before testing on rodents. Importantly, the model can be readily applied in academic or pharmaceutical laboratories because it does not require specialized techniques or personnel training.

This model can also be applied to study the impact of vitamin B6 deficiency, which is induced by ginkgotoxin treatment. It has been difficult to create nutrient deficiency in zebrafish because they eat plankton and baby shrimp, which are rich in various nutrients. Thus, this model will enable zebrafish studies of issues such as how certain metabolic enzymes are altered when vitamin B6 is deficient and how GABA receptors respond to vitamin B6 deficiency.

formyltetrahydrofolate was determined by subtracting the tetrahydrofolate peak area of the sample without adding FDH from the tetrahydrofolate peak area with FDH.

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COMPETING INTERESTS

The authors declare that they do not have any competing or financial interests.

AUTHOR CONTRIBUTIONS

G.-H.L., S.-Y.S., W.-N.C. and T.-F.F. conceived and designed the experiments. G.-H.L., T.-T.K. and H.-C.D. performed the experiments. G.-H.L. and T.-F.F. analyzed the data. M.K.S. provided ginkgotoxin and ginkgotoxin phosphate.

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SUPPLEMENTARY MATERIAL

Supplementary material for this article is available at <http://dmm.biologists.org/lookup/suppl/doi:10.1242/dmm.009449/-/DC1>

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