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Identification of compounds with anti-convulsant properties in a zebrafish model of epileptic seizures

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SUMMARY

The availability of animal models of epileptic seizures provides opportunities to identify novel anticonvulsants for the treatment of people with epilepsy. We found that exposure of 2-day-old zebrafish embryos to the convulsant agent pentylenetetrazole (PTZ) rapidly induces the expression of synaptic-activity-regulated genes in the CNS, and elicited vigorous episodes of calcium (Ca^{2+}) flux in muscle cells as well as intense locomotor activity. We then screened a library of ~2000 known bioactive small molecules and identified 46 compounds that suppressed PTZ-induced transcription of the synaptic-activity-regulated gene *fos* in 2-day-old (2 dpf) zebrafish embryos. Further analysis of a subset of these compounds, which included compounds with known and newly identified anticonvulsant properties, revealed that they exhibited concentration-dependent inhibition of both locomotor activity and PTZ-induced *fos* transcription, confirming their anticonvulsant characteristics. We conclude that this in situ hybridisation assay for *fos* transcription in the zebrafish embryonic CNS is a robust, high-throughput in vivo indicator of the neural response to convulsant treatment and lends itself well to chemical screening applications. Moreover, our results demonstrate that suppression of PTZ-induced *fos* expression provides a sensitive means of identifying compounds with anticonvulsant activities.

INTRODUCTION

Epilepsy is a common neurological disorder that is frequently characterised by recurrent, unprovoked seizures that result from excessive and hypersynchronous electrical discharges in the brain. Depending on the location and extent of the abnormal electrical activity in the brain, epileptic seizures can manifest in many different ways, which can include temporary loss of consciousness, or abnormal motor activity that can range from minor involuntary movements to whole body convulsions. Many different epilepsy syndromes are recognised, each of which affects the nervous system in distinct ways, and in which seizures are prominent phenotypic components (Reid et al., 2009). Seizures can be either localized to specific parts of the brain, or distributed more broadly as generalized seizures. Epileptic seizures can affect people of all ages. They can occur in the absence of structural brain abnormalities or be a manifestation of an underlying brain lesion, such as a brain tumour or changes attributable to a head injury. Genetic factors also play important roles in many forms of epilepsy (Reid et al., 2009). A wide range of structurally diverse anti-epileptic drugs is currently available for treatment of this disorder (Stafstrom, 2010). These drugs act in a variety of distinct ways. For example, benzodiazepines act as direct agonists of $GABA_A$ receptors, whereas carbamazepine and lamotrigine block sodium (Na^+) and calcium (Ca^{2+}) channels, the normal opening of which enables the firing of neuronal action potentials in response to excitatory neurotransmitters. Moreover, valproic acid (VPA) has been shown to inhibit the activities of histone deacetylases and GABA transaminase, as well as to reduce the production of phosphoinositides (Nalivaeva et al., 2009; Chang et al., 2012), so its therapeutic effects might result from a combination of these modes of action. Despite the wide variety of available treatments, approximately 30% of people with epilepsy fail to respond satisfactorily to first-line anti-epileptic drugs (Remy and Beck, 2006). Furthermore, many prescribed anti-epileptic drugs exhibit substantial side effects (Cramer et al., 2010). There is, therefore, an important unmet clinical need for new antiepileptic therapeutics with more specific mechanisms of action, fewer side effects and increased potency.

In order to develop new antiepileptics and understand the pathogenetic mechanisms underlying seizure disorders, animal models of epilepsy are invaluable. Pharmacological and genetic models of epilepsy have been developed in rodents, using known convulsant agents to induce seizures, as well as through the phenotypic analysis of mutations that cause seizures (Löscher, 2011). Rodent models have been used extensively both for elucidating seizure mechanisms and characterising the mechanisms of action of anti-epileptic drugs. However, the relatively high costs and labour-intensive nature of drug discovery work using rodents limit their usefulness as organisms in which large numbers of compounds can be efficiently screened to identify compounds with anticonvulsant activities. Consequently, seizure models have been developed in a range of non-mammalian organisms that are more amenable to high-throughput analysis (Baraban, 2007). Among these species, the zebrafish is emerging as a pre-eminent model vertebrate for the in vivo analysis of many developmental and disease mechanisms. Moreover, within the last few years, the usefulness of this organism for in vivo drug discovery has become

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increasingly apparent. Several recent studies have demonstrated that the zebrafish is particularly well suited to the analysis of epilepsy mechanisms and anti-epileptic drug discovery (Baraban et al., 2005; Berghmans et al., 2007; Hortopan et al., 2010a; Hortopan et al., 2010b; Stewart et al., 2012). At 7 days of age, the free-swimming, independently feeding zebrafish larva exhibits seizures when treated with chemical convulsants (Baraban et al., 2005; Winter et al., 2008), and these seizures can be ameliorated by administration of known anti-epileptic drugs (Baraban et al., 2005; Berghmans et al., 2007). Other behavioural phenotypes can also be readily analysed in embryos, larvae, or juvenile or adult zebrafish by immersion in fish water containing neuroactive compounds such as addictive, anxiolytic or anxiogenic agents (Darland and Dowling, 2001; Kokel et al., 2010; Cachat et al., 2010; Cachat et al., 2011; Steenbergen et al., 2011; Norton and Bally-Cuif, 2010). One of the key experimental advantages of the zebrafish stems from the transparency of its embryo, which facilitates three-dimensional analysis of gene expression patterns using whole-mount in situ hybridisation. We took advantage of this technique to establish a robust and sensitive gene-expression-based in vivo assay for seizures in 2-day-old [2 days post-fertilisation (dpf)] zebrafish embryos. We then utilised this assay in a medium-throughput screen of a collection of 2000 bioactive small molecules to identify a subset of compounds with previously unknown anticonvulsant activities. This group of compounds includes molecules that could represent new candidate therapeutics for treating epilepsy.

RESULTS

Pharmacological induction of convulsions in zebrafish embryos

Exposure of 7 dpf zebrafish larvae to chemical convulsants such as pentylenetetrazole (PTZ) induces seizures that exhibit many of the features of epilepsy, including clonic-like convulsions and ictal-like electrographic discharges (Baraban et al., 2005; Winter et al., 2008; Berghmans et al., 2007). In both rodents and larval zebrafish, induction of seizures is accompanied by robust expression of the synaptic-activity-dependent neuroprotective gene *fos* in the brain (Zhang et al., 2002; Baraban et al., 2005). By 7 dpf, zebrafish larvae swim fast, respond to a wide variety of sensory stimuli and exhibit a broad range of behaviours. However, by 7 dpf the larval integument is heavily pigmented and the nervous system is highly complex, which makes the analysis of CNS-specific gene expression patterns challenging. By contrast, 2 dpf embryos have a simple, transparent integument, which makes them amenable to whole-mount analysis of gene expression patterns in the developing CNS. Furthermore, by 2 dpf, embryos exhibit locomotor responses to extrinsic mechanical stimuli, implying the existence of neural circuits that could mediate locomotor responses of embryos to treatment with seizure-inducing chemicals. Previous studies have demonstrated that exposure of zebrafish larvae to PTZ elicited concentration-dependent increases in locomotor activity (Berghmans et al., 2007). We analysed the locomotor behaviour of 4 dpf larvae exposed to concentrations of PTZ ranging from 1.25 mM to 80 mM. Robust, concentration-dependent increases in locomotor activity were observed in response to treatment with PTZ, which was greatest after exposure to 20 mM PTZ (Fig. 1A). This concentration of PTZ was selected for all subsequent experiments. Evidence suggests that convulsants such as PTZ elicit seizures by inhibiting the $GABA_A$ receptor (Huang et al., 2001). In situ hybridisation analysis of 48 hpf embryos for expression of the $GABA_A$ receptor subunit genes *gabra1* and *gabrg2* revealed widespread expression of both genes in the brain (Fig. 1B), implying the existence of large numbers of GABA-responsive neurons in the CNS at this early stage of development. Consistent with these observations, treatment of 48 hpf embryos with 20 mM PTZ also induced a robust increase in locomotor activity characteristic of seizure induction (Fig. 1C, panel 1). To characterise the PTZ-induced increase in locomotor activity further, we microinjected a muscle-specific transgene encoding the fluorescent calcium reporter GCaMP-3 into embryos at the one-cell stage. 50 hpf embryos that transiently expressed GCaMP-3 in muscle cells were then exposed to PTZ. Within 5 minutes of exposure, intense muscular contractions could be readily detected as robust, transient increases in GCaMP-3 fluorescence (Fig. 1C), each of a duration of approximately 10 seconds, with an amplitude up to five times that of baseline fluorescence and separated by longer periods of relaxation (also see supplementary material Movie 1). These spasms are characteristic features of clonic seizures, indicating that PTZ induces neuromuscular convulsions in 2-day-old zebrafish embryos that are similar to those observed in some people with epilepsy.

A programme of seizure-associated gene expression in the CNS and muscle of convulsant-treated 2-day-old zebrafish embryos

Having established that convulsions could be reliably induced in 2-day-old zebrafish embryos by exposure to the seizure-inducing chemical PTZ, we sought to determine whether exposure to convulsant elicited a corresponding programme of seizure-associated gene expression. First, we investigated whether the synaptic-activity-regulated gene *fos* was induced by exposure of 2-day-old embryos to PTZ and another $GABA_A$ receptor inhibitor, picrotoxin. Analysis of embryos after 30, 60 or 90 minutes of treatment with either of these convulsants resulted in the induction of *fos* transcripts in an intricate pattern of multiple discrete domains within the ventral telencephalon, ventral diencephalon, hindbrain, spinal cord and trunk muscle. Expression of *fos* was detectable in the brain within 30 minutes of administering either PTZ or picrotoxin, and had increased by 60 and 90 minutes after initial administration (Fig. 2A). Closer inspection of the expression domains of *fos* revealed that the robust induction of *fos* by PTZ in the forebrain is restricted to two distinct territories: the ventral telencephalon, including the subpallium, and the ventral diencephalon, encompassing the preoptic area, posterior tuberculum and hypothalamus (Fig. 2B). Taken together, these results confirmed that the induction of *fos* expression in specific regions of the developing CNS and trunk muscle of 2-day-old zebrafish embryos after treatment with convulsant compounds is a robust transcriptional response, consistent with the initiation of seizures in the CNS and the onset of convulsions in the trunk musculature. In order to determine whether PTZ- and picrotoxin-mediated induction of *fos* expression in these domains could be suppressed by a known anticonvulsant, we exposed embryos to the anti-epileptic drug sodium valproate (VPA) and either PTZ or picrotoxin, which resulted in the complete suppression of *fos* expression (Fig. 2C).

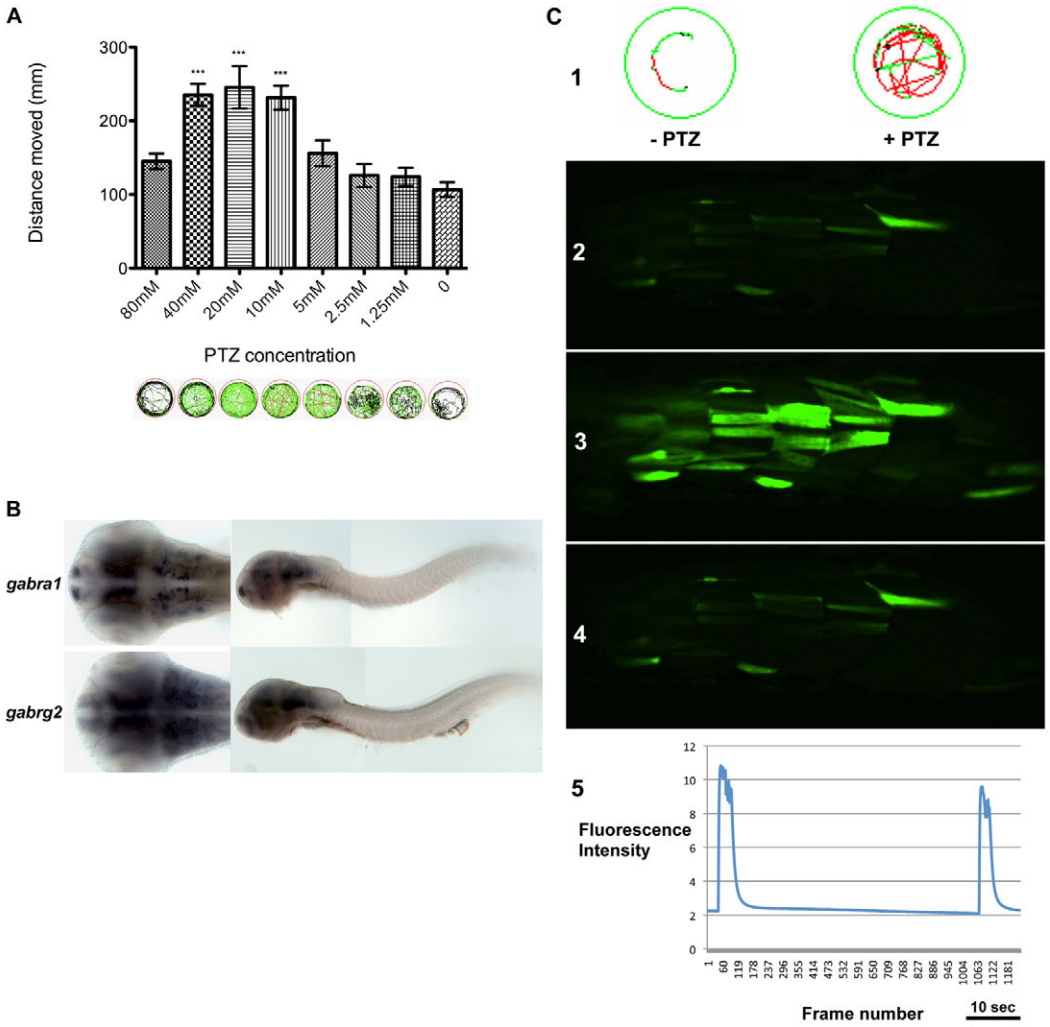

Fig. 1. Treatment of embryos and larvae with the GABA pathway inhibitor pentylenetetrazole induces convulsions that are characterised by increased locomotor activity and calcium influx into muscle cells. (A) 4 dpf larvae were exposed to 1.25, 2.5, 5, 10, 20, 40 or 80 mM PTZ for 10 minutes, then movements were recorded over the next 10 minutes. Values are given as means (*n*=4); error bars indicate s.e.m. ***Significantly different (*P*<0.001) from untreated embryos, using one-way ANOVA with Dunnett's post-test. Representative traces of movements after exposure to each PTZ concentration lie beneath each corresponding value for PTZ concentration in the histogram. Black, green and red traces indicate swimming speeds of 0-1.5, 1.5-6 and >6 mm/second, respectively. (B) In situ hybridisation analysis reveals strong expression of $GABA_A$ receptor component genes *gabra1* and *gabrg2* throughout the brain of 50 hpf embryos. (C) Induction of convulsions in embryos by exposure to PTZ. Panel 1: representative traces of locomotor activity of 48 hpf embryos untreated (–PTZ) and exposed to 20 mM PTZ (+PTZ). Black, green and red traces indicate swimming speeds of 0-1.5, 1.5-6 and >6 mm/second, respectively. Panels 2, 3 and 4: live confocal imaging of GCaMP-3 fluorescence in the muscle of a 50 hpf zebrafish embryo transiently expressing a *mylz2:GCaMP3* reporter transgene. Images are taken from a movie after 5 minutes of exposure to 20 mM PTZ, before (Panel 2), during (Panel 3) and after (Panel 4) a single convulsion. Panel 5: graph of GCaMP-3 fluorescence from the embryo in Panels 2-4, over a period in which two consecutive convulsions occurred.

Previous studies of the transcriptional consequences of exposing in vitro cultured neurons to convulsants and other inducers of synaptic activity have demonstrated that a broad programme of gene expression is elicited, components of which might perform neuroprotective functions that limit neuronal depolarization and help prevent calcium influxes reaching excitotoxic levels (Greer and Greenberg, 2008). Among these genes, neuroprotective functions are indicated for the bHLH-PAS-domain-containing transcription factor gene *Npas4* and the neurotrophic factor *BDNF* (Lin et al., 2008; Greenberg et al., 2009). When expression of the zebrafish orthologues of these two genes was analysed by whole-mount in situ hybridisation, both *npas4* and *bdnf* transcripts were induced in the ventral telencephalon and diencephalon of PTZ-treated embryos, in patterns that are similar to that of *fos* (Fig. 3). 3 dpf zebrafish embryos that are homozygous for the recessive *mind bomb* mutation were previously shown to exhibit seizure behaviour and aberrantly increased expression of a close relative of the endogenous anti-convulsant and anxiolytic Neuropeptide Y gene, *pyya* (Hortopan et al., 2010a). Interestingly, we also observed that PTZ treatment increased *pyya* expression within the CNS of 50 hpf wild-type embryos, which was most readily apparent within the posterior hindbrain (Fig. 3).

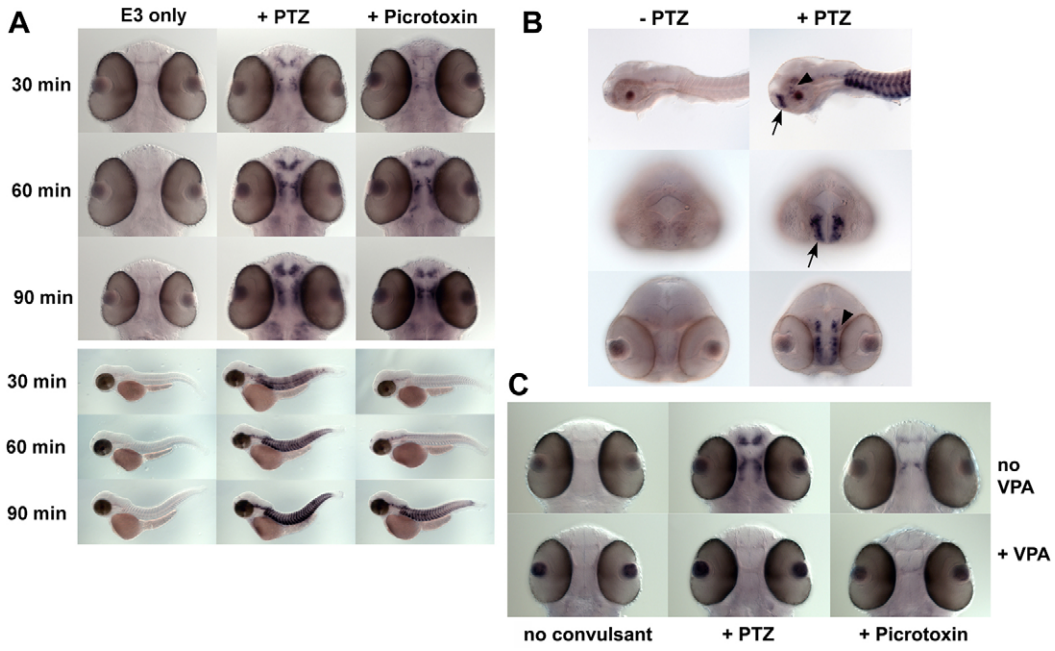

Fig. 2. Pentylenetetrazole and picrotoxin treatments induce expression of the synaptic-activity-regulated gene *fos* in 2-day-old zebrafish embryos, and this expression is suppressed by the anticonvulsant drug VPA. (A) Treatment of 50 hpf embryos with the $GABA_A$ receptor inhibitors PTZ (20 mM) or picrotoxin (300 μM) caused rapid induction of *fos* expression in the forebrain within 30 minutes of addition of drug, which was increased at 60 and 90 minutes after initial addition. Treatment of 50 hpf embryos with PTZ (20 mM) also induced strong *fos* expression in the trunk muscle after 30, 60 and 90 minutes of treatment, whereas treatment with picrotoxin (300 μM) only induced detectable *fos* expression after 90 minutes of treatment. (B) PTZ-induced expression of *fos* in the developing CNS is greatest in the ventral telencephalon (subpallium; arrows) and ventral diencephalon (arrowheads) within the forebrain of 50 hpf embryos. In the ventral diencephalon, the *fos* expression domain induced by PTZ encompasses the preoptic area, posterior tuberculum and hypothalamus. (C) Treatment of embryos with the anticonvulsant VPA suppresses PTZ- and picrotoxin-induced expression of *fos* in the ventral telencephalon and ventral diencephalon. 50 hpf embryos were incubated for 60 minutes in E3 medium containing 1 mM VPA or E3 medium only (no VPA), after which either 20 mM PTZ (+PTZ) or 300 μM picrotoxin (+Picrotoxin) was added and embryos were incubated for a further 90 minutes. Embryos were then fixed and analysed for expression of *fos* transcripts.

An in situ hybridisation screen of a library of bioactive small molecules for compounds with anti-convulsant activity in zebrafish embryos

Our results indicated that a programme of CNS-specific and muscle-specific gene expression accompanies the induction of neuromuscular convulsions by PTZ in 2-day-old embryos, and that this could be suppressed by co-administration of the known anticonvulsant VPA, thus providing a robust method of monitoring induction and suppression of convulsion-associated synaptic activity in the CNS. Of the genes analysed, *fos* was the most sensitive indicator of seizure onset. We therefore developed a high-throughput in situ hybridisation assay for *fos* transcription as the basis for identifying potential anti-convulsants, in a screen of 2000 known bioactive compounds in the Microsource Spectrum Collection. Embryos were first exposed to these compounds in 96-well plates, then PTZ was added, after which samples were fixed and analysed for expression of *fos* by whole-mount in situ hybridisation. A total of 46 compounds were identified that caused strong attenuation or extinction of PTZ-induced *fos* expression in the developing brain (Table 1 and supplementary material Table S1). These compounds included several natural and synthetic steroids, as well as steroid-related compounds and compounds known to interact with other types of nuclear hormone receptors. Other molecules with known antifungal, anti-inflammatory, antioxidant, vasodilatory, pesticide, herbicide or neuroactive properties were also identified. Three structurally related antifungal agents, sulconazole, bifonazole and oxiconazole, were identified among the 46 hits, along with three calcium channel blockers and two inhibitors of monoamine receptors. However, the mechanisms of action of many of the identified compounds are unknown. A subset of 12 compounds was then selected from the initial list of 46 putative anti-convulsants for in-depth analysis of their dose-response characteristics in the *fos* expression assay and the locomotor activity assay. These compounds were initially selected on the basis of their relatively high potency in suppressing *fos* expression, viability of embryos exposed to compound at 10 μM, and their availability from suppliers other than the source of the compound library. Six of these compounds are known to act on the endocrine or nervous systems and include the steroids ethinyl estradiol and allopregnanolone, the calcium channel inhibitors nimodipine and nitrendipine, and the monoamine receptor inhibitors methiothepin and pimozide. The other six compounds, which were selected for further evaluation on the basis of their apparent potency in the initial screen and their structural diversity, were sulconazole, suloctidil, nerolidol, dioxybenzone, hexylresorcinol and retinyl acetate. Figs 4 and 5 show that each of these compounds suppressed PTZ-induced *fos* expression in 50 hpf embryos. Moreover, all compounds apart from retinyl acetate exhibited a graded, concentration-dependent inhibition of locomotor activity in the Viewpoint tracking assay

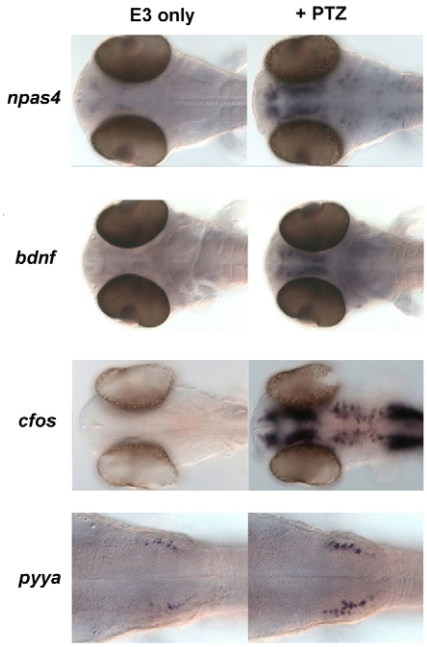

Fig. 3. Increased expression of CNS-specific genes accompanies transcriptional activation of *fos* by pentylenetetrazole. Exposure of 50 hpf embryos to 20 mM PTZ induced expression of genes encoding the transcription factor Npas4 and the secreted neurotrophic factor Bdnf in the ventral forebrain, both of which are known to be synaptic-activity-regulated, within 90 minutes of exposure. Expression of *fos* in the CNS was strongest in the regions of the forebrain that also expressed *npas4* and *bdnf*. Increased expression of *pyya*, encoding a homologue of Neuropeptide Y, was also observed at multiple locations within the CNS, most prominently within the posterior hindbrain. Left panels, embryos incubated in E3 medium only; right panels, embryos incubated in E3 medium containing 20 mM PTZ for 90 minutes.

Table 1. Identification of compounds that suppress PTZ-induced expression of *fos* in 50 hpf zebrafish embryos

Sub-group	Compounds
Steroid/steroid-like	Megestrol acetate, ethinylestradiol, epiandrosterone, progesterone, allopregnanolone, formestane, hexestrol (related to diethylstilbestrol), triptophenolide
Non-steroidal nuclear receptor ligand	Gemfibrozil, retinyl acetate
Anti-infective	Sulconazole, bifonazole, oxiconazole, acrisorcin, fenamisal, hexylresorcinol, diallyl sulphide, exalamide
Anti-inflammatory	Naproxen, mefenamic acid
Calcium channel blocker	Nimodipine, nitrendipine, suloctidil
Monoamine receptor ligand	Methiothepin maleate, pimozide
Antioxidant	Theaflavin monogallates, 2-isopropyl-methoxycinnamic acid
Vasodilator	Molsidomine
Pesticide/herbicide	Lindane, deguelin, rotenonic acid, endrin, propanil
Mechanism of action unknown	Mundulone, dioxybenzone, nonoxynol-9, larixol, haematommic acid, avocadyne, ethyl everninate, isosafrole, oxyquinoline hemisulphate, 8-β-hydroxycarapin 3,8-hemiacetal, peucedanin, nerolidol, senecrassidiol 6-acetate

46 compounds were identified from the Spectrum Collection of 2000 bioactive small molecules, each of which strongly suppressed PTZ-induced expression of *fos* in 2-day-old zebrafish embryos. Compounds were classified into sub-groups as indicated in the table.

(Figs 4, 5), which was confirmed by quantitative analysis of locomotor activity (Fig. 6). The most potent anticonvulsants identified in this screen are ethinyl estradiol, sulconazole, suloctidil, nerolidol, dioxybenzone and hexylresorcinol. Each of these compounds exhibited comparable potency at 8.2 μM to treatment of embryos with the clinically prescribed anti-epileptic drug VPA at a concentration of 1 mM. When the effect of each compound on apoptosis was evaluated, none induced appreciable cell death (supplementary material Fig. S1), confirming that the relatively brief exposure to these compounds did not elicit any significant cell toxicity. To determine whether the identified compounds might impair the general viability of larvae, cardiac function of larvae was monitored after 90 minutes of exposure to each compound at 8.2 μM and 24.7 μM (Table 2). At 24.7 μM, nimodipine, pimozide, nitrendipine, sulconazole and suloctidil caused a slowing of the heart rate, and nimodipine and pimozide elicited cardiac arrest in a proportion of treated larvae. However, only nimodipine appreciably inhibited cardiac function when assayed at a concentration of 8.2 μM, causing a slowing of heart rate (but not cardiac arrest) in treated larvae. Taken together, our results demonstrate that the use of an in situ hybridisation screen for *fos* expression is an efficient route to identifying compounds with potent in vivo anti-convulsant activities.

DISCUSSION

We sought to develop a zebrafish model of epileptic seizures that would be amenable to efficient screening of chemical libraries for compounds with anticonvulsant activities. We found that exposure of 2-day-old zebrafish embryos to the convulsant PTZ rapidly induced intense neuromuscular activity, increased locomotor behaviour and initiated a programme of synaptic-activity-induced gene expression in specific regions of the brain, all of which are characteristics of seizures in mammals. The widespread expression of *gabra1* and *gabrg2* in the brain of 2-day-old zebrafish embryos, together with our observation that a second, structurally distinct GABA pathway inhibitor, picrotoxin, induced a pattern of *fos* transcription in the CNS and trunk muscle that is similar to that induced by PTZ, further suggested that the effects of PTZ in the embryo are probably mediated by inhibition of the GABA pathway. Our results are consistent with previous observations of the seizure-inducing effects of PTZ on 7-day-old zebrafish larvae, which can be suppressed by a wide variety of known antiepileptic drugs (Baraban et al., 2005; Berghmans et al., 2007). The induction of *fos* transcription in response to excitatory neurotransmission, particularly as an indicator of seizure onset, has been documented extensively in mammals and in 7 dpf larval zebrafish, but we show here for the first time that the PTZ-induced *fos* expression pattern

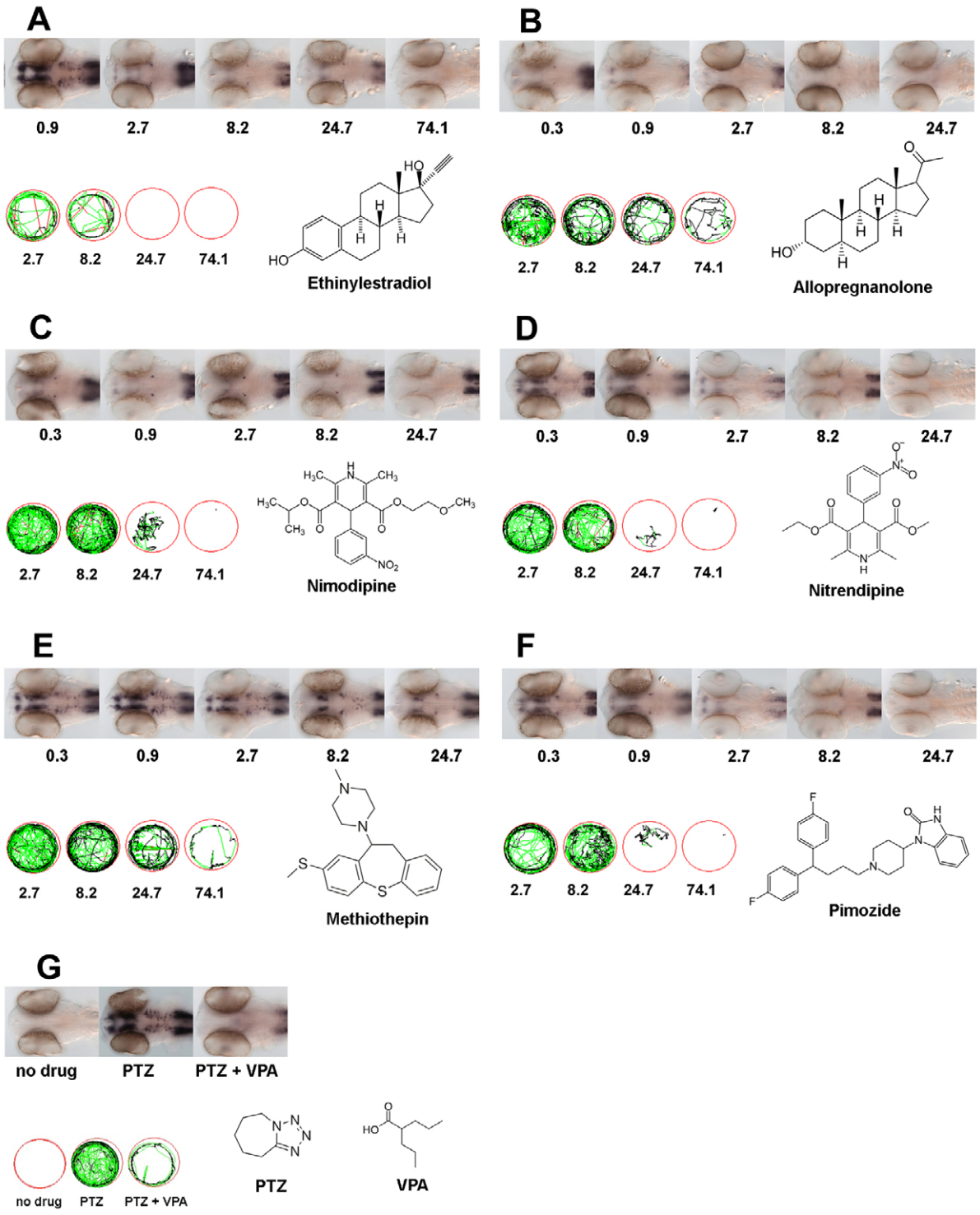

Fig. 4. Identification of known neuroactive compounds with anticonvulsant activity in the zebrafish embryo. Known inhibitors of neural activity were identified within the Microsource Spectrum Collection on the basis of their concentration-dependent suppression of PTZ-induced *fos* expression and inhibition of PTZ-induced locomotor activity. Compounds were administered to 50 hpf embryos for 90 minutes in 96-well plates, at a final concentration of 0.9, 2.7, 8.2, 24.7 or 74.1 μM (as indicated), then 20 mM PTZ was added and embryos were incubated for a further 60 minutes, before being fixed and analysed for expression of *fos*. In parallel, 4-day-old zebrafish larvae were exposed to the same range of compound concentrations in 48-well plates for 90 minutes, then PTZ was added and larvae were incubated for a further 10 minutes. 48-well plates were then transferred to the Viewpoint Zebrabox for live tracking of locomotor activity over a 10 minute period. Black, green and red traces indicate swimming speeds of 0-1.5, 1.5-6 and >6 mm/second, respectively. (A) Ethinylestradiol; (B) allopregnanolone; (C) nimodipine; (D) nitrendipine; (E) methiothepin; (F) pimozide. (G) The anticonvulsant activity of the positive control compound VPA is shown, for comparison.

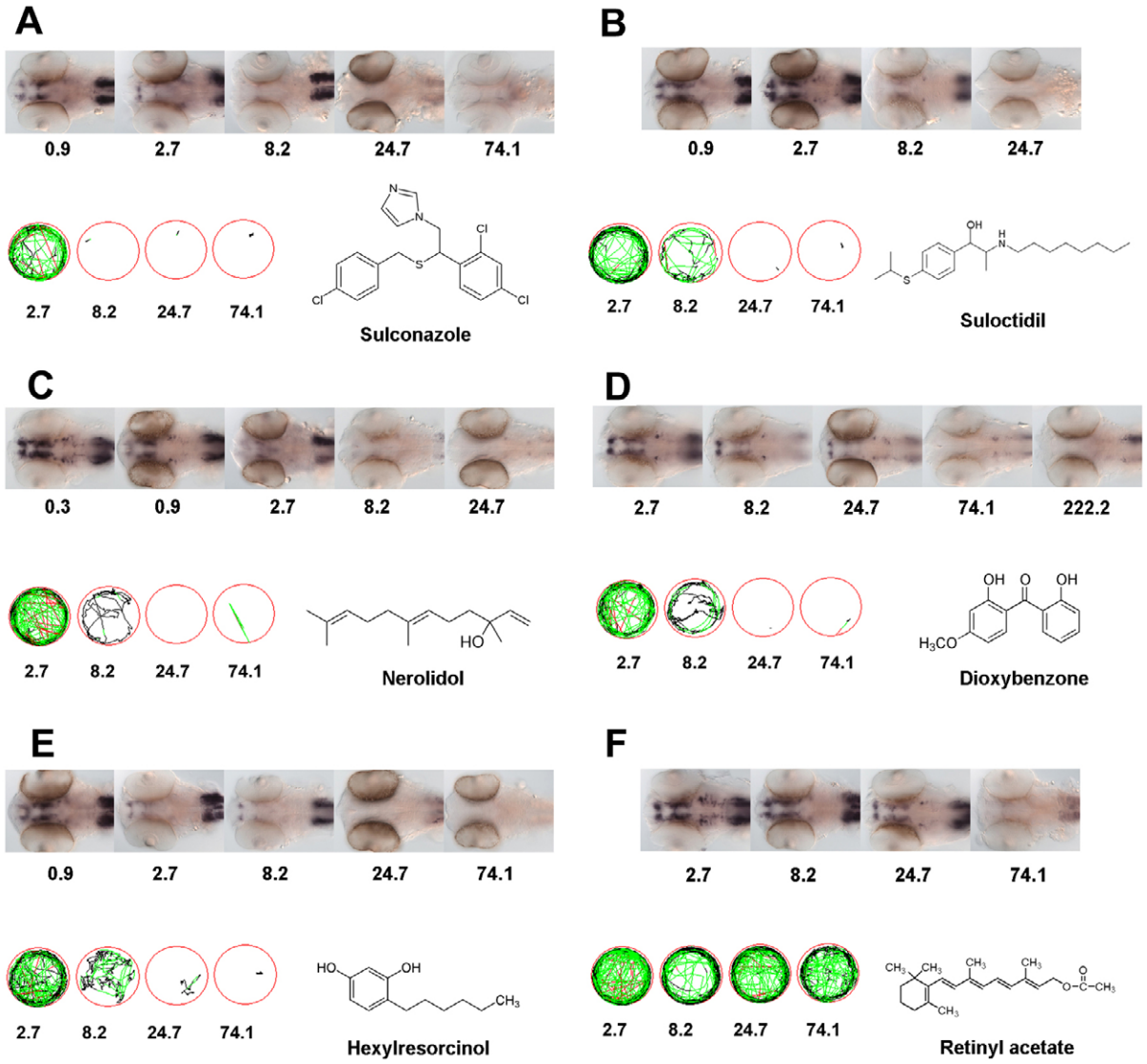

Fig. 5. Identification of compounds with anticonvulsant activity in the zebrafish embryo. Previously unknown inhibitors of neural activity were identified within the Microsource Spectrum Collection, on the basis of their concentration-dependent suppression of PTZ-induced *fos* expression and inhibition of PTZ-induced locomotor activity. Compounds were administered to embryos for 90 minutes in 96-well plates, at a final concentration of 0.9, 2.7, 8.2, 24.7 or 74.1 μM (as indicated), then 20 mM PTZ was added, and embryos were then incubated for a further 60 minutes before being fixed and analysed for expression of *fos*. In parallel, 4-day-old zebrafish larvae were exposed to the same range of compound concentrations in 48-well plates for 90 minutes, then PTZ was added and larvae were incubated for a further 10 minutes. 48-well plates were then transferred to the Viewpoint Zebrabox for live tracking of locomotor activity over a 10 minute period. Black, green and red traces indicate swimming speeds of 0-1.5, 1.5-6 and >6 mm/second, respectively. (A) Sulconazole; (B) suloctidil; (C) nerolidol; (D) dioxybenzone; (E) hexylresorcinol; (F) retinyl acetate.

in 50 hpf embryos is complex even at this relatively early stage, and includes several spatially distinct structures within the brain. In rodents, transcription of *fos* is induced by convulsants such as kainic acid and in response to a range of other excitatory stimuli, including anxiogenic or addictive compounds, as well as behavioural stimuli such as fear-inducing or other stressful experiences. Because *fos* is such a versatile marker of synaptic activity in the mammalian CNS, it will be of interest to explore further its responsiveness to a similar range of stimuli in zebrafish. The recent identification of a large number of synaptic-activity-regulated genes raises the possibility that some of these genes are specifically activated in distinct neuronal subsets by distinct neurotransmitter subtypes (Loebrich and Nedivi, 2009). Such markers might also be useful in distinguishing different types of neuroactive drugs, e.g. psychostimulants and convulsants, through their effects on regulating the activity of distinct neuronal subtypes. Genetic analysis of *fos* function in the mouse indicates that this gene performs a neuroprotective role as part of the response to the onset of seizures (Zhang et al., 2002), although the relevant downstream target genes for the Fos transcription factor, and its mechanism of action, remain unidentified. The dramatic induction of *fos* transcription in the embryonic trunk muscle was surprising and has not previously

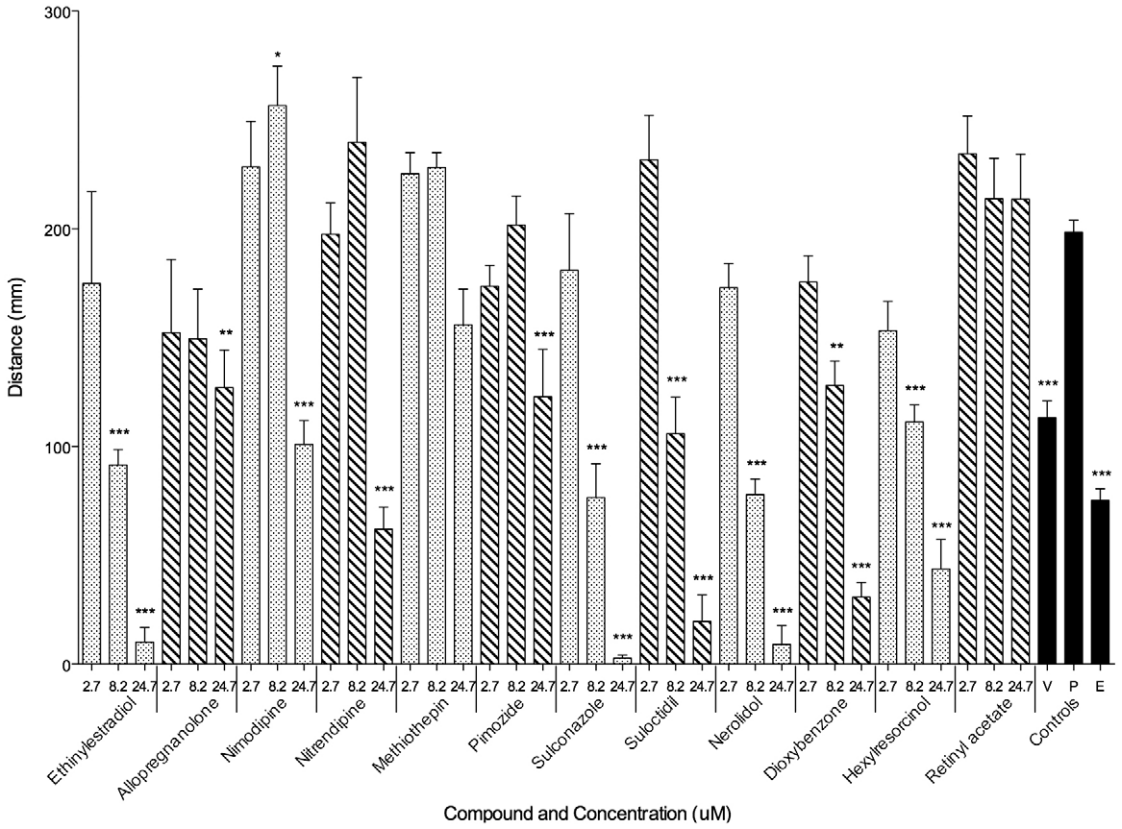

Fig. 6. Quantitative comparison of the concentration-dependent effects of identified anticonvulsants on PTZ-induced locomotor activity of 4-day-old zebrafish larvae. Compounds were administered to larvae for 90 minutes in 48-well plates, at a final concentration of 2.7, 8.2 or 24.7 μM, then 20 mM PTZ was added and larvae were then incubated for a further 10 minutes before being transferred to the Viewpoint Zebrabox for live tracking of locomotor activity over a 10 minute period. For each test compound, n=9; for VPA controls (V), n=71; for PTZ controls (P), n=108; for E3-only controls (E), n=96. Error bars show s.e.m. Asterisks indicate values that are significantly different from the value for PTZ-treated embryos (P), using a one-way ANOVA analysis with Dunnett's post-test. Level of statistical significance is indicated: ***P<0.001; **P<0.01; *P<0.05.

been documented as a response to seizures. This abundant transcription of *fos* within the developing somites of convulsant-treated embryos could indicate a protective function for this gene in muscle cells, which might help to buffer these cells against the potentially damaging effects of the strong calcium influx that occurs during seizures. Our use of a muscle-specific GCaMP-3 transgene revealed that convulsant-induced calcium-influx-dependent increases in fluorescence could be readily detected in muscle cells within 5 minutes of PTZ administration, and suggests that measurement of GCaMP-3 fluorescence could provide the basis for a high-throughput in vivo screen for detecting anticonvulsant activities in the future. Equally, in situ hybridisation assays to detect *fos* and other gene expression, or quantitative analysis of fluorescence changes using a GCaMP-3 transgenic line, could be used for the efficient detection of seizure liabilities in compounds as part of safety pharmacology programmes (Winter et al., 2008).

In neurons, the onset of synaptic activity induces transcription of a specific set of genes encoding transcription factors, intercellular signalling molecules and peptide hormones (Loebrich and Nedivi, 2009). To date, *fos* is perhaps the best characterised of these synaptic-activity-regulated genes. We found that genes encoding the bHLH transcription factor Npas4, the secreted intercellular signal Bdnf and the stress hormone PYYa exhibited increased transcription in the CNS of PTZ-treated embryos. Npas4 has previously been shown to promote the formation of GABAergic inhibitory synapses in mammalian cultured neurons (Lin et al., 2008), so its induction within the ventral forebrain of PTZ-treated embryos could reflect the activation of inhibitory neural circuits within this region to mitigate the excitatory effects of PTZ exposure. The neuroprotective properties of Bdnf include roles in protecting neurons against axonal injury and apoptosis, as well as in facilitating experience-dependent synaptic remodelling and promoting neurogenesis (Lipsky and Marini, 2007). We also observed that PTZ exposure led to increased expression of *pyya*, a close relative of *NPY*, which has been implicated both as an endogenous inhibitor of epileptic seizures (Baraban et al., 1997) and as a neuroprotective component of the endocrine response to stress and mood disorders, mediating anxiolytic effects on neuronal targets within the mammalian CNS (Heilig, 2004; Thorsell, 2010). The complexity of the *fos* expression pattern induced by PTZ treatment suggests that transcription of *fos* is activated in many different types of neurons within the CNS. PTZ-induced expression of *fos* is most prominent in the ventral telencephalon and ventral diencephalon, as is expression of *npas4* and *bdnf*, suggesting that neurons within these

Table 2. Effects of exposure to *fos*-suppressor compounds on viability of 4-day-old larvae

	% with heartbeat	
Compound	**8.2 μM**	**24.7 μM**
Sulconazole	100	100 S
Suloctidil	100	100 S
Ethinyl estradiol	100	100
Nimodipine	100 S	66 S
Nerolidol	100	100
Allopregnanolone	100	100
Dioxybenzone	100	100
Hexylresorcinol	100	100
Methiothepin	100	100
Nitrendipine	100	100 S
Pimozide	100	44 S
Retinyl acetate	100	100

Larvae were exposed to each identified *fos*-suppressor compound at a concentration of either 8.2 μM or 24.7 μM for 90 minutes, after which the number of larvae with a regular heartbeat was determined. For each treatment, a total of nine larvae were tested. Treatments that slowed the heartbeat of larvae in comparison with the untreated larvae are indicated by S.

regions are particularly sensitive to loss of GABA-mediated inhibitory neurotransmission. It is interesting to note that, in mammals, the ventral telencephalon and ventral diencephalon give rise to components of the limbic system and hypothalamic-pituitary-adrenal (HPA) axis, respectively, which mediate neural and endocrine responses to behavioural and pharmacological stressors, including factors that trigger the onset of seizures. Future studies will explore the possibility that GABAergic neurons lying within embryonic precursors of the limbic system and HPA axis regulate susceptibility to seizures. Phenotypic analysis of zebrafish *mind bomb* mutant embryos, which lack Notch signalling pathway activity, previously demonstrated that expression levels of *fos*, *bdnf* and *pyya* transcripts were increased in the CNS of 3-day-old mutant embryos, which also exhibit spontaneous seizures (Hortopan et al., 2010a). In light of the phenotypic similarities between PTZ-treated wild-type embryos and *mind bomb* mutants, it might be of interest to examine the possibility that Notch pathway activity regulates GABAergic signalling. The ease with which PTZ-induced expression of *fos* was detected in a specific pattern within the zebrafish embryonic brain, together with its robust inhibition by exposure of embryos to VPA, suggested that suppression of PTZ-induced *fos* transcription in 2-day-old embryos could provide a means for identifying putative anticonvulsants in chemical libraries. Our results demonstrate the validity of this approach, in which a hit rate of 2.3% was observed. The two largest classes of compounds that exhibited anticonvulsant activity were steroids and anti-infectives (Table 1). Among the steroids with anticonvulsant activity, progesterone and its derivatives allopregnanolone and megestrol were identified, as were the weak androgens epiandrosterone and formestane. In addition, the synthetic estrogens ethinyl estradiol and hexestrol exhibited robust anticonvulsant activities. Epiandrosterone is an isomer of the known anticonvulsant androsterone (Kaminski et al., 2005), whereas megestrol was previously shown to be neuroprotective against oxidative stress (Sarang et al., 2002). Endogenous neurosteroids such as allopregnanolone and progesterone have previously been demonstrated to exhibit potent anticonvulsant activities in rodent models, and evidence suggests that circulating neurosteroids regulate seizure frequency and severity in some individuals with epilepsy (Reddy, 2010; Pack et al., 2011). Allopregnanolone functions as a direct allosteric agonist of the $GABA_A$ receptor, suppressing seizures by potentiating the receptor's signalling function and thereby over-riding the inhibitory effects of PTZ (Hosie et al., 2006). Progesterone could exert its anticonvulsant effect in the zebrafish embryo as a result of its conversion to allopregnanolone, as has been observed in mice (Reddy et al., 2004). Our discovery of an anticonvulsant effect for ethinyl estradiol is intriguing because this drug is known to reduce aggression and reproductive success in zebrafish (Colman et al., 2009) and it is neuroprotective against kainic-acid-induced excitotoxicity (Picazo et al., 2010), but it has not previously been shown to stimulate inhibitory neurotransmission as a $GABA_A$ receptor agonist or to act as an anticonvulsant. Nevertheless, ethinyl estradiol was a more potent inhibitor of PTZ-induced locomotor activity than was allopregnanolone, raising the possibility that it might have an inhibitory effect on movement that could be independent of, and in addition to, an interaction with the $GABA_A$ receptor. Three calcium channel blockers, nimodipine, nitrendipine and suloctidil, were identified as potent inhibitors both of PTZ-induced *fos* expression and locomotor activity, perhaps reflecting their abilities to directly inhibit neural activity and muscular contraction by blocking calcium influx. Exposure to nimodipine (24.7 μM) caused cessation of heartbeat in a proportion (~33-36%) of treated embryos (Table 2). This effect might be due to inhibition of calcium channel function in cardiomyocytes, rather than a non-specific effect on cell viability, because no increase in apoptosis was observed after exposure to this compound. The identification of the antipsychotics methiothepin and pimozide, both of which inhibit dopamine and serotonin receptors (Seeman, 2002; Mahé et al., 2004), as suppressors of PTZ-induced *fos* expression and locomotor activity, is particularly interesting and strongly suggests that, as observed in other organisms (Bozzi et al., 2011), these monoamine neurotransmitters are involved in regulating seizures in zebrafish.

The second largest group of functionally related compounds that we identified in our screen was a class of anti-infectives that includes sulconazole, bifonazole and oxiconazole. Each of these compounds contains an imidazole ring and is related to ketoconazole, which is used to suppress excess glucocorticoid production in people with Cushing's syndrome, in which activity of the HPA axis is abnormally high (Feldman, 1986). The mode of action of ketoconazole involves inhibition of stress hormone synthesis, by an incompletely understood mechanism, raising the possibility that its close relatives might also act similarly, modulating neuroendocrine components of the HPA axis that regulate stress responses and thereby suppressing seizures. Interestingly, the imidazole-based anti-epileptic drugs denzimol and nafimidone inhibit sodium channels (Mishra and Ganguly, 2012), suggesting another mechanism by which sulconazole, bifonazole and oxiconazole could elicit their anticonvulsant effects. Two other anti-infective hits identified in our screen are hexylresorcinol and acrisorcin (which is a combination of 9-aminoacridine and hexylresorcinol). Hexylresorcinol has anaesthetic properties and was previously shown to bind to sodium channels,

inhibiting their function (Buchholtz et al., 2009), which could account for its anticonvulsant effect in our experiments. Several other compounds identified in our screen exhibit anticonvulsant activity in rodents. Previous studies of the COX inhibitors naproxen and mefenamic acid revealed that they both possessed anticonvulsant or related activities in PTZ-treated mice (Dhir et al., 2005; Wallenstein, 1991). Moreover, mefenamic acid acts as an agonist of the $GABA_A$ receptor (Coyne et al., 2007). Interestingly, molsidomine enhanced the anticonvulsant properties of VPA in PTZ-treated mice (Tutka et al., 2002), and peucedanin is closely related to oxypeucedanin, which exhibited anticonvulsant activity in electroshock-treated mice (Luszczki et al., 2010). Among the compounds identified in our screen, the flavonoids rotenonic acid and deguelin, as well as the organochlorines endrin, propanil and lindane, were identified as anticonvulsants. Given that each of these compounds has a history of use as a neurotoxic pesticide, it is perhaps unsurprising that they were identified in our screen for neuroactive anticonvulsants using a gene expression assay for synaptic activity. Interestingly, lindane is a direct inhibitor of $GABA_A$ receptors and exhibits convulsant activity in mice (Tochman et al., 2000; Vale et al., 2003), so its ability to suppress *fos* expression might reflect neurotoxicity, rather than an intrinsic anticonvulsant activity.

Overall, our results describe the establishment and validation of an in vivo model of epileptic seizures in the 2-day-old zebrafish embryo and its utilisation in a re-profiling screen of a bioactive small molecule library to identify compounds with anticonvulsant properties. Some of the compounds identified in this screen, such as allopregnanolone, nitrendipine, nimodipine, pimozide and methiothepin, have known biochemical specificities that readily account for their anticonvulsant behaviour in our assays. By contrast, the mechanisms of action of sulconazole, suloctidil and hexylresorcinol are poorly understood, and those of dioxybenzone and the sesquiterpene nerolidol are completely unknown. These compounds thus represent novel starting points for the development of new anti-epileptic drugs. Future studies will aim to elucidate the mechanisms of action of these molecules and to employ the in vivo assays we have developed to identify further compounds with anticonvulsant properties.

METHODS

Pharmacological induction and suppression of seizures in zebrafish embryos

Stocks of PTZ (200 mM, in water) or picrotoxin (100 mM in DMSO) were diluted to the required concentration in fresh E3 medium. Embryos were exposed to PTZ or picrotoxin then analysed as required. For in situ hybridisation analysis, embryos were transferred immediately to fixative containing 4% paraformaldehyde and incubated at 4°C overnight. As a positive control for anticonvulsant activity, a 250 mM stock of VPA was made up in E3 medium and administered to embryos at a final concentration of 2.5 mM before the addition of the convulsant. All procedures involving experimental animals were performed in compliance with local and national animal welfare laws, guidelines and policies.

Compound library aliquoting, storage and administration to embryos

The Spectrum Collection (Microsource Discovery Systems) of 2000 compounds was stored with each compound at a concentration of 5 mM in DMSO in 25 v-bottomed 96-well microtitre plates (Matrix) at −80°C. Assay plates contained compounds diluted to 10 μM in E3 media for drug screening. For the anticonvulsant assay, embryos were raised to 50 hpf and treated with Pronase (Sigma) to remove the chorions. Embryos were aliquoted at four embryos per well into Multiscreen mesh-bottomed plates (100 mm; Millipore) and transferred to Multiscreen 96-well culture receiver trays (Millipore) containing the Spectrum library compounds at 10 μM in columns 2-11, with control wells containing either 1 mM VPA, E3 or DMSO only in columns 1 and 12. Assay plates were incubated in the dark at 28°C for 90 minutes followed by addition of PTZ to a final concentration of 20 mM to all the compound wells and half of the control wells. Assay plates were incubated for 1 hour at 28°C and the embryos were then transferred to fixative containing 4% PFA and stored at 4°C overnight. Embryos were bleached according to the standard protocol (Thisse and Thisse, 2008) and stored at −20°C in methanol until required for in situ hybridisation. In order to facilitate screening and eliminate the need to transfer embryos between plates at any stage of the process, samples were maintained in the same 96-well mesh-bottomed Multiscreen plates (Millipore) during drug treatment, in situ hybridisation and hit detection. Hits that were identified in the primary screen were selected from the Spectrum library and rescreened using the method above. Selected compound stocks were then purchased separately from Sigma and tested in dose-response assays to confirm that the Spectrum Collection stocks had been assigned the correct identities.

Whole-mount in situ hybridisation analysis of gene expression

Digoxigenin-labelled RNA probes were prepared as recommended by the manufacturer (Roche). Details of the *gabra1*, *gabrg2*, *fos*, *npas4*, *bdnf* and *pyya* probes utilised are available on request. Whole-mount in situ hybridisation was performed using standard procedures (Thisse and Thisse, 2008) with modifications. All steps were performed using Multiscreen mesh-bottomed plates. In order to increase throughput, the Biolane HTI 16V In situ robot (Intavis) was used. Stained embryos were imaged in Multiscreen plates using a Nikon AZ100 microscope fitted with an automated stage (Prior). Extended depth of focus (EDF) images from *z*-sections through the embryos were compressed using the NIS-Elements software (Nikon) to show the areas of staining and the results were scored. Wells that contained embryos with no *fos* expression in the brain were taken forward for further analysis.

Analysis of locomotor activity using the Viewpoint Zebrabox system

The distance moved by larvae over a 10 minute period was recorded using the Zebrabox system (Viewpoint, France). AB larvae at 4 dpf, with swim bladders inflated, were transferred to a 48-well microtitre plate, one larva per well in 445 μl of E3 medium. Dilutions of compounds over the range of 74-0.3 μM were added to embryos in triplicate, and control wells containing VPA and E3 alone were also included. Plates were incubated at 28°C in the dark for 90 minutes. Larvae were then tested for the presence of a heartbeat and motor response before addition of PTZ to wells containing compounds and half of the wells containing E3 only. After 5 minutes exposure to PTZ, larval movements were recorded with the Zebrabox for 10 minutes using a light cycle of 2

TRANSLATIONAL IMPACT

Clinical issue

Epilepsy is a common neurological disorder affecting ~1% of the world's population. People with epilepsy experience seizures that vary in severity, frequency and physical location in the nervous system. A range of genetic factors play important etiologic roles in many forms of epilepsy, reflecting the heterogeneity of the pathogenetic mechanisms that underlie these disorders. Various structurally diverse anti-epileptic drugs are available for the treatment of epilepsy, but ~30% of people with epilepsy do not respond satisfactorily to many of the first-line treatments, and substantial side effects are widely documented. Thus, there is a large unmet clinical need for new anti-epileptic medicines with improved specificity and potency, and with new mechanisms of action. Recent progress in identifying new drugs has been slow, at least in part because high-throughput methods for in vivo discovery of new drugs with anti-convulsant activity are limited.

Results

Taking advantage of the transparency and developmental simplicity of the 2-day-old zebrafish embryo, the authors developed and validated a tractable, in vivo, high-throughput assay based on expression of *fos*, which is upregulated in response to chemically induced seizures. They applied this assay in a screen of 2000 bioactive small molecules and identified 46 compounds with anti-convulsant activity. Further analysis of a subset of these compounds, which included both known and newly identified anticonvulsants, showed that they exhibited concentration-dependent inhibition of convulsant-induced locomotor activity and *fos* transcription in 2-day-old zebrafish embryos.

Implications and future directions

These data introduce a robust, scaleable approach for in vivo screening for compounds with anti-convulsant activity in a vertebrate model organism, and show how it can be used it to identify anti-convulsants from a library of small molecules. This strategy provides a relatively low-cost, high-throughput route to discovering new molecules with potential clinical application for the treatment of epilepsy

minutes:100% light; 2 minutes:0% light. Each experiment was repeated in triplicate to give nine data points for every drug concentration and the total distance moved by each larva was calculated.

Statistical analysis of locomotor activity data

For each group of embryos exposed to compounds, the mean distance moved and the standard error of mean (s.e.m.) were calculated. The effects on locomotor activity of exposing embryos to a range of PTZ concentrations were evaluated using a one-way ANOVA with Dunnett's post-test. The effects of administering hit compounds were also evaluated using a one-way ANOVA statistical test to determine whether each compound suppressed PTZ-induced locomotor activity at 3, 8 and 24 μM concentrations.

Analysis of GCaMP-3 fluorescence in muscle cells of PTZ-treated embryos

A muscle-specific GCaMP-3 reporter plasmid was constructed by inserting the GCaMP-3 open reading frame (Tian et al., 2009) downstream of zebrafish *mylz2* promoter sequences (von Hofsten et al., 2008). The circular plasmid DNA was injected into zebrafish embryos at the one-cell stage at a concentration of 0.1 ng/nl to generate mosaic embryos, which were incubated at 28.5°C until they reached 50 hpf. Embryos with detectable GFP fluorescence in muscle cells were transferred to E3 medium containing blebbistatin (50 μM) for 30-60 minutes at 28.5°C. Embryos were transferred singly to a 35 mm Wilco glass-bottomed Petri dish (22 mm glass diameter) and mounted for lateral viewing in a drop of 1% low-melting-point agarose containing 50 μM blebbistatin and 20 mM PTZ. Convulsant-induced increases in GCaMP-3 fluorescence were apparent within 3-5 minutes after mounting, and embryos were imaged with a Perkin-Elmer Spinning Disk microscope and Volocity (Improvision) software, using the GFP channel and a frame-rate of 20-40 Hz.

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COMPETING INTERESTS

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

AUTHOR CONTRIBUTIONS

S.B., P.W.I. and V.T.C. conceived and designed the experiments. S.B., C.J.H., P.L.M.S., M.R.M.H., J.F., C.A.P. and V.T.C. performed the experiments. S.B., C.J.H., P.L.M.S., M.R.M.H., J.F., C.A.P. and V.T.C. analysed the data. S.B. and V.T.C. wrote the paper.

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SUPPLEMENTARY MATERIAL

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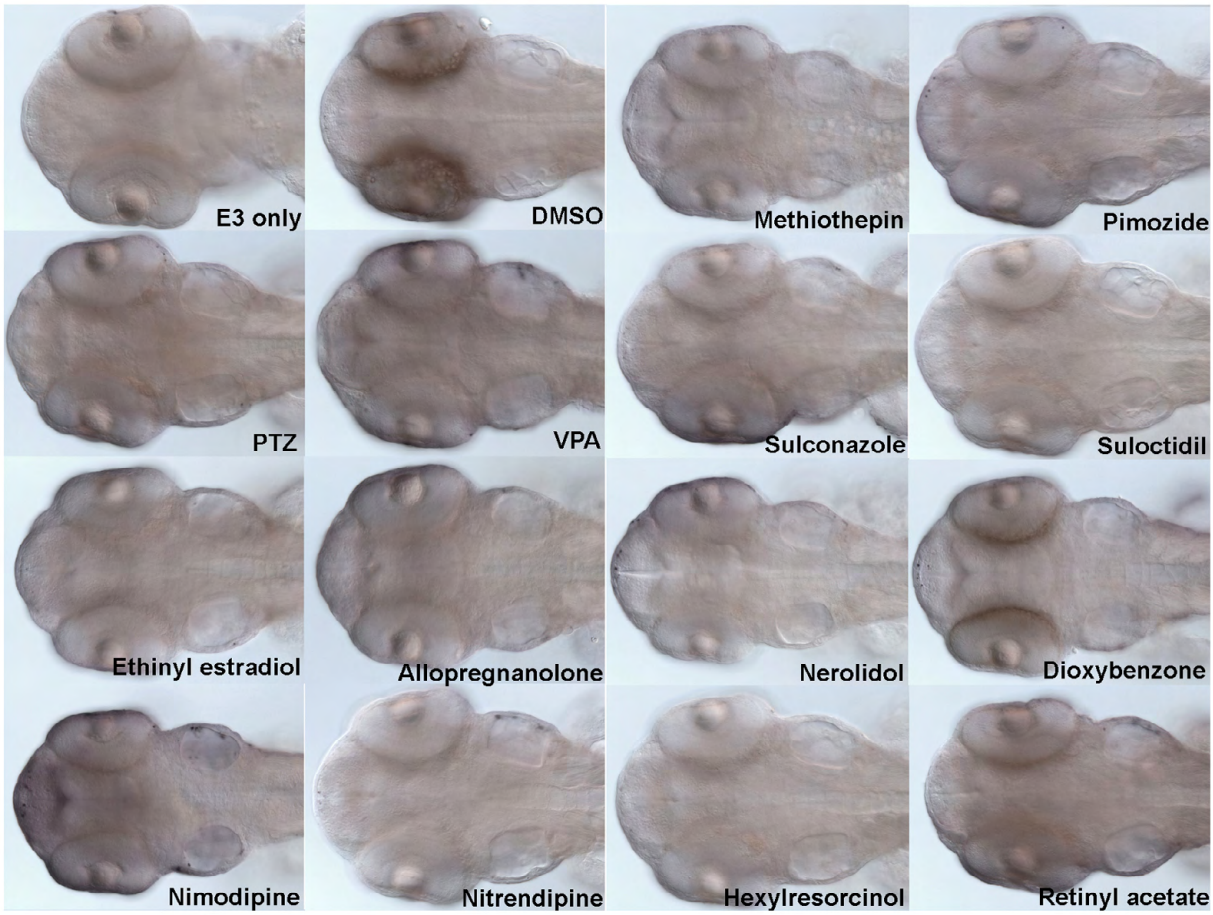
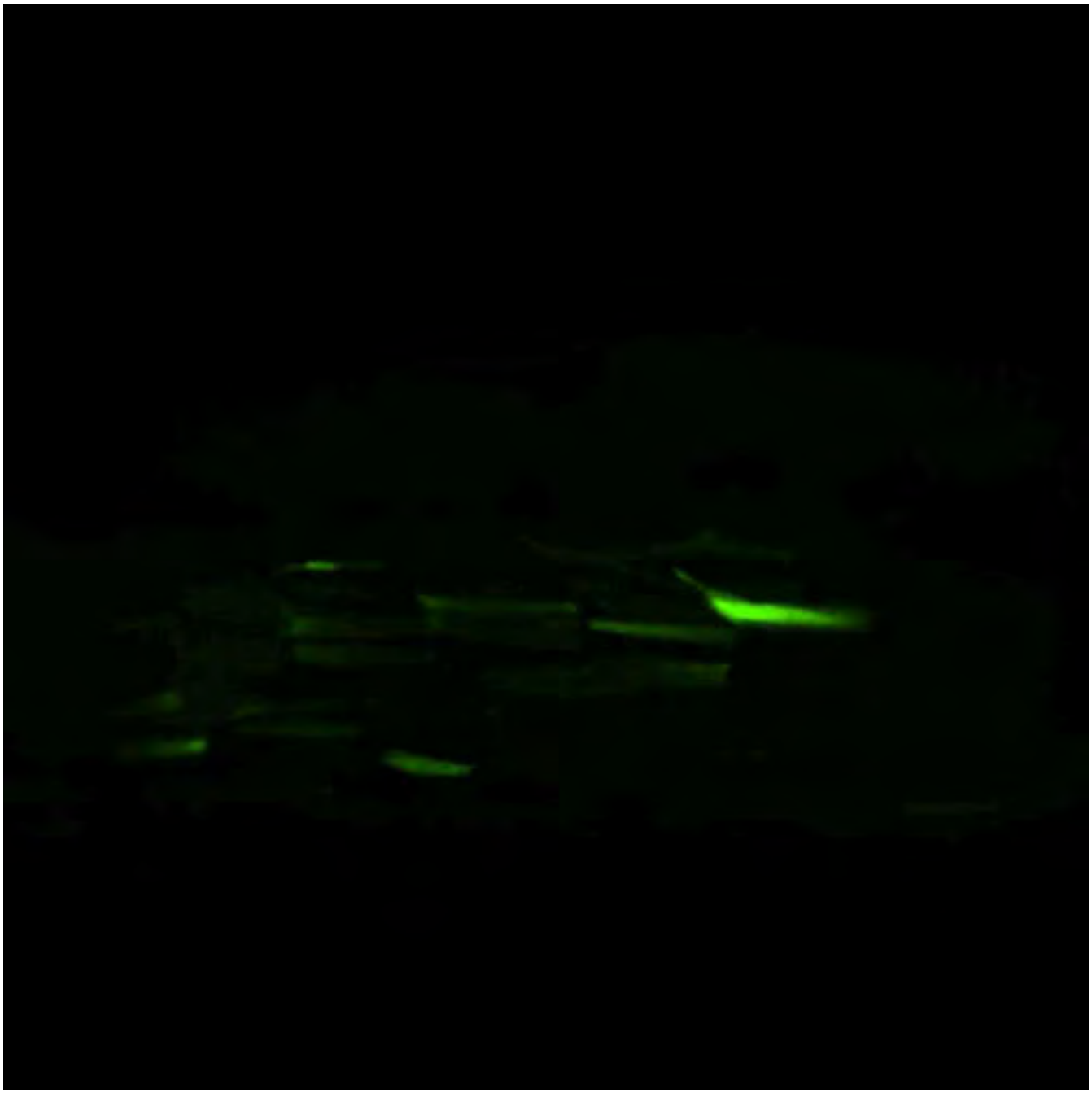


Fig. S1. Analysis of apoptosis in the CNS of drug-treated embryos using TUNEL. Anticonvulsant compounds were administered to 50 hpf embryos for 90 minutes in multi-well plates, at a final concentration of 10 uM. PTZ was administered at a final concentration of 20 mM and VPA was administered at a final concentration of 1 mM. Embryos were fixed and analysed for the presence of apoptotic cells using TUNEL. Each panels shows a dorsal view of the brain of an embryo exposed to the indicated compound. No significant induction of apoptosis was observed after treatment of embryos with any of the selected compounds.



Movie 1. Live confocal imaging of GCaMP-3 fluorescence changes in muscle cells of 50 hpf zebrafish embryo transiently expressing a *mylz2:GCaMP3* reporter transgene, after 5 minutes of exposure to 20 mM PTZ. Still images taken from movie are included in Fig. 1C.

Supplementary Table 1

Neuroactive properties of anticonvulsant compounds identified in zebrafish embryos.

Compound	*Previously reported neuroactivity in mammalian systems*	*References*
Megestrol acetate	Neuroprotective against oxidant injury	Sarang et al., 2002
Ethinylestradiol	Neuroprotective against kainic acid treatment	Picazo et al., 2010
Epiandrosterone	Isomer of anticonvulsant androsterone	Kaminski et al., 2005
Progesterone	Precursor of anticonvulsant allopregnanolone	Reddy et al., 2004
Allopregnanolone	Anticonvulsant	Reddy, 2010
Formestane	None reported	
Hexestrol	None reported	
Triptophenolide	None reported	
Gemfibrozil	None reported	
Retinyl acetate	None reported	
Sulconazole	None reported, related to sodium channel inhibitors	
Bifonazole	None reported, related to sodium channel inhibitors	
Oxiconazole	None reported, related to sodium channel inhibitors	
Acrisorcin	Contains hexylresorcinol, sodium channel blocker	Buchholz et al. 2009
Fenamisal	None reported	
Hexylresorcinol	Sodium channel blocker	Buchholz et al. 2009
Diallyl sulphide	None reported	
Exalamide	None reported	
Naproxen	Anticonvulsant, COX inhibitor	Dhir et al. 2005
Mefenamic Acid	Neuroprotective, anticonvulsant, $GABA_A$ receptor agonist	Coyne et al. 2007
Nimodipine	Calcium channel blocker	
Nitrendipine	Calcium channel blocker	
Suloctidil	None reported	
Methiothepin	Antipsychotic, monoamine receptor antagonist	Mahe et al. 2004
Pimozide	Antipsychotic, monoamine receptor antagonist	Seeman et al. 2002

Theaflavin	Neuroprotective	Anandhan et al. 2011
2-isopropyl-methoxycinnamic acid	None reported	
Molsidomine	Anticonvulsant adjuvant	Tutka et al. 2002
Lindane	Convulsant, $GABA_A$ and Glycine Receptor ligand	Vale et al. 2003
Deguelin	None reported	
Rotenonic Acid	Neurotoxin	
Endrin	Neurotoxin	
Propanil	Neurotoxin	
Mundulone	None reported	
Dioxybenzone	None reported	
Nonoxynol-9	None reported	
Larixol	None reported	
Haematommic Acid	None reported	
Avocadyne	None reported	
Ethyl everninate	None reported	
Isosafrole	None reported	
Oxyquinoline hemisulphate	None reported	
8-beta-Hydroxycarapin 3,8-Hemiacetal	None reported	
Peucedanin	Related to anticonvulsant Oxypeucedanin	Luszczki et al., 2010
Nerolidol	None reported	
Senecrassidiol 6-acetate	None reported	