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Multidimensional chemobehavior analysis of flavonoids and neuroactive compounds in zebrafish

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ABSTRACT

The comparative analysis of complex behavioral phenotypes is valuable as a reductionist tool for both drug discovery and defining chemical bioactivity. Flavonoids are a diverse class of chemicals that elicit robust neuroactive and hormonal actions, though bioactivity information is limited for many, particularly for neurobehavioral endpoints. Here, we used a zebrafish larval chemomotor response (LCR) bioassay to comparatively evaluate a suite of 24 flavonoids, and in addition a panel of 30 model neuroactive compounds representing diverse modes of action (e.g. caffeine, chlorpyrifos, methamphetamine, nicotine, picrotoxin). Naïve larval zebrafish were exposed to concentration ranges of each compound at 120 hour post-fertilization (hpf) and locomotor activity measured for 5 h. The model neuroactive compounds were largely behaviorally bioactive (20 of 30) with most effects phenotypic of their known modes of action. Flavonoids rapidly and broadly elicited hyperactive locomotor effects (22 of 24). Multidimensional analyses compared responses over time and identified three distinct bioactive groups of flavonoids based on efficacy and potency. Using GABAergics to modulate hyperactive responses, two flavonoids, (*S*)-equol and kaempferol were tested for $GABA_A$ receptor antagonism, as well as a known $GABA_A$ receptor antagonist, picrotoxin. Pharmacological intervention with positive allosteric modulators of the $GABA_A$ receptor, alfaxalone and chlormethiazole, ameliorated the hyperactive response to picrotoxin, but not for (*S*)-equol or kaempferol. Taken together, these studies demonstrate that flavonoids are differentially bioactive and that the chemobehavioral effects likely do not involve a $GABA_A$ receptor mediated mode of action. Overall, the integrative zebrafish platform provides a useful framework for comparatively evaluating high-content chemobehavioral data for sets of structurally- and mechanistically-related flavonoids and neuroactive compounds.

1. Introduction

Flavonoids are a large class of structurally diverse compounds that exhibit highly bioactive and structure-dependent properties (Fig. 1). A number of naturally occurring food sources contain flavonoids (i.e. fruits, vegetables), though relatively high levels are found in processed soy-based products and over-the-counter dietary supplements. As a result, flavonoids are commonly studied for their widely varied and potentially beneficial nutraceutical properties, including anti-oxidant, anti-cancer, anti-inflammatory, and hormonal bioactivities (reviewed by: Nijveldt et al., 2001; Cornwell et al., 2004; Kumar and Pandey, 2013). Due to the ubiquity of these compounds in the diets of certain populations, flavonoids are frequently detected in biological fluids, such as urine (Valentin-Blasini et al., 2003; Valentin-Blasini et al., 2005) and blood plasma (Peeters et al., 2007). Several bioactive flavonoids are also detected in amniotic fluid and umbilical cord blood (Adlercreutz et al., 1999; Foster et al., 2002; Todaka et al., 2005; Mustafa et al., 2007), as well as breast milk (Choi et al., 2002; Song et al., 2013). Relatively high levels are also detected in soy-based baby formulas (Setchell et al., 1997; Setchell et al., 1998; Choi et al., 2002; Song et al., 2013), thus raising concerns regarding adverse developmental effects from the potent neuroactive and hormonal bioactive properties, though potential consequences of such exposures remain unclear. Taken together, there is a need to comparatively evaluate and comprehensively define the multifaceted bioactive effects of flavonoids.

In higher vertebrates, diverse types of neurobehavioral effects have been reported for select flavonoids, including modulation of anxiety (Lund and Lephart, 2001), depression (Wang et al., 2010), fear (Garey

Abbreviations: GABA, gamma-aminobutyric acid; HCA, hierarchical clustering analysis; hpf, h post-fertilization; LCR, larval chemomotor response; LEL, lowest effect level; PCA, principle component analysis

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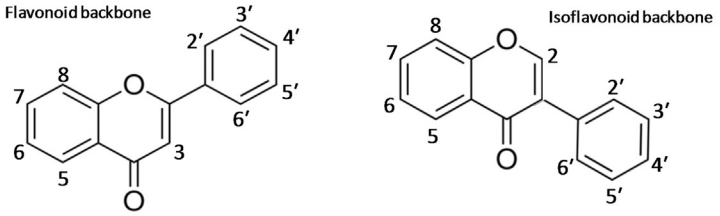

	R3	R5	R6	R7	R8	R2′	R3′	R4′	R5′	Class	Substitution
Flavone										Flavonoid	none
3′-hydroxyflavone							-OH			Flavonoid	mono-OH
6-hydroxyflavone			-OH							Flavonoid	mono-OH
7-hydroxyflavone				-OH						Flavonoid	mono-OH
Chrysin		-OH		-OH						Flavonoid	di-OH
Apigenin		-OH		-OH				-OH		Flavonoid	tri-OH
Galangin	-OH	-OH		-OH						Flavonoid	tri-OH
Naringenin		-OH		-OH				-OH		Flavonoid	tri-OH
Kaempferol	-OH	-OH		-OH				-OH		Flavonoid	tetra-OH
Fisetin	-OH			-OH				-OH	-OH	Flavonoid	tetra-OH
Luteolin		-OH		-OH			-OH	-OH		Flavonoid	tetra-OH
Morin	-OH	-OH		-OH		-OH		-OH		Flavonoid	penta-OH
Quercetin	-OH	-OH		-OH			-OH	-OH		Flavonoid	penta-OH
Quercetin 3-β-D glucoside	$-OC_6H_{12}O_5$	-OH		-OH			-OH	-OH		Flavonoid	tetra-OH, -glucoside
Myricetin	-OH	-OH		-OH			-OH	-OH	-OH	Flavonoid	hexa-OH
α-Naphthoflavone				Fused	Benzene					Flavonoid	*ortho* -benzene
β-Naphthoflavone		Fused	Benzene							Flavonoid	*ortho* -benzene
Daidzein				-OH				-OH		Isoflavonoid	di-OH
Puerarin				-OH	$-C_6H_{12}O_5$			-OH		Isoflavonoid	di-OH, -glucoside
(S)-Equol				-OH				-OH		Isoflavandiol	di-OH
Formononetin				-OH				$-OCH_3$		Isoflavonoid	mono-OH, -OMe
Genistein		-OH		-OH				-OH		Isoflavonoid	tri-OH
Biochanin A		-OH		-OH				$-OCH_3$		Isoflavonoid	di-OH, -OMe

Fig. 1. Backbones and substituent groups for all tested flavonoids and isoflavonoids. Also tested but not shown is resveratrol, a flavonoid-like stilbenoid.

et al., 2001), learning (Kohara et al., 2014), and seizure-like activity (Medina et al., 1990; Avallone et al., 2000). For some flavonoids (e.g. phytoestrogens), the behavioral effects in adults may in part be the result of hormonal action affecting endocrine pathways throughout the HPG axis and effects on reproductive activity (Lephart et al., 2002; Ball et al., 2010). However, many of the widely varying and complex behavioral effects of flavonoids can be attributed to interactions with a variety of important neuroreceptors throughout the nervous system. Neurotransmitter signaling and neuroreceptor function can be directly modulated by flavonoids in vitro, including acetylcholinesterase (reviewed by: Uriarte-Pueyo and Calvo, 2011), adenosine receptors (Moro et al., 1998), glycine receptors (Huang and Dillon, 2000), opioid receptors (Katavic et al., 2007), serotonin and dopamine receptors (Katavic et al., 2007), and nicotinic acetylcholine receptors (Lee et al., 2011). Perhaps most well understood are the mixed interactions of several flavonoids with $GABA_A$ receptors in vitro, particularly as antagonists (reviewed by: Wang et al., 2010; Hanrahan et al., 2011). Previously, we demonstrated that developmental exposure to several flavonoids elicits seizure-like spasms and hyperactive behaviors (spastic pectoral fin movement and body twitching), though the etiology of this neurodevelopmental effect is unclear (Bugel et al., 2016). For the vast majority of flavonoids, in vivo bioactivity information for integrated apical endpoints, such as behavior, is limited.

The zebrafish has many advantages that provide a useful framework to comparatively investigate chemical bioactivity in vivo (reviewed by: Bugel et al., 2014; Caro et al., 2016). The zebrafish genome also shares 70% homology with the human genome, which is valuable for translational medicine, drug discovery, and large-scale chemical screens (Howe et al., 2013). The development of zebrafish-based chemobehavioral assays have shown potential for improving our understanding of how chemicals affect neurobehavior in vivo, and has shown considerable promise for translating discoveries to clinical applications (reviewed by: Kokel and Peterson, 2008; Bruni et al., 2014). Low-complexity chemobehavioral bioassays in zebrafish are also highly amenable to high-throughput interrogation of large sets of compounds (Kokel et al., 2010). These reductionist approaches have led to powerful applications in drug discovery for profiling previously unknown compounds for neuroactive modes of action that could lead to either undesirable toxic side effects or potentially beneficial applications as antipsychotic (Ellis and Soanes, 2012; Bruni et al., 2016) and anti-seizure treatments (Baraban et al., 2005; Ellis et al., 2012). Overall, the zebrafish provides a powerful platform that permits the comparative evaluation of chemobehavior for structurally-related and mechanistically-related compounds.

Here, we present a comparative evaluation of locomotor behavioral responses for a diverse group of neuroactive compounds in zebrafish using a low-complexity larval chemomotor response (LCR) bioassay. We first tested 30 model neuroactive chemicals as proof-of-principle to validate the capacity of the bioassay to detect behavioral responses for known neuroactive substances. This panel included 14 drugs and toxicants representing a variety of known modes of action, and 16 GABAergic drugs (Table 1). Second, we evaluated the chemobehavioral responses of 24 flavonoid and flavonoid-like chemicals to determine chemical relationships (Fig. 1, Table 1). Finally, we used two $GABA_A$ receptor specific drugs (alfaxalone and chlormethiazole) to test the involvement of $GABA_A$ receptors as a potential mode of action for stimulatory compounds found to elicit hyperactivity, specifically picrotoxin, (*S*)-equol, kaempferol.

2. Materials and methods

2.1. Chemicals

The structural backbones and substituents for the flavonoids and flavonoid-like chemicals tested are shown in Fig. 1. A list of all model neuroactive chemicals and flavonoids tested are provided in Tables 1