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Research Report

Distinct models of induced hyperactivity in zebrafish larvae

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ABSTRACT

The analysis of behavioural hyperactivity can provide insights into how perturbations in normal activity may be linked to the altered function of the nervous system and possibly the symptoms of disease. As a small vertebrate zebrafish have numerous experimental advantages that are making them a powerful model for these types of studies. While the majority of behavioural studies have focused on adult zebrafish, it has become apparent that larvae can also display complex stereotypical patterns of behaviour. Here we have used three compounds (pentylenetetrazole (PTZ), aconitine and 4-aminopyridine) that have different neuronal targets (GABA, sodium and potassium channels), to induce distinct patterns of hyperactivity in larvae. Our studies have revealed that each compound produces a number of distinct concentration-dependent activity patterns. This work has shown for the first time that at sub-convulsive concentrations, PTZ can reverse the normal behavioural response to alternating periods of light and dark in zebrafish larvae. It also appears that both PTZ and 4-aminopyridine produce distinct changes in the normal startle response patterns immediately following light/dark transitions that may be the result of an elevation in stress/anxiety. Aconitine produces a general elevation in activity that eliminates the normal response to light and dark. In addition to differences in the patterns of behaviour each compound also produces a unique pattern of *c-fos* (an immediate early gene) expression in the brain. While more work is required to make direct links between region specific neuronal activity and individual behaviours, these models provide a framework with which to study and compare mechanistically different types of inducible behaviours.

1. Introduction

The zebrafish has emerged as an excellent model system for studying mechanisms of altered behaviour (Baraban et al., 2005; Blaser and Gerlai, 2006; Burgess and Granato, 2007; Irons et al., 2010; Li et al., 2009; Steenbergen et al., 2010; Winter et al., 2008) and for screening potential therapeutics (Chakraborty et al., 2009; Goldsmith, 2004; Irons et al., 2010; Kokel and Peterson, 2008; Kokel et al., 2010; Rihel et al., 2010; Seibt et al., 2010; Zon and Peterson, 2010). In addition to high fecundity and low rearing costs, the stereotypical patterns of behaviour and conserved drug responses have made the zebrafish a creditable alternative to traditional mammalian models. Neuroactive compounds such as ketamine, dizocilpine, phencyclidine, kainic acid, caffeine, pentylenetretrazole (PTZ), cocaine and picrotoxin (Berghmans et al., 2007; Kim et

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al., 2010; Seibt et al., 2010; Wong et al., 2010; Zakhary et al., 2011) have been used to induce behavioural hyperactivity that appears to mimic some of the symptoms of disease states, such as schizophrenia, epilepsy and anxiety. These models have primarily assessed altered patterns of behaviour in adult zebrafish, while the majority of larval studies have been used to evaluate seizure activity induced by high levels of neuroactive compounds (Burgess and Granato, 2007; Kim et al., 2010).

Recent work has shown that larvae can display complex, stereotypical patterns of behaviour and the analysis of these behaviours can provide valuable information regarding the therapeutic potential and target specificity of new compounds (Kokel et al., 2010; Rihel et al., 2010). Similar to adult studies, induced hyperactivity in larvae may also produce phenotypes that could be used to mirror symptoms of disease. While the adult models of hyperactivity have suggested links to disease states, these associations are based solely on the interpretation of the induced patterns of behaviour and correlations with mammalian behaviours, with little information regarding mechanism. In order to make stronger links between these types of models and the symptoms of a disease more work is required to define the regions of the zebrafish brain that are activated and determine if these regions are comprised of the same types of neurons and are regulated in the same fashion (i.e. neurotransmitters) as those linked to specific disease states.

Here we have used zebrafish larvae to conduct a detailed analysis of the patterns of behaviour produced by neuroactive compounds that are known to act on different targets. Three compounds, one well-characterized and two with undefined phenotypic effects in zebrafish larvae were selected because of the ability of the compound to elevate neuronal activity. We have shown that PTZ, which has primarily been used as a convulsant in zebrafish (Baraban et al., 2005, 2007; Berghmans et al., 2007; Kim et al., 2010; Lee et al., 2010), along with aconitine and 4-aminopyridine (4-AP) can induce complex changes in behaviour. Each compound induces a distinct concentration-dependent behavioural profile in zebrafish larvae that becomes obvious once the behaviour is analyzed using multiple parameters. An initial analysis of *c-fos* expression has revealed that each compound produces a distinct, regionalized, pattern of elevated neuronal activity. Being able to link specific behaviours with the activation of specific regions of the brain and ultimately with specific neuronal subtypes will allow us to begin to understand how these distinct types of behaviours are regulated.

The abbreviations used are: 4-AP, 4-aminopyridine; PTZ, pentylenetretrazole; *c-fos*, FBJ murine osteosarcoma viral oncogene homolog; LIGHT/DARK, light/dark; hpf, hours post fertilization; dpf, days post fertilization; qPCR, quantitative polymerase chain reaction; EF1α, Elongation factor 1alpha; SSC, Saline–Sodium Citrate; NBT-BCIP, nitro-blue tetrazolium and 5-bromo-4-chloro-3'-indolyphosphate; AP, alkaline phosphatase; ISH, *in situ* hybridization; HPI, hypothalamic-pituitary-interrenal; Pa, pallium; Sp, subpallium, Po, preoptic; Cb, cerebellum; VT, ventral thalamus; PT, posterior tuberculum; Hy, hypothalamus; OB, olfactory bulb; forebrain, Fb; Te, tegmentum; Hb, hindbrain.

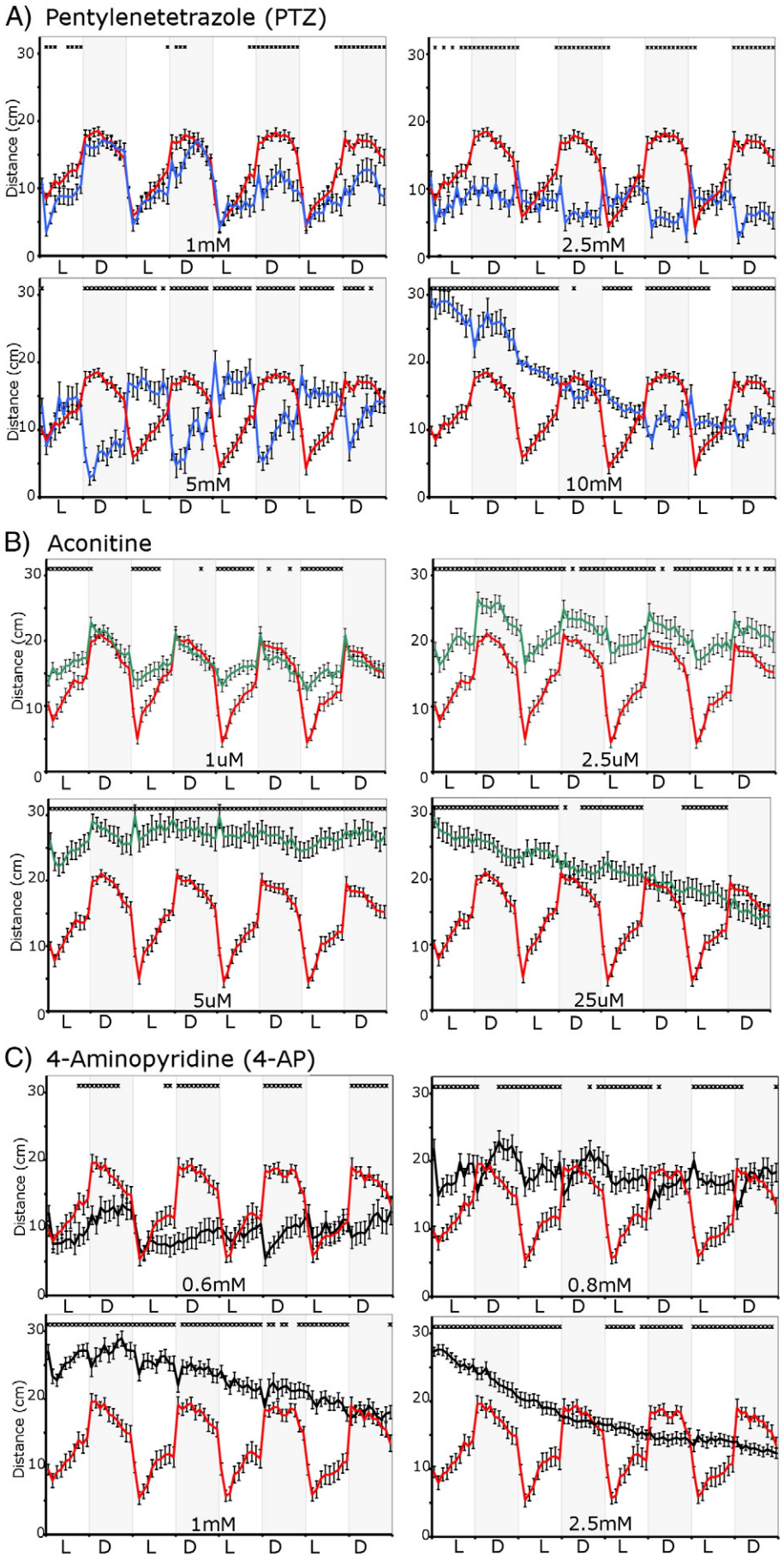
2. Results

2.1. *Characterization of altered light/dark response patterns*

The effect of each compound on larval activity was initially evaluated across a broad concentration range in order to establish a biologically relevant range beginning at a concentration that produced no measurable change in activity to the highest level that was not toxic. Following this initial visual assessment larval activity (defined as distance traveled in cm) was measured in a 96-well plate with the ViewPoint ZebraLab tracking software. In order to evaluate sensory responsiveness larvae were treated for 30 min with an individual compound and then presented with four 10-minute periods of light then dark (Irons et al., 2010) (Fig. 1). The concentration range was further narrowed following the initial response profile to between a level that produced a detectable change in activity in either the light or dark phase and a level that resulted in a drop in activity over a 2-hour period, but did not lead to lethality. Following this initial assessment the concentrations that produced unique phenotypic patterns were selected to highlight the differences between each compound.

The γ-Aminobutyric acid (GABA) antagonist PTZ has been one of the most widely studied excitatory compounds and has been used in the past to evaluate the effects of anticonvulsants on zebrafish (Baraban et al., 2005; Berghmans et al., 2007). Previous studies have evaluated increased larval activity generated by PTZ at concentrations between 5 and 30 mM {Baraban, 2005 #2;Berghmans, 2007 #37}. In the current study, we initially evaluated the activity produced by PTZ using a ¼ log dilution series between 500 μM and 25 mM. There was no significant change in activity at 500 μM and at concentrations above 10 mM larvae displayed sporadic tremors followed by lateral recumbency similar to the profile previously described as a transition from a phase II to phase III seizure state (Baraban et al., 2005). Between 1 and 10 mM PTZ produced a concentration-dependent profile that appears to consist of two distinct components, a gradual reversal in the normal light/dark response profile followed by an overall increase in activity at higher concentrations.

Beginning at 1 mM there was a reduction in activity during the dark phase, leading to a significantly lower activity level than controls by the 3rd dark cycle (asterisks Fig. 1A; *t*-test, $p<0.05$). Incremental increases in PTZ (of 1 mM) produced a profile in which the activity during the dark phase was further decreased and the light cycle activity began to increase. At levels of PTZ above 2.5 mM (Fig. 1A) the light phase activity became larger than the dark phase activity. As the concentration of PTZ was increased further, the reversal of the normal light/dark response became more prominent leading to what appeared to be a complete reversal of the light/dark response pattern at 5 mM. At concentrations above 5 mM this pattern of activity was retained, however the overall activity level for both the light and dark phases continued to increase until 10 mM, at which time the response to changes in light/dark was all but eliminated. This concentration-dependent profile has revealed a phenotypic change in activity induced by subconvulsive levels of PTZ that has not been previously reported for zebrafish.


A) Pentylenetetrazole (PTZ)
Distance (cm)
1mM
2.5mM
5mM
10mM
B) Aconitine
1uM
2.5uM
5uM
25uM
C) 4-Aminopyridine (4-AP)
0.6mM
0.8mM
1mM
2.5mM
L D L D L D L D

The sodium channel antagonist aconitine induced a concentration-dependent behavioural profile that differed from PTZ. An initial ¼ log dilution series revealed that below 1 μM there was no significant change in activity and at levels above 25 μM, an initial large increase in activity was followed by a complete loss of movement. At 1 μM the first changes in activity were noted as an increase in the light phase (Fig. 1B). Both the light and dark activity became significantly higher than controls at and above 2.5 μM (*t*-test, $p<0.05$). The activity profile of aconitine was different than that of PTZ as there was a general increase in activity that resulted in an elevation in both the light and dark responses until there was no longer a response to light/dark changes and the activity appeared to plateau (at and above 5 μM). At 20 μM, the initial elevation in activity subsequently decayed back to levels near controls over the 2-hour experiment. The decrease in activity at high concentrations of aconitine was not accompanied by tremors but lead to inactivity.

The potassium channel antagonist 4-AP also induced a distinct concentration dependent behavioural profile. The initial ¼ log dilution series revealed that below 500 μM there was no significant effect on activity and levels above 2.5 mM eliminated larval activity. The concentration range was narrowed to levels between the no observable effect level of 500 μM and 1 mM where the elevated activity began to decay over the course of the experiment. At 600 μM 4-AP produced an initial decrease in dark phase activity similar to that of PTZ (Fig. 1C). However, unlike PTZ as the concentration of 4-AP was elevated to 800 μM and 1 mM there was an elevation in both light and dark phase activity that did not produce the reversal in the pattern of light/dark response that was seen for PTZ. The elevated activity produced at 2.5 mM was found to decay, however this did not result in the general inactivity found at higher levels of 4-AP.

2.2. *Defining the pattern of hyperactivity based on velocity*

In order to further analyze the changes in larval activity following treatment with the hyperactive compounds, we separated movement based on velocity. Previous studies have evaluated hyperactivity by measuring the distance travelled above a specified threshold velocity (20 mm/s; Winter et al., 2008). For our studies we evaluated the entire activity profile. A high (20 mm/s) and low (3 mm/s) threshold were set that divided the activity into 3 types of movement. The total distance traveled at velocities above the high threshold is defined as fast movement, the distance traveled between the two thresholds is referred to as slow movement and the distance traveled at velocities below the low threshold is inactive movement. The inactive category was comprised mainly of small changes in orientation and since the levels did not change for any of the treatments or controls during the light/dark cycling this movement category was not analyzed further. The fast movement accounted for between 4 and 5% of the total movement above the inactive threshold for control larvae and there was no significant difference between the fraction of fast movement between the light and dark phases.

At all concentrations of PTZ the slow movement pattern appeared similar to the overall activity pattern, with a gradual increase in movement associated with the light phase and a decrease in the amount of activity during the dark phase at 2.5 and 5 mM (Fig. 2A). The fast movement pattern became significantly larger than controls for both the light and dark phases at 2.5 mM (t-test, $p<0.05$, Fig. 2A). The pattern of fast and slow movement at 2.5 and 5 mM were similar for both the light and dark phases and did not appear to change during the 2-hour experiment. The reversal of the normal light/dark response then appears to be the result of a combination of both fast and slow movement compared to the controls where most of the light/dark specific behaviour consists of slow movements. The decay in activity that occurs at and above 10 mM is also produced by a drop in both fast and slow movement with the fast movement dropping at a faster rate than the slow movement.

Analysis of the aconitine induced activity types revealed a profile that was distinct from PTZ (Fig. 2B). Similar to PTZ the slow movement pattern appeared to mirror that of the total activity. However, the level of slow movement appeared to represent a larger component of the total activity than was seen for PTZ. The level of fast movement increased with concentration, with the peak occurring at 10 μM. At 10 μM the initial elevation in fast movement dropped sharply and leveled off after the first hour of exposure, while the slow movement remained elevated for the entire 2 h.

4-AP induced a different pattern of fast and slow activity than either PTZ or aconitine (Fig. 2C). At 600 and 800 μM 4-AP the overall activity pattern showed a drop in activity following a light to dark transition (Fig. 1C). When the activity was separated, this drop in activity was more apparent for the fast movement pattern than it was for the slow movement (Fig. 2C). Additionally, much of the decay in the overall activity at 800 μM and 1 mM can be attributed to a drop in the fast movement. One of the interesting components of the 4-AP induced activity occurred at 2.5 mM where the fast movement was all but eliminated while the slow movement remained elevated. This produced a phenotype consisting of a continuous state of slow movement in which the larvae no longer responded to light/dark cycling. It then appears that the fast movement contributes a large component to the overall activity pattern found at low to moderate doses of 4-AP, while at higher doses the fast movement is eliminated.

Fig. 1 – Light/dark response of larval zebrafish following chemically induced hyperactivity. Following 30 min in the dark four 10-minute light/dark cycles were used to assess changes in activity based on distance traveled (data presented as average distance traveled ± standard error; 60 s bins; n = 36/concentration). (A) Four different activity patterns are produced by PTZ at 1, 2.5, 5 and 10 mM (blue lines, red lines—controls). (B) Aconitine treatment results in a graded increase in activity with concentration at 1, 2.5, 5 and 25 μM (green lines). (C) 4-AP produces an initial drop in the average activity followed by a complex elevation in activity at 0.6, 0.8, 1 and 2.5 mM (blue lines). * Denotes significant differences from controls (*t*-test, $p<0.05$).

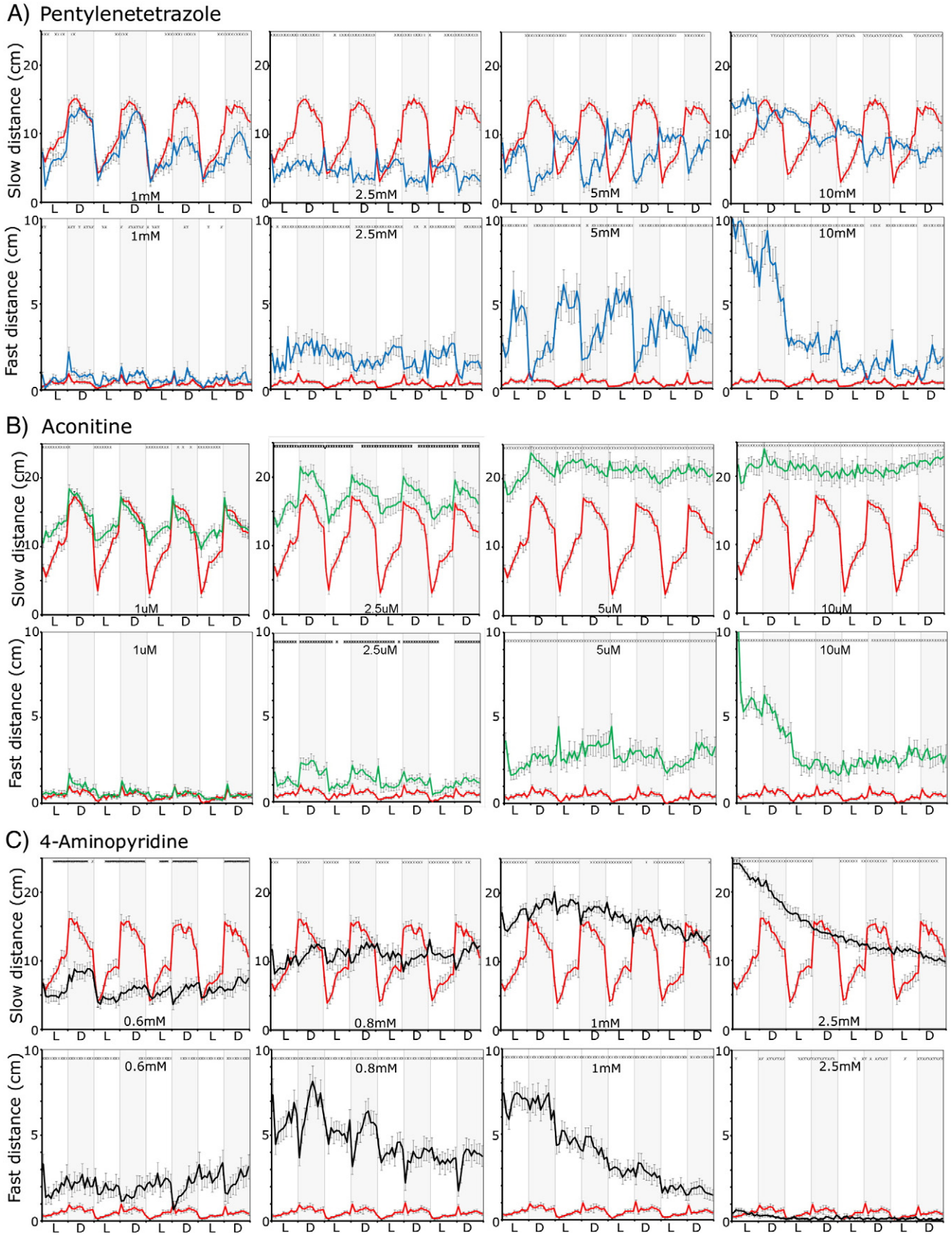


Fig. 2 – Characterization of hyperactivity based on velocity. Two thresholds were used to separate activity into fast (over 20 mm/s) and slow (3–20 mm/s) components. Dose dependent changes in the fast and slow activity for (A) PTZ, (B) aconitine and (C) 4-aminopyridine are presented as average distance traveled ± standard error (60 s bins; n = 36/concentration). * Denotes significant differences from controls (t-test, $p < 0.05$).

2.3. *Activity changes immediately following a transition*

It was previously demonstrated that zebrafish show characteristic responses within the first few seconds of the transition from either light to dark or dark to light (Emran et al., 2008). In order to further profile the induced activity changes we analyzed the activity pattern the minute before and the minute after a light to dark or dark to light transition. Similar to the pattern previously described (Emran et al., 2008) the transition from dark to light produced a short-duration (<5 s) spike in activity that decayed to below the pre-transition activity levels. The transition from light to dark also produced a short-duration spike in activity that did not decay back to baseline but remained above the pre-transition levels for the entire 60 s.

A number of interesting activity patterns were revealed following further analysis of the activity patterns produced by the neuroactive compounds. Since the baseline activity level produced by the neuroactive compounds was often higher than controls, in order to evaluate changes in the short-duration activity patterns the post-transition activity (5-second windows) was compared to the activity for the 60 s prior to the transition (1-way ANOVA, 95% confidence level). As mentioned, at 5 mM PTZ showed a complete reversal of the normal light/dark activity pattern. At 5 mM PTZ the transition from dark to light lead to a large initial response not found for controls producing a peak in total activity approximately 4 fold higher than controls during the first 5 s following the transition (Fig. 3A). Following the initial spike, larval activity remained above the pre-transition levels. The transition from light to dark also produced a short-duration spike in activity, however the peak was not significantly different than controls ($p > 0.05$, *t*-test). Following the initial peak in activity (0–10 s) the activity level of the light to dark transition fell below the pre-transition levels. It would then appear that the switch in the normal response to light/dark transitions produced by 5 mM PTZ is apparent even during the initial minute following the transition.

At 5 μM, aconitine produces an elevation in overall activity and appeared to only respond to dark to light, but not light to dark transitions (Fig. 1B). This activity pattern was confirmed when the transitions were evaluated more closely (Fig. 3B). While there was an increase in activity following the transition from dark to light, unlike the controls, there was no initial

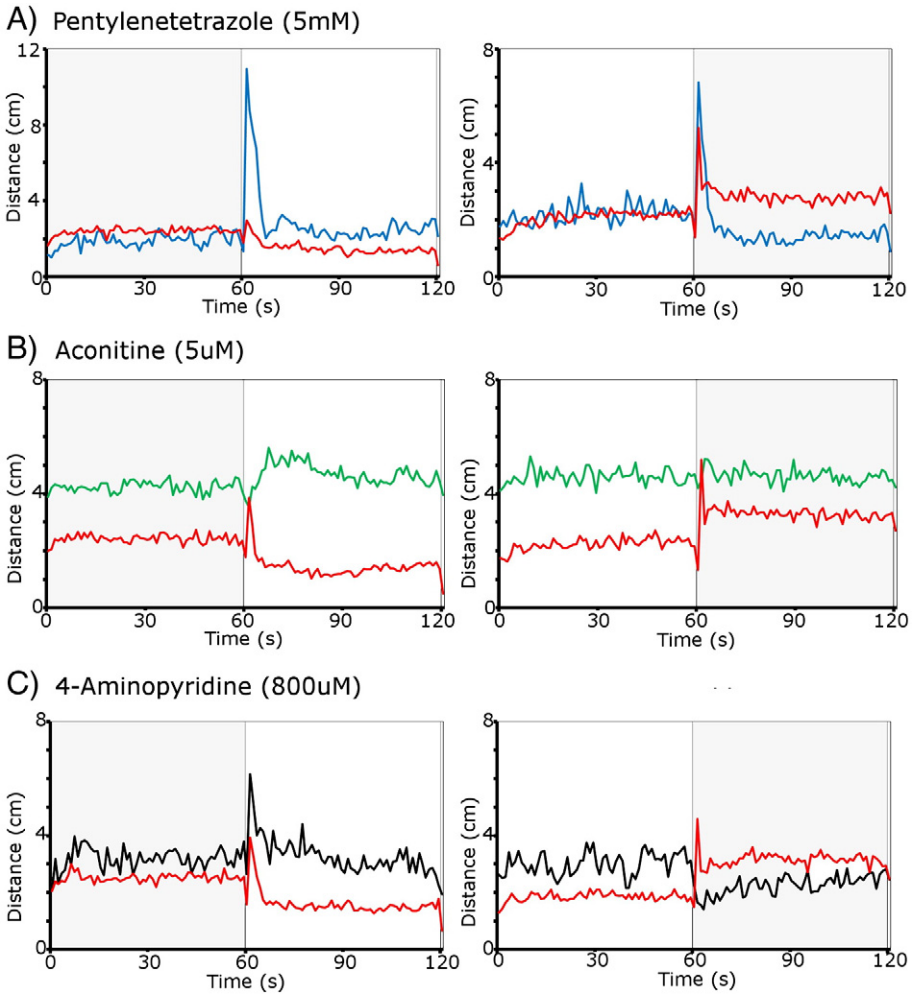


Fig. 3 – Activity immediately following transition. The activity 60 s prior to and 60 s following a dark to light or light to dark transition following treatment with (A) PTZ (5 mM), (B) aconitine (5 μM) or (C)4-aminopyridine (800 μM). Data presented as average activity for 3 consecutive light/dark and dark/light transitions (1 s bins; n=36 larvae).

spike in activity during the first 5 s after the transition, with the activity increase becoming significantly larger than the pre-transition levels 5 to 20 s following the transition (comparison of 5-second bins with the 60 s before the transition, 1-way ANOVA, 95% confidence interval). There was no change in activity during the light to dark transition even though the levels of activity were higher than controls at all points around the transition.

At 800 μM 4-AP appeared to produce a response pattern similar to controls during dark to light transitions, while the transitions from light to dark produced a drop in activity (Fig. 1). When the transition activity was evaluated more closely, it appeared that the transition from dark to light was similar to controls, with an initial spike in activity. However, the activity returned to levels that were not significantly different than the pre-transition activity, unlike the control activity which dropped below the pre-transition levels following the initial spike (Fig. 3C). Confirming the previous results, the light to dark transition resulted in an immediate drop in activity that returned to baseline 25–30 s following transition (Fig. 3C). It then appears that larvae exposed to 800 μM 4-AP produce different response patterns for light to dark or dark to light transitions.

2.4. *Assessing thigmotaxis*

It has been shown that stress inducing situations, such as exposure to a novel environment, cause adult zebrafish to stay close to the side of the tank (Champagne et al., 2010). This could be considered a measure of thigmotaxis or a preference for physical contact, which is thought to be part of normal stress-response pattern in a number of species. In order to test if this behaviour exists in 5 day old larvae and could be quantified we made use of the Viewpoint behavioural analysis software and 48 well plates in order to localize the activity within a well. Each well (containing a single larva) was separated into a defined central area and an outer ring containing the outside edge of each well. In our experiments larvae were loaded into plates the day before the experiment, which should eliminate most of the novelty response that could increase the level of thigmotaxis. The total activity during the first light or dark phase was assessed as a 10-minute bin. The activity that occurred within the central area was quantified and presented as a percentage of the total activity that occurred within the entire well. Stereotypical larval activity is mainly comprised of movement around the perimeter of the well with occasional exploration of the center. Notably, there are differences in the level of larval activity in the center area in the light versus dark phases (Fig. 4). The percentage of activity for control larvae was significantly larger in the center during the light phase (18.5±1.4%), than during the dark phase (7.6±1%, *t*-test, $p<0.05$, control values are the average of all control larvae from the 3 treatment groups). In contrast the total activity level for the entire well was higher during the dark phase (Fig. 1). Since thigmotaxis is considered a quantifiable measure of anxiety in zebrafish this activity pattern would seem to indicate a more exploratory nature during the light phase and perhaps a higher level of stress/anxiety (wall hugging) in the dark.

The activity patterns of control larvae within the center of the well was further analyzed by examining the percentage of distance covered by fast movement for both light and dark phases and then expressed as a percentage of the total activity (distance) within the center of the well. The activity generated by fast movement in the center of the well during the light phase was lower than during the dark phase (average of all controls; 13.1±1.7% vs. 18.7±1.7%, *t*-test, $p<0.05$, Fig. 4), possibly indicating a higher level of exploration during the light phase with more slow scanning type of movements and less fast-darting activity seen during the dark phases. Again this may indicate a heightened level of stress during the dark phase as the main type of movement across the center of the well is rapid darting with the larvae quickly returning to the "safety" of the well wall.

PTZ treatment lead to a significant reduction in the amount of larval activity in the center of the well for the light phases beginning at 1 mM (*t*-test, $p<0.05$) with a decrease to levels similar to the dark phases, by 2.5 mM (Fig. 4A, light 4.1±1.1%, dark 5.0±1.1%). There was no appreciable change in the amount of activity in the center of the well during the dark phases however, the type of activity changed. The percentage of activity (distance) covered by fast movement increased with concentration for both the light and dark phases peaking at 89.2±4.5% of the light and 89.5±3.7% of the dark activity.

Aconitine treated larvae displayed an increase in activity during the dark phase within the center of the well between 5 and 10 μM but resulted in a decrease in larval activity during both the light and dark phases at 20 μM (*t*-test, $p<0.05$, Fig. 4B). The percentage of activity covered by fast movement in the center of the well during the dark phase increased between 5 and 10 μM, peaking at 44.8±5.4% of the total center activity, while the light phase activity showed an increase between 10 and 20 μM, peaking at 35.6±9.4%.

The 4-AP treated larvae showed a concentration-dependent decrease in center activity for both the light and dark phases between 800 μM and 2.5 mM (Fig. 4C). While there was no increase in the total activity in the center of the well at 600 μM the percentage of fast movement in the center increased significantly for the light (47.5±9.1%) and dark phases (44.8±6.6%). Interestingly, although the fraction of fast movement was higher than controls at 2.5 mM, the fraction of activity within the center of the well dropped below 1% of the carrier control.

2.5. *Measuring elevations in neural hyperactivity*

Since the 3 hyperactive compounds produced distinct, concentration dependent, patterns of behaviour, we were interested in how this would correlate with the overall levels of neural activity at each concentration. In order to quantify increased neural activity, changes in the levels of the immediate early gene *c-fos*, which has been shown to become elevated in zebrafish in response to increased neuronal activity (Baraban et al., 2005), were evaluated by qPCR using whole larvae (Fig. 5). Exposure to each compound for 2 h (the length of the behaviour protocol) produced a distinct concentration-dependent elevation in *c-fos*. At 2.5, 5 and 10 mM PTZ elevated *c-fos* levels by 11.3±1, 52.8±1 and 279.7±1 fold, respectively. Aconitine produced a smaller

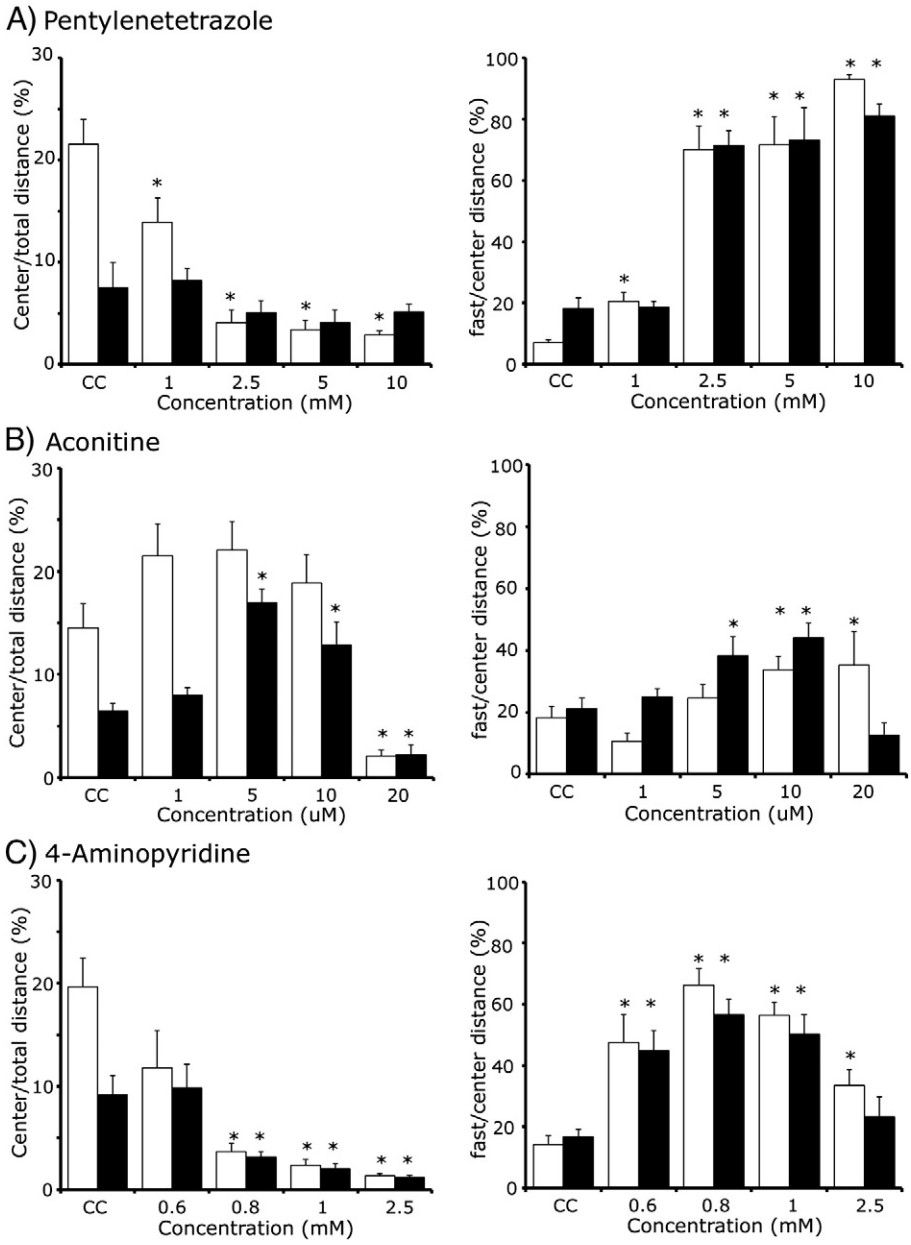


Fig. 4 – Assessing thigmotaxis. The amount of activity (distance traveled) that occurred in the center of each well of a 48-well plate for the first light/dark cycle (L—white bars, D—black bars; 10-minute bin) was assessed for (A) PTZ, (B) aconitine and (C) 4-AP. The activity (distance) within the center of the well was further analyzed by examining the distance covered by larvae during fast movement for both light and dark phases. Activity is presented as the percentage of distance moved ± standard error (n = 18). CC—carrier control (0.1% DMSO). * Denotes significant difference from treatment controls (t-test, $p < 0.05$).

increase in *c-fos* at 2.5, 5 and 10 μM of 3.6 ± 1, 11.9 ± 1 and 19.7 ± 1 fold. 4-AP showed an elevation in *c-fos* of 38.9 ± 1, 130 ± 1 and 222.7 ± 1 fold at 600, 800 μM and 1 mM.

2.6. *Mapping the pattern of induced activity to specific brain regions*

In order to confirm that the increase in *c-fos* measured by qPCR represented an increase in neural activity within the brain we mapped the *c-fos* expression pattern through *in situ* hybridization (ISH). It was found that each compound elevated *c-fos* levels in the brain and each appeared to produce a regionalized expression pattern at concentrations that matched a distinctive phenotype produced by each compound (PTZ-5 mM, aconitine-5 μM, 4-AP-800 μM).

While high levels of PTZ (15 mM) have previously been shown to lead to a global elevation in *c-fos* expression in the brain (Baraban et al., 2005), here we have found that at lower, sub-convulsive, concentrations (5 mM) PTZ leads to a regionalized increase in *c-fos* expression. *c-fos* became elevated in the pallium and subpallial regions of the forebrain, the preoptic area, the rhombencephalon, and the midbrain tegmentum, along with what appeared to be small pockets of cells in the ventral thalamus, posterior tuberculum, and

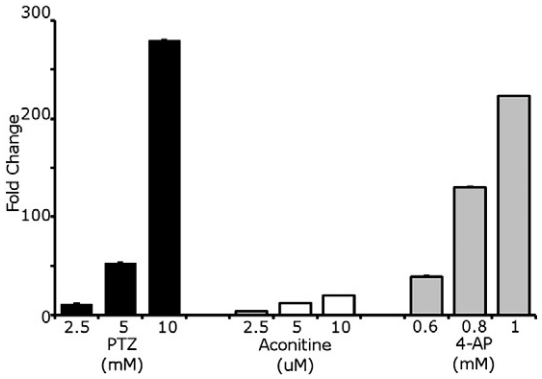


Fig. 5 – Quantitative PCR. Change in *c-fos* level following 2-hour exposure to PTZ (2.5, 5, 10 mM), aconitine (2.5, 5, 10 μM) or 4-AP (0.6, 0.8, 1 mM). Data presented as fold change ± standard error.

hypothalamus (Fig. 6A). Aconitine treatment produced a restricted *c-fos* expression pattern primarily localized to the preoptic area and regions of the forebrain, namely, the olfactory bulb, pallium and subpallium (Fig. 6B). 4-AP treatment (800 μM) displayed an elevation in *c-fos* expression in the preoptic region, posterior tuberculum, tegmentum and the hindbrain. Carrier control treated larvae (0.1% DMSO) showed low-level diffuse *c-fos* staining (Fig. 6D).

While it is difficult to quantify the *c-fos* expression through whole-mount *in situ* hybridization, the expression patterns do reveal apparent similarities and differences between larvae treated with each compound. Further work is required to evaluate the *c-fos* expression patterns in more detail.

3. Discussion

Here we have shown that mechanistically different neuroactive compounds can produce distinct changes in larval behaviour that are both concentration and compound dependent. Using short-duration cycles of light and dark we have confirmed previous work that showed an elevated level of activity during 10-minute dark phases and a drop in activity during light phases (Irons et al., 2010; Staddon et al., 2010). In order to compare and contrast the phenotypes produced by the neuroactive compounds it was important to further define the normal response patterns. During the initial minute following the transition from dark to light there appeared to be an overall drop in activity, however, when the data was evaluated at a closer level (1-second bins) there was actually an initial, short-duration (<5 s), spike in activity. This activity pattern was similar to what has been previously described for dark to light transitions (Emran et al., 2008). It then appears that zebrafish larvae show a complex pattern of normal responses to light/dark transitions: both dark to light and light to dark transitions show an initial spike in activity during the first 5 s, with the dark to light activity declining to below pre-transition levels, while the light to dark activity remains above pre-transition levels. Interestingly the light and dark response patterns are even more complex as the elevated dark activity has been shown to decay and the light activity

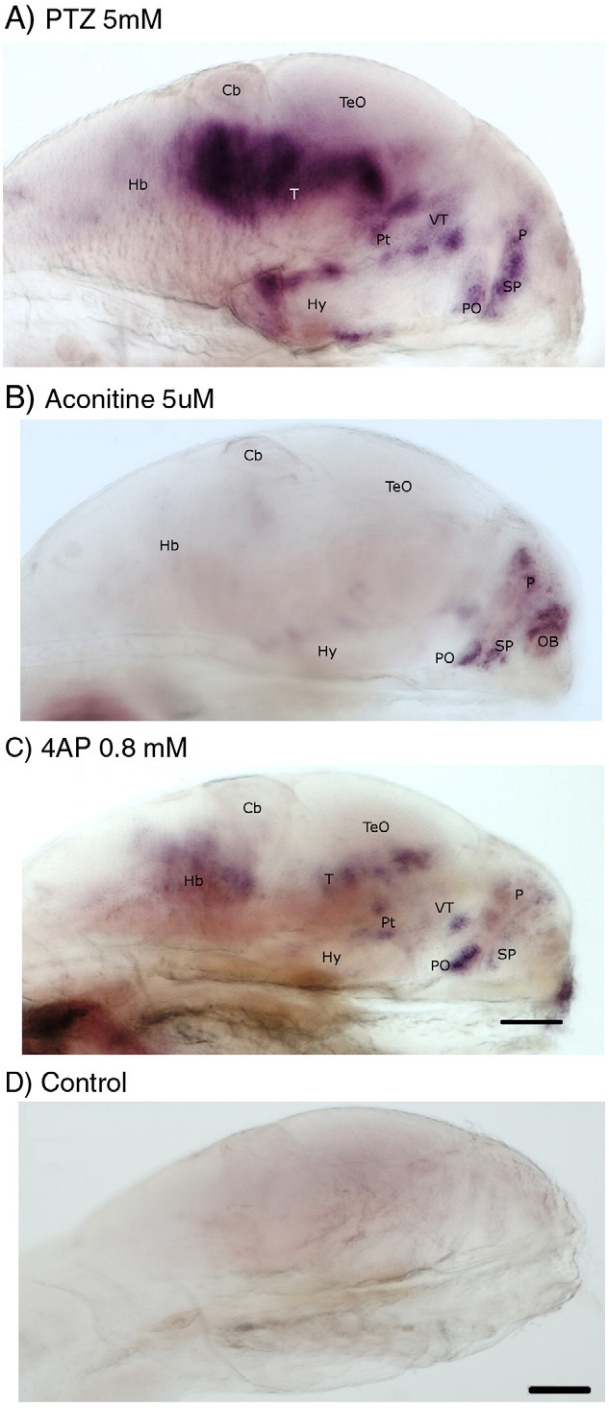


Fig. 6 – *c-fos* expression pattern following treatment with neuroactive compounds. (A) Lateral view of 5 dpf larvae following treatment with 5 mM PTZ showing elevated *c-fos* expression in the pallium (Pa), subpallium (Sp), preoptic area (Po), hypothalamus (Hy), the ventral midbrain below the cerebellum (Cb) along with regions of the ventral thalamus (VT) and posterior tuberculum (PT). (B) Aconitine treated larvae (5 μM) showing elevated *c-fos* expression in the Po, Sp, Pa and olfactory bulb (OB) areas of the forebrain (Fb). (C) 4-AP treated larvae (800 μM) showing elevations in *c-fos* in the Po, Pt , tegmentum (Te) and the hindbrain (Hb). Scale bar = 1 mm.

increases during normal circadian activity (Prober et al., 2006). These normal circadian patterns of behaviour result in the light activity eventually rising above the activity produced during the dark periods. This highlights the complexity of normal light/dark response patterns in zebrafish.

One unique aspect of this study is the assessment of each hyperactive compound across a broad concentration range. The $GABA_A$ antagonist PTZ has previously been used to model both anxiety and seizures in mammals, including humans, (Jung et al., 2002; Loscher, 2009; Snyder-Keller and Keller, 2001) and zebrafish (Baraban et al., 2005; Berghmans et al., 2007; Goldsmith et al., 2007; Winter et al., 2008; Wong et al., 2010). While anxiety is generated by a lower concentration of PTZ compared to the seizure models in mammals, the zebrafish studies have primarily made use of high concentrations of PTZ to model seizure like activity. Here we have confirmed previous work that has used PTZ as a seizure model (Berghmans et al., 2007; Hortopan et al., 2010; Kim et al., 2010; Wong et al., 2010) by showing that high levels of PTZ produced bursts of rapid circling and darting followed by inactivity that was associated with lateral recumbency and/or tremors. Importantly, we have now also shown that at lower levels (1–5 mM) the PTZ induced activity pattern is distinct from the response observed at higher concentrations (above 10 mM). As the concentration of PTZ is increased from "no observable effect levels", two interesting behavioural changes occur. The first is an elevated spike in activity during the first 60 s following the transition from dark to light and the second is a reversal of the normal light/dark activity levels (Figs. 1–3).

The exact nature of the normal initial response to light and dark changes has not been fully defined. However, it has recently been demonstrated that 1-week-old larvae display dark avoidance that can be altered by anxiolytic/anxiogenic compounds (Steenbergen et al., 2011) and it has been suggested that changes in the typical response to light and dark may be a component of a stress response (scototaxis, (Maximino and Herculano, 2010; Maximino et al., 2010a, 2010b, 2010c, 2010d). The initial spike in activity following a transition that is elevated by low levels of PTZ may then represent an elevated startle/stress response. During the assessment of location preference we found that although the activity level during the light phase was elevated by low levels of PTZ, the amount of time spent in the central area of the well was reduced and the center activity changed from slow scanning to rapid darting. The wall-hugging response could be considered a measure of thigmotaxis, which is also a characteristic of stress in the larval system (Berghmans et al., 2007; Maximino et al., 2010b, 2010c; Steenbergen et al., 2010). In support of the idea that low concentrations of PTZ elicit a stress response, the preliminary mapping studies have suggested that PTZ elevates the levels of *c-fos* in the preoptic area and hypothalamus, two components of the hypothalamic-pituitary-interrenal (HPI) stress-response axis (Fuzzen et al., 2010). If these potential measures of a stress response could be modified by anxiolytic compounds this would help to strengthen the hypothesis that the low-concentration PTZ model displays an elevation in anxiety and/or stress.

As the level of PTZ was increased, in addition to the spike in activity following the transitions, a complete reversal in the normal response to light or dark occurred. This activity pattern was not seen following either aconitine or 4-AP exposure at any concentration tested (data not shown). Preliminary evidence suggests that these levels of PTZ may also alter normal circadian activity (data not shown), however, the toxicity and decrease in activity associated with long-term PTZ exposure has made the analysis of this data difficult. Interestingly, one of the criteria for a diagnosis of bipolar disorder in humans is altered circadian activity (Jones, 2001) and it has been suggested that activation of the mammalian homologue of the HPI axis (HP-Adrenal axis) is involved in the switch from mania to depression in bipolar patients (Salvadore et al., 2010). A number of recent studies have shown that adult zebrafish can model complex human neurological and psychological disorders such as schizophrenia (Morris, 2009; Seibt et al., 2010; Zakhary et al., 2011), anxiety/stress (Champagne et al., 2010; Maximino et al., 2010b, 2010c; Steenbergen et al., 2010; Stewart et al., 2010), addiction (Mathur and Guo, 2010), and learning deficits (Lee et al., 2010). Taken together the increase in stress/anxiety levels paired with the reversal of the normal light/dark response pattern may suggest that activation of the zebrafish HPI axis produces a phenotype that may mirror some of the symptoms of bipolar disorder. While it is far too early to suggest direct links between aberrant behaviour in larvae and complex psychological disorders, developing models of this nature may help to discern how the activation of distinct brain regions in zebrafish can produce symptoms similar to those seen in human disorders and how this activity may be targeted by potential therapeutics. Work is currently underway to characterize both the molecular and behavioural effect of known therapeutics against the low-concentration PTZ model.

Similar to PTZ, 4-AP altered the response to light and dark transitions and decreased the time spent in the center of the well (scototaxis and thigmotaxis) indicating a stress response. Both compounds produced a spike in activity following a dark to light transition, however, during a light to dark transition 4-AP showed a rapid-sustained drop in activity. It then appears that PTZ and 4-AP may produce distinct stress-response patterns, with PTZ treated larvae producing a darting type of stress response, while 4-AP appears to produce more of a freeze-type response. This may indicate that zebrafish stress-response patterns are complex, even in larvae, and since PTZ and 4-AP produce distinct patterns of *c-fos* activation, these responses may be controlled by distinct areas of the larval brain. As noted, PTZ has previously been shown to produce anxiety in humans, and one of the side effects of 4-AP overdose is also anxiety (Schwam, 2009).

Not only did 4-AP treatment of larvae produce behaviours reminiscent of a stress response, but at higher concentrations 4-AP treated larvae also displayed slow continuous circling activity. Since one of the major areas of *c-fos* expression induced by 4-AP was in the reticulospinal neuron regions of the hindbrain and these neurons contain Kv1 potassium channels (Nakayama and Oda, 2004) that can be targeted by 4-AP, this response may be the result of a sustained level of activity in these neurons. Additionally, 4-AP has been shown to broaden the motor neuron action potential and lead to continuous firing (Buss and Drapeau, 2001), which may also contribute to the behavioural profile. One may then predict that the anxiety/stress component of the 4-AP induced activity found at low to

moderate concentrations may be produced by the activation of the preoptic area and midbrain, while the subsequent overall elevation in activity found at higher concentrations may be the result of increased activity in the hindbrain.

Aconitine produced an overall concentration-dependent elevation in activity that was comprised of a larger fraction of slow activity, with a lower level of fast-darting activity than was seen for PTZ or 4-AP. The light/dark response appeared to be almost eliminated and the fraction of time spent in the center of the well did not decrease until there was a major drop in the overall activity levels. This suggests that aconitine does not lead to scototaxic or thigmotaxic behaviours. Interestingly, aconitine showed a regionally restricted *c-fos* expression pattern, with an increase in *c-fos* in the preoptic area and forebrain. Elevated activity appeared regionalized to the olfactory bulb, caudal pallium and subpallium. The pallium has been proposed to be analogous to the amygdala and hypothalamus of mammals (Salas et al., 2006), regions involved in learning and memory. Further analysis of alterations in normal behaviour that are produced by aconitine may determine if aconitine has an effect on zebrafish learning and help to discern if the pallial regions are involved in such events.

When comparing the induced activity and *c-fos* expression patterns, PTZ, 4-AP and aconitine all showed a prominent elevation of *c-fos* in the preoptic area. As mentioned the preoptic area forms the initiation point of the HPI stress-response axis. While PTZ and 4-AP both appear to produce anxiety/stress type behaviours, aconitine also activates the preoptic area but does not appear to produce a similar type of anxiety/stress response. It would then appear that downstream activity must also be required to generate and/or mould the response pattern. Further work will be required to elucidate the cell types within the preoptic area that are activated by each hyperactive model and the downstream areas of the brain that are responsible for the various components of the complex response patterns.

Our study has demonstrated that chemically induced behaviour in zebrafish larvae can produce complex phenotypes. While more work is required to further define the regionalized *c-fos* expression patterns, by comparing similarities and differences in both the behaviour and *c-fos* expression patterns generated by these three mechanistically distinct neuroactive compounds, we have shown that it may be possible to link components of behaviour with regionalized neural activity in the larval system. Recapitulating a particular disease state with a single compound is idealistic, but perhaps further comparisons of the phenotypes generated by multiple models of hyperactivity with the symptoms of disease states may help unravel disease etiology and possibly provide a novel platform to evaluate potential therapeutics.

4. Experimental procedures

4.1. Animals

Zebrafish (*Danio rerio*) were maintained according to standard animal care protocols (Westerfield, 1995) and in accordance with the Canadian Council on Animal Care guidelines. AB/Tubingen adults, embryos and larvae were maintained on recirculating water at 28 °C+/−1 °C, pH 7.0–7.5 on a 14/10-hour light/dark cycle using a high density rack system (Tecniplast and Aquatic habitats). Embryos were collected from multiple AB/Tubingen breeding pairs and pooled. Following 4–6 h in an incubator in E3 media (5 mM NaCl, 0.17 mM KCL, 0.33 mM $CaCl_2 \cdot 2H_2O$, 0.33 mM $MgSO_4$) unfertilized embryos were removed and the remainder placed in a mesh-bottom baby basket in a larger flow through tank. This allowed the embryos to be maintained under consistent water conditions and with the same light/dark cycling as the adults. Raising embryos using the basket system resulted in more consistent activity patterns than rearing embryos in Petri dishes in an incubator.

4.2. Video tracking

Larvae were loaded into multi-well plates between 100 and 106 hpf (hours post fertilization), 1 fish/well with either 270 μL (96 well) or 450 μL (48 well) HEPES buffered E3 (pH 7.2) and acclimated at 28 °C overnight. By 120 hpf zebrafish larvae no longer rely on the yolk sac for sustenance and must become active to begin to search for food. As such, larval behaviour is elevated and more consistent than at previous stages of development. Larvae were treated between 120 and 128 hpf with neuroactive compounds by spiking each well with a 10× concentration of the specified treatment (30 μL for 96 well plates and 50 μL for 48 well plates). The plates were placed immediately into the Zebrabox plate holder comprised of a recirculating water bath used to heat the plates, maintaining the plate temperature at 28 °C. Larval activity was immediately tracked using the Viewpoint video tracking system and software (Viewpoint Life Sciences Inc., Montreal, Quebec, Canada). All experiments consisted of 30 min of acclimation in the dark followed by 4–20 min cycles containing a light and dark phase (10 min each—110 min total, see (Irons et al., 2010)). Images were scanned at a rate of 40 fps and binned into 1 or 60-second windows. To dissect out the different types of activity a high threshold was set at a level that was rarely crossed by controls (20 mm/s) accounting for, on average, less that 5% of control activity. A low threshold was also set in order to eliminate activity that did not contribute to the behavioural phenotype, such as small changes in orientation. Based on the profiles of the control animals this “inactive” activity accounted for ~20% of the total activity, leaving the remaining ~75% in the “slow” category. For the 48 well experiments the activity within the center of the well is defined as the distance traveled in the central area of the well and is presented as a percentage of the total activity for the entire well.

4.3. Chemical modulation of activity

Unless otherwise stated, all drugs and toxins were purchased from Sigma-Aldrich. Initially the larval responses to the three hyperactive compounds, PTZ, aconitine and 4-AP were assessed across a wide concentration range (1/4 log dilutions) in order to assess toxicity and to establish a biologically relevant range to use during experiments. The biologically relevant range defined concentrations that resulted in the first significant change in the normal pattern of activity to a high concentration that produced a significant decay in activity between the 1st and 4th light/dark cycles (*t*-test; data pooled for entire phase). Video tracking experiments were performed

with 96 well plates. Each row of 12 larvae was exposed to a single concentration of a compound and a carrier control group on each plate. Experiments were performed on three separate days with larvae from different clutches. There was no significant difference between experiments run on separate days (ANOVA, $p<0.05$). The data was subsequently pooled for further analysis (n=36). Following automated video tracking larvae were visually assessed for seizure like activity and toxicity.

4.4. *Quantitative PCR*

Larvae were loaded into a 24 well plate, 20 larvae/well with 900 μL of HEPES buffered E3 media at 100 to 106 hpf and placed in an incubator at 28 °C overnight. Each well was spiked with 100 μL of 10× drug stock at 120 hpf and placed in an incubator at 28 °C for 2 h which corresponds to the end of the behavioural tracking experiment. Larvae were rapidly chilled on ice until there was no longer a heartbeat, the media was removed and they were frozen on dry ice in a 1.5 mL tube and tissues were stored at −80 °C until required. RNA was extracted from larvae taken from duplicate wells on two separate days (n=4 groups of 20) with a Noragen RNA purification kit (Noragen Biotek). cDNA was synthesized from 1 μg RNA with the Superscript III ® first strand SuperMix for qPCR from Invitrogen. qPCR was performed on the Roche LightCycler 480 system with Kappa SYBR fast qPCR master mix (KAPABiosciences). Each qPCR reaction was performed in triplicate to measure *c-fos* against EF1α and β-Actin reference genes. Primers were as follows: *c-fos* F—5′GTGCAGCACGGCTTCACCGA 3′, R—5′ TTGAGCTGCGCCGTTGGAGG 3′, EF1α F—5′ GATGCACCACGAGTCTCTGA 3′, R—5′ CGGTCGATCTTCTCCTTGAG 3′, β-Actin F—5′ CCGTTGCCCCGAGGCTCTCT 3′, R—5′ CGCATCCTGAGTCAATGCGCCA 3′.

4.5. *In situ hybridization*

All *in situ* experiments were performed in duplicate with larvae from different clutches. Larvae were loaded into a 12 well plate, 10–15 larvae/well with 3 mL of HEPES buffered E3 media at 100 to 106 hpf and placed in an incubator at 28 °C overnight. Each well was spiked with the appropriate volume of drug stock at 120 hpf and placed in an incubator at 28 °C for 30 min which corresponds to the timing of the first light/dark cycle of the behavioural tracking experiments. Whole-mount *in situ* hybridizations for *c-fos* were performed on 6–10 larvae from 2 separate treatment groups (minimum n=10) according to established an protocol (Thisse and Thisse, 2008), with the following modifications: Hydrogen peroxide pigment removal was performed for 2 h after methanol storage and progressive rehydration, fresh peroxide was added after 1 h. Proteinase K digestion was for 10 min at room temperature. Post-hybridization washes were performed at 70 °C as follows: 2×30 min in 50% formamide/2× SSC, 1×15 min 2× SSC, 2×30 min 0.2× SSC. Embryos were transferred to tissue baskets in 48-well plates and washed 3×5 min in maleic acid buffer (MABT) (100 mM maleic acid, 150 mM NaCl, 0.1 M Tris pH 9.5, 0.1% Tween-20, pH adjusted to 7.5 with NaOH) and blocked for 2 h in blocking buffer (MABT with 10% FBS, 400 μg/mL BSA). Anti-Digoxygenin-AP antibody (Roche #11 093 274 910) was diluted 1/15,000 and allowed to bind overnight at 4 °C. Embryos were washed at room temperature 1×30 min in blocking buffer, 2×30 min in MABT and 4×5min in alkaline Tris buffer. NBT-BCIP color development was between 1 and 2.5 h. The template for the *c-fos* riboprobe was created by cloning a 1500 bp region, amplified with the following primers: F-GACAGGATGATGTTACCAGCCTTAACG; R-GGCTATGGAAGTGACTCTAAAGCAATGC.

4.6. *Data analysis*

Data was analyzed using GraphPad Prism software. Replicate 96 well dilution series experiments were run on three separate days (n=12/concentration/experiment). Larval activity (distance traveled) was pooled into 60 second bins and a one-way ANOVA was used to test for variability between the 3 trials (95% confidence level). No significant differences between trials was found, allowing for the data from the replicates to be pooled for subsequent analysis (n=36/treatment). Each 60-second time point was initially compared to controls (*t*-test, $p<0.05$) followed by a subsequent analysis of the data as 10-minute bins for the thigmotaxis assays and compared to controls (*t*-test, $p<0.05$). In order to evaluate activity decay, which may indicate toxicity or a phenotypic seizure like state (lateral recumbency and tremors), each concentration was assessed by comparing either the 4 light or the 4 dark cycles as 10-minute windows (ANOVA, 95% confidence level). The thigmotaxis experiments were performed in 48 well plates and evaluated the activity during the first light and dark cycle compared to controls for the combined duplicate trials (n=24, *t*-test, $p<0.05$). For the transition analysis each 5-second window following the transition was compared to the 60 s immediately prior to the transition (ANOVA, 95% confidence level, Tukey's post-hoc analysis). There was no significant difference between control activities of each treatment group when compared by a one-way ANOVA (95% confidence level).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.brainres.2012.02.022.

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