Developing a Zebrafish Model of Autism:

Valproic Acid and the Mediating Effects of KCC2

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Introduction

In developmental neurobiology, researchers seek to examine the importance of small, short-term events during fetal development, be they exposures to teratogens or other stressors that could potentially have impacts on the future development of an organism. When these sorts of events occur during critical periods in development, they can have profound long-term effects on the outcome of the organism.

Much research has sought to examine the relationship between over-excitation in the developing brain and its subsequent effect on social behavior in children. Over-excitation can occur as a result of maternal use of antiepileptics, or due to other abnormal prenatal events (Ben-Ari, 2014). Studies investigating this phenomenon have consistently found that too much excitation in a developing brain results in deficits in social behavior (Wood et al., 2015; Tyzio et al., 2014; Silvestrin et al., 2013). Subsequently, researchers have tried to explore the relationships between the physical mechanisms within the developing brain, and how these processes can be mediated or changed to produce more normal development (Jantzie et al., 2014; Tang et al., 2016). The most important neurotransmitter that has been implicated in all of the literature on this topic is GABA.

The GABA Switch

Gamma aminobutyric acid (GABA) is one of the most important neurotransmitters in the brain, and is often referred to casually as the primary inhibitory neurotransmitter. Along with its excitatory counterpart, glutamate, GABA helps to regulate cellular firing by inhibiting neural

networks, hyperpolarizing neurons and decreasing the likelihood of them sending a signal (Krnjevic and Phillis, 1963).

However, GABA has different effects in the brain of a developing fetus. Rather than serving as an inhibitory neurotransmitter, it actually produces excitatory effects (Obata, 1978; Ben-Ari, 2002). The purpose for this is not well-understood, but it is a phenomenon that can be demonstrated across species as a conserved mechanism of development. Around the time of birth, a surge of oxytocin released by the mother effects the "GABA switch" in which GABA's role changes from being excitatory to inhibitory (Tyzio et al., 2006).

In much of developmental biology, timing is crucial. The GABA switch must occur during a defined "critical period" in order for normal development to proceed (Kim et al., 2011). It sometimes happens that the GABA switch is delayed by some sort of prenatal teratogen or genetic malfunction, producing abnormalities in social behavior characteristic of autism spectrum disorders (Wood et al., 2015).

Why is GABA excitatory during development?

To better understand the excitatory and inhibitory actions of neurotransmitters in the brain, it is important first to understand the physical relationships between cells, and how chemical signals are transmitted between them. The two neurotransmitters glutamate and GABA are not inherently excitatory or inhibitory--they simply allow for the opening of different types of channels in brain cells when they bind to receptors (Ben-Ari, 2014).

The charge of a resting neuron is -70 mV, meaning that it is negatively charged relative to the surrounding environment. When glutamate binds to a receptor on a postsynaptic neuron, it

opens sodium channels in the brain. Since sodium is a positively-charged ion in the human body, and since there is a lower concentration of sodium ions inside a neuron, this opening causes sodium to flood the cell, producing a depolarization and subsequent firing of an action potential.

GABA, on the other hand, controls a chloride channel. In an adult brain, there is a lower concentration of chloride inside the cell relative to its outside environment, so the opening of a chloride channel results in an influx of negatively-charged chloride. This hyperpolarizes the cell, decreasing its likelihood of firing and thereby inhibiting the transmission of neural messages.

In a developing fetus, however, there is a much higher concentration of intracellular chloride than in an adult brain (Ben-Ari, 2002; Cherubini et al., 1991). Consequently, when GABA binds to a receptor and opens a chloride channel, the result is an efflux of negatively-charged chloride from the cell, resulting in a depolarization rather than a hyperpolarization. In this manner, GABA has excitatory effects in the developing brain rather than inhibitory.

What mechanisms create this differential environment?

A good question to ask is, “what causes the elevated levels of intracellular Cl- during development?”

Cells are always hard at work to maintain their own natural resting balance. Neurons maintain their concentrations of ions through the use of co-transporter proteins embedded in the surfaces of their cellular membranes. These co-transporters use ATP to “pump” certain ions in or out of a cell depending upon their concentrations at any given moment.

Two co-transporters, NKCC1 and KCC2, regulate the levels of chloride in a cell (Blaesse et al., 2009). NKCC1 works to pump chloride into the cell, while KCC2 exports chloride out of the cell. The relative numbers of chloride importers compared to chloride exporters is crucial to the concentration of chloride in the cell.

Since these co-transporters are composed of proteins, they are built in the cells by ribosomes and coded for by genes. The genes that produce these two proteins have been uncovered and named nkcc1 and kcc2--so it is logical that affecting these genes can affect the levels of intracellular chloride and, ultimately, the effects of GABA in the brain (Zhang et al., 2013).

Animal models

Researchers have been curious about the effects of a delayed GABA switch for quite some time, and have performed many different experiments to investigate its implications. In order to examine this phenomenon, many researchers used animal models such as rats and mice, and antiepileptic drugs such as valproic acid in order to prolong the excitatory effects of GABA in the brain and see what the results are (Bambini-Junior et al., 2011; Cohen et al., 2013; DuFour-Rainfray et al., 2010; Silvestrin et al., 2013).

Many studies sought to simply uncover the "critical period" during which the GABA switch normally occurs in the brains of rats (Kim et al., 2010; Levav-Rabkin et al., 2010). Several pointed to specific days during embryonic development, and over the course of many studies, it was possible to develop a fairly reliable window of time in which valproic acid should

be administered in order to produce the characteristic social deficits associated with a delayed GABA switch.

Other studies have used genetic lines of animals that will express social deficits, such as Fragile X or Rett's Syndrome and tried to rescue normal social functions through the administration of oxytocin or bumetanide (He et al., 2014; Lemonnier et al., 2012;Tang et al., 2015). Although the results were not overwhelmingly effective, there has been some success with these treatments in alleviating the autistic symptoms that would typically have resulted from these genotypes.

Importance of the Proposed Research

At present, no model exists of a zebrafish GABA switch and its effects on social behavior. Over the past ten years or so, zebrafish (*danio rerio*) have been on the rise as a preferred research model. This is because the species confer several advantages for researchers.

First, zebrafish provide a simple system while still being able to serve as homologs for humans, thanks to the fact that they're vertebrates. Unlike other simple models like *C. Elegans*, zebrafish can more accurately inform us of how activities would take place in a human brain

Second, their physical characteristics make them convenient to study. Zebrafish larvae are transparent, allowing us to view their nervous systems *in vivo* through a microscope without disrupting them in an invasive way. Furthermore, their rapid rate of reproduction allows for researchers to raise large clutches of fish in a short period of time.

Finally, a crucial advantage to using zebrafish is that the entire zebrafish genome has been sequenced. As more neuroscience labs have begun adopting zebrafish as a model organism

for study, labs across the world have worked to produce genetically-modified lines of zebrafish with fluorescence labeling or other modifications to suit target areas of research. In particular, the zebrafish lab at Humboldt State University houses a line of GCaMP zebrafish, which have been modified to express a green fluorescent protein in the presence of calcium ions (Ca2+). Since Ca2+ is produced each time a cell fires an action potential, this modification allows us to track the activity of neural communication by evaluating brightness of the fluorescence and, by proxy, the amount of activity occurring at a given place and time in the brain.

It would be useful to develop a zebrafish model of autism similar to the ones that have been developed using rats and mice, in order to provide a basis for further research into the mechanisms underlying the GABA switch and its implications for later social development.

Primary Hypotheses/Research questions

In order to begin establishing this model, a few important questions must be asked. First, a model of "normal" social behavior in zebrafish must be established in order to assess the degree of deficit in the fish. Several researchers at the University of Oregon in Eugene have been working to develop robust descriptions of normal zebrafish social behavior, using software to track movements, and observing schooling behavior. In rat models, several different tests have been used, mostly to measure the time rats prefer to spend in groups, as well as other distinct characteristics such as the degree to which pups call for their mothers (Reynolds et al., 2012). I plan to use information from the researchers who study zebrafish behavior in order to have a scale by which to judge my own subjects.

A second challenge is determining the timing of the "normal" GABA switch in zebrafish, in order to alter it from its course. Since zebrafish develop much more rapidly than rats, it is not useful to compare them. Luckily, research into the switch has pointed to 2.5 to 3 days post fertilization (dpf) as the critical period for the GABA switch in zebrafish (Zhang et al., 2013). Administration of valproic acid at that time should induce the same prolongation of GABA's excitatory effects as it does in rat models.

My research design will include three conditions--one control condition, one group of fish that will be exposed to valproate during the critical period, and a third group that will be genetically altered to knockdown the expression of the kcc2 gene, which encodes the chloride exporter.

I plan to observe all three conditions shortly after the time of the "normal" switch, using a microscope to track the Ca2+ expression. I then plan to allow the fish to mature to adulthood, and then observe their social behavior in order to determine whether they show any deficits.

I hypothesize that the groups of fish exposed to valproate and those with knocked-out KCC2 genes will both show a significantly higher level of Ca2+ expression compared to the control group. I also hypothesize that these two groups will demonstrate abnormal social behavior in adulthood when compared to the control group.

Rationale

While many studies of the GABA switch with rats have exclusively used valproic acid to induce behavioral abnormalities, many researchers have been more curious about how valproic acid works to have its effects. Studies have suggested that valproic acid actually serves as a

histone deacetylase inhibitor, which prevents the expression of several specific genes--including the kcc2 gene (Kostrouchova & Kostrouch, 2007). In this manner, the effects of the KCC2 exporter seem to be more of the driving force between the GABA switch, rather than valproate having a direct effect.

Rather than using solely valproic acid to complete my experiment or solely using knockdown of the kcc2 gene, it would be more interesting and informative to include both conditions. If the kcc2 gene truly is the mediating factor between valproic acid and the GABA switch, we ought to see very similar results in both these groups of fish.

Moreover, a difference in these two groups could suggest several different directions for future research. For example, if the group exposed to the valproate failed to show the same over-excitation or social deficits as the knockdown group, it may indicate that valproate should be administered at a different time period in order to have the expected effects.

No matter the nature of the results, this study would provide a first exploration into using zebrafish as a model organism for research into the GABA switch and autism. Further studies could use its flaws or successes as a jumping point for new questions or directions.

Summary

While the GABA switch has been shown as a reliable phenomenon in rat and mice models, it has not yet been documented in zebrafish. Since this organism is on the rise as a preferred model organism, it would be useful to have a baseline model of this action to use for further investigation into this critical period's effect on social behavior.

Since past research has used both drug-induced methods with valproic acid and genetic examinations, it would be useful to employ both of these techniques in a baseline study in order to compare the results. Furthermore, this collaboration with researchers who are examining social behavior of zebrafish could facilitate the development of a reliable model for “normal” social behavior in the fish. This could be useful for a number of different types of studies, apart from those simply examining autism.

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