



Review

Flavonoid nutraceuticals and ionotropic receptors for the inhibitory neurotransmitter GABA



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ABSTRACT

Flavonoids that are found in nutraceuticals have many and varied effects on the activation of ionotropic receptors for GABA, the major inhibitory neurotransmitter in our brains. They can act as positive or negative modulators enhancing or reducing the effect of GABA. They can act as allosteric agonists. They can act to modulate the action of other modulators.

There is considerable evidence that these flavonoids are able to enter the brain to influence brain function. They may have a range of effects including relief of anxiety, improvement in cognition, acting as neuroprotectants and as sedatives. All of these effects are sought after in nutraceuticals.

A number of studies have likened flavonoids to the widely prescribed benzodiazepines as 'a new family of benzodiazepine receptor ligands'. They are much more than that with many flavonoid actions on ionotropic GABA receptors being insensitive to the classic benzodiazepine antagonist flumazenil and thus independent of the classic benzodiazepine actions. It is time to consider flavonoids in their own right as important modulators of these vital receptors in brain function.

Flavonoids are rarely consumed as a single flavonoid except as dietary supplements. The effects of mixtures of flavonoids and other modulators on GABA_A receptors need to be more thoroughly investigated.

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Contents

1. Introduction	120
2. Do dietary flavonoids enter the brain?	121
3. Flavonoids as neuroprotectants and antioxidants	121
4. Memory and cognition	121
5. Receptors for the inhibitory neurotransmitter GABA	122
6. Flavonoids and GABA _A receptors	122
7. Apigenin, hispidulin and luteolin	122
8. (–)-Epigallocatechin gallate, (+)-catechin, (+)-taxifolin and dihydromyricetin	123
9. Conclusion	123
References	124

1. Introduction

Flavonoids have long been regarded as nutraceuticals due to

their widespread occurrence in our diet and their diverse pharmacological properties. Some individual flavonoids are used as dietary supplements. There are numerous reviews on flavonoids as nutraceuticals (Georgiev et al., 2014; Jain et al., 2010; Tapas et al., 2008), some highlighting the effects of flavonoids on the central nervous system particularly as neuroprotectants (Dajas et al., 2013; Devore et al., 2012; Grosso et al., 2013; Jager and Saaby, 2011; Latif,

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2015; Spencer, 2008a; Vauzour, 2014) and for their effects on memory and cognition (Fruson et al., 2012; Letenneur et al., 2007; Mastroiacovo et al., 2015; Spencer, 2008b, 2010).

This mini-review targets the effects of flavonoids in our diet and as dietary supplements on the central nervous system via modulation of ionotropic receptors for the neurotransmitter GABA. As GABA is the major inhibitory neurotransmitter in the brain it is involved in many aspects of brain function. Flavonoids have been suggested to be useful in the treatment of Alzheimer's disease, Parkinson's disease, epilepsy, depression and schizophrenia (Grosso et al., 2013), all conditions in which GABA is likely to play an important part, including the treatment of anxiety and sleep disorders (Chebib and Johnston, 2000).

Many ionotropic GABA receptors are modulated by widely used therapeutic drugs such as benzodiazepines (Chebib and Johnston, 2000). It was the pioneering work of Marder, Medina, Paladini and their co-workers in Argentina during the 1990s that drew attention to flavonoids as 'a new family of benzodiazepine receptor ligands' (Medina et al., 1997; Paladini et al., 1999).

As benzodiazepines were at that time amongst the most widely prescribed pharmaceuticals, an extensive range of natural and synthetic flavonoids were investigated *in vitro* and *in vivo* as potential leads for new benzodiazepine ligands. Structure activity studies led to development of synthetic flavonoid ligands with the high affinity for the classical benzodiazepine binding sites on GABA_A receptors that are sensitive to antagonism by flumazenil (Nilsson and Sterner, 2011). In addition, it has emerged that flavonoids can influence GABA_A receptors in a flumazenil-insensitive manner independent of classical benzodiazepine binding sites (Hanrahan et al., 2011, 2015).

2. Do dietary flavonoids enter the brain?

Flavonoids are found in almost all terrestrial plants and are thus

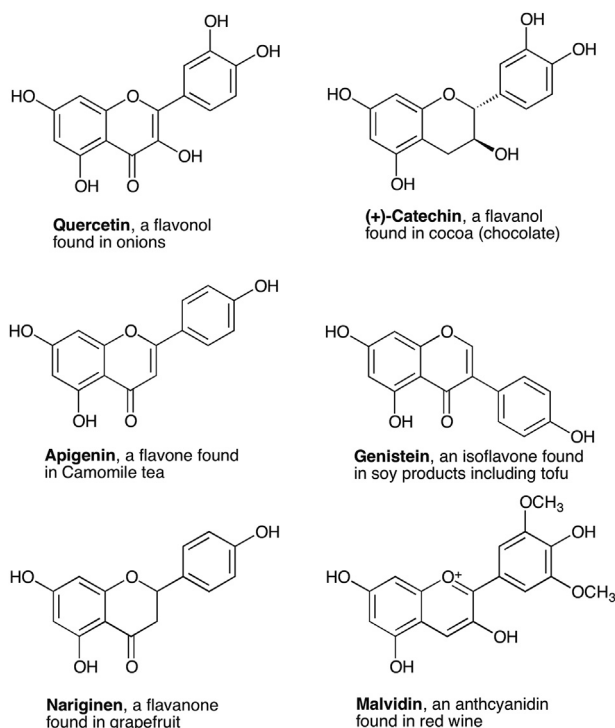


Fig. 1. Representatives of different classes of naturally-occurring flavonoids that have been shown to influence ionotropic GABA receptors.

important constituents of many foods (Jäger and Saaby, 2011). Major dietary sources of flavonoids include flavanols (onions and broccoli), flavanols (green tea, red wine and chocolates), flavones (parsley and celery), isoflavones (soy), flavanones (citrus fruit and tomatoes) and anthocyanidins (red wine, berries) (Latif, 2015). Examples of these different classes of flavonoids that occur in foodstuffs are shown in Fig. 1. Each example is known to influence GABA receptors (Hanrahan et al., 2011, 2015). The main differences in the basic flavonoid structures of the compounds involve the presence or absence of a ketone function at C4, a double bond at C2–C3 and a hydroxyl moiety at C3.

The intake of flavonoids in the diet ranges from 60 to 350 mg/day (Bai et al., 2014; Beking and Vieira, 2011). For dietary flavonoids to act on central GABA receptors they must enter the brain. That flavonoids influence brain function on systemic administration indicates that they and/or their metabolites enter the brain penetrating the blood–brain barrier (Jäger and Saaby, 2011; Youdim et al., 2004). This has been shown using hCMEC/D3 cells as a human blood–brain barrier model (Faria et al., 2014). Administered orally, radioactive (–)-epigallocatechin gallate has been detected in mouse brain (Suganuma et al., 1998). Similarly radioactive hispidulin has been found to cross the blood–brain barrier into rat brain (Kavvadias et al., 2004). More work needs to be done relating ingested levels of individual and mixtures of flavonoids to the levels achieved in the brain to influence brain function.

3. Flavonoids as neuroprotectants and antioxidants

The ability of flavonoids to protect neurones from cell death is well documented (Dajas et al., 2013; Devore et al., 2012; Grosso et al., 2013; Jäger and Saaby, 2011; Latif, 2015; Spencer, 2008a; Vauzour, 2014). A variety of mechanisms appear to underlie such neuroprotective actions. They appear to target key proteins involved in Alzheimer's disease (Baptista et al., 2014) acting against beta-amyloid toxicity (Bastianetto et al., 2006). Dietary flavonoids are known to induce cytoprotective proteins (Leonardo and Dore, 2011). They interact with many neurotransmitter systems including adenosine (Alexander, 2006), dopamine (Kita et al., 2014), GABA (Hanrahan et al., 2011) and glycine (Raafat et al., 2010) that may be involved in neuroprotection.

The ability of flavonoids, in common with many polyphenols, to act as antioxidants is widely considered to be an important aspect of the ability to act as neuroprotectives (Bubols et al., 2013). Interestingly, methylated polyphenols that are poor antioxidants protect cells from hydrogen peroxide-induced cytotoxicity indicating that their protectant activity may be largely unrelated to their chemical antioxidant capacity (Deng et al., 2006). Indeed the health claims of antioxidant capacity regarding polyphenol-containing nutraceuticals has been described as 'fiction' (Espin et al., 2007). In a study of structural features of the neuroprotective actions of flavonoids it was concluded that neuroprotection appears to be linked to specific structural motifs beyond those involved in antioxidation (Dajas et al., 2013).

4. Memory and cognition

Dietary flavonoids are considered to be beneficial in enhancing human memory and cognition (Spencer, 2008b, 2010). Flavonoid intake in the diet has been shown to decrease cognitive decline over a 10-year period (Letenneur et al., 2007). Cocoa flavonoid consumption has been shown to improve cognitive function in the elderly (Mastroiacovo et al., 2015). A study in snails has shown that a flavonoid present in cocoa improves long term memory (Fruson et al., 2012).

The flavonoid luteolin improved performance in an animal

model of spatial memory; an effect associated with increased brain-derived neurotrophic factor and acetylcholine, and decreased lipid peroxidation (Yoo et al., 2013). Many neurotransmitter systems are involved in memory and cognition including GABA (Möhler, 2009). Luteolin is known to modulate GABA receptors that are involved in animal models of memory (Chebib et al., 2009; Gibbs and Johnston, 2005).

5. Receptors for the inhibitory neurotransmitter GABA

There have been significant advances in our understanding of receptors for GABA, the major inhibitory transmitter in the brain due largely to the ability to study human cloned receptors *in vitro*. Generally this involves expressing combinations of the protein subunits in *Xenopus* oocytes and studying the effects of various chemicals by two-electrode voltage clamp techniques. In this way the effects of pure chemicals can be studied on the function of clearly defined receptors (Chebib and Johnston, 2000). The effects of plant extracts can also be studied in this way although this can be complicated by the presence of GABA, a significant constituent of many plants, needs to be taken into account (Abdelhalim et al., 2014).

GABA receptors are found pre- and post-synaptically as well as extra-synaptically in the central nervous system. They may be divided into two major types. Ionotropic GABA receptors act via GABA-activated chloride channels while metabotropic GABA receptors act via G-protein couple receptor complexes. Ionotropic GABA receptors may be classified pharmacologically on the basis of their sensitive to selective antagonists, with GABA_A receptors being antagonised by the convulsant alkaloid bicuculline (Johnston, 2013) and GABA_C receptors being antagonised by TPMPA (tetrahydropyridin-4-yl)methylphosphinic acid) (Murata et al., 1996). Metabotropic GABA receptors are designated as GABA_B receptors and are antagonised by phaclofen and insensitive to either bicuculline or TPMPA (Bowery, 2006). They consist of a combination of isoforms (Möhler et al., 2001).

Ionotropic GABA receptors are members of the cys-loop superfamily of ligand-gated ion channels that include both cationic (nicotinic acetylcholine and 5HT₃) and anionic (GABA_A, GABA_C and glycine) receptors. They are made up of pentameric protein subunits. GABA_A receptors are heteromeric made up complex combinations of subunits resulting in a variety of receptors that have distinct pharmacological and physiological properties (Möhler, 2011). GABA_C receptors are in the main homomeric and relatively simple (Bormann, 2000; Johnston et al., 2003). GABA_A receptors are of special interest in that their activation can be modulated by a variety of structurally diverse chemicals (Johnston, 2005). While most interest has been in the benzodiazepines that can act as positive, negative and neutralising modulators (Rudolph and Möhler, 2004) many natural products including flavonoids can act in a similar manner (Johnston et al., 2009).

6. Flavonoids and GABA_A receptors

Flavonoids were first linked to GABA_A receptors when three isoflavans isolated from bovine urine were shown to inhibit benzodiazepine binding to brain membranes (Luk et al., 1983). These isoflavans were most probably derived from plant sources in the bovine diet. Subsequently, many flavonoids directly isolated from plants were shown to influence benzodiazepine binding (Johnston et al., 2009). As noted earlier it was the pioneering work of Marder, Medina, Paladini and their co-workers in Argentina during the 1990s that drew attention to flavonoids as 'a new family of benzodiazepine receptor ligands' (Medina et al., 1997; Paladini et al., 1999). We and others have used bioassay-guided assays of

GABA_A receptor modulation to investigate such activity in plant extracts (Abdelhalim et al., 2014; Yang et al., 2011).

Flavonoids can act on GABA_A receptors at low concentrations in either a flumazenil-sensitive or flumazenil-insensitive manner as modulators of these receptors (Hanrahan et al., 2015) in a somewhat similar manner to benzodiazepines that have been described as acting via 'two distinct and separable mechanisms' (Walters et al., 2000). Furthermore, many flavonoids act in a biphasic manner, potentiating GABA actions at low concentrations and inhibiting at high concentrations. In addition, some flavonoids have agonist actions on certain GABA receptors, directly gating the receptor in the absence of GABA. Clearly flavonoids can interact with a variety of specific active sites on GABA_A receptors.

To illustrate the range of activities that closely structurally-related flavonoids can have on ionotropic GABA receptors the effects of three flavanones (apigenin, hispidulin and luteolin) two flavonols ((-)-epigallocatechin gallate and (+)-catechin) and two flavanonols (taxifolin, dihydromyricetin) are discussed. It is apparent that certain flavonoids act selectively on subtypes of GABA_A receptors.

7. Apigenin, hispidulin and luteolin

Apigenin (Fig. 1) has complex modulatory actions on GABA_A receptors. It has a negative modulatory action on the effect of GABA on cultured cerebellar granule cells and is a weak inhibitor on the binding of flumazenil to cerebellar membranes (Avalone et al., 2000). Unlike benzodiazepines, apigenin had no anxiolytic effects in rats according to these authors, despite clear anxiolytic effects being reported by others in mice (Viola et al., 1995). This may reflect the different levels of anxiety in rats and mice. The inhibitory action of apigenin on locomotor activity in rats was however not influenced by pretreatment with flumazenil and it was concluded that the sedative action of apigenin 'cannot be ascribed to an interaction with GABA–benzodiazepine receptor, since it is not counteracted by the benzodiazepine antagonist flumazenil' (Zanoli et al., 2000). That was before the flumazenil-insensitive action of flavonoids on GABA_A receptors was discovered (Hanrahan et al., 2011).

Subsequently it was found that apigenin could influence the flumazenil-sensitive modulatory effects of benzodiazepine ligands on GABA_A receptors under conditions where the flavonoids alone

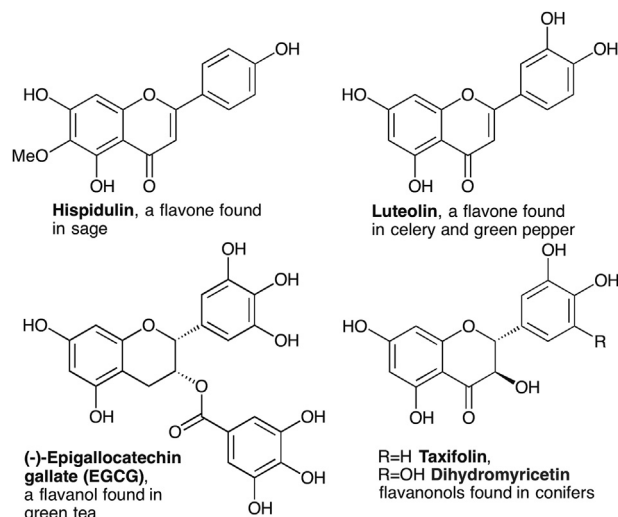


Fig. 2. Structurally related flavonoids that modulate ionotropic GABA receptors.

had no detectable modulatory effects on GABA responses. This gave rise to the concept of second order modulation by flavonoids of first order modulation by benzodiazepines (Campbell et al., 2004; Fernandez et al., 2005; Vignes et al., 2006). The second order positive modulation of diazepam enhancement of GABA responses by apigenin was observed under conditions of the maximal flumazenil-sensitive enhancement of the action of low doses of GABA. It is unlikely that apigenin acts by enhancing diazepam binding as it is known to inhibit such binding. Furthermore, apigenin does not influence the binding of muscimol, a potent GABA_A agonist. The observed second order modulation may result from alterations in the coupling of the flumazenil-sensitive benzodiazepine allosteric sites with the orthosteric GABA sites on GABA_A receptors. This action was selective for diazepam modulation as it was not observed for enhancement by pentobarbitone or allopregnanolone. Second order modulation (or metamodulation) of receptor activation has been noted in other systems (Mescue, 2002; Ribeiro and Sebastiao, 2010) and may represent a new, albeit obscure, mechanism of drug action deserving of further investigation. It is not easy to study as it involves the dose-dependant interactions between three ligands and thus may be difficult to observe. Synergistic interactions have been described between other flavonoids and GABA receptors (Choi et al., 2013), and between flavonoids, strychnine and glycine receptors (Raafat et al., 2010). Thus complex tertiary interactions between flavonoids and other substances may be a subtle feature of cys-loop ligand gated ion channels.

Hispidulin (Fig. 2), which differs from apigenin only by the addition of a 6-methoxy substituent, is a potent ligand at benzodiazepine binding sites and has demonstrated anticonvulsant activity in a model of epilepsy in seizure-prone Mongolian gerbils (Kavvadias et al., 2004). In functional studies on recombinant receptors expressed in oocytes hispidulin is inactive when applied alone and found to be approximately equipotent and exhibit a biphasic activity at $\alpha 1,2,3,5,6\beta 2\gamma 2S$ receptors, enhancing at low concentrations (EC₅₀ 0.8–5 μ M) and inhibiting at higher concentrations (>30 μ M), was only partially blocked by flumazenil but was inactive at $\alpha 1\beta 2$ receptors at a concentration of 10 μ M (Kavvadias et al., 2004). The fact that hispidulin is active at $\alpha 6$ -containing receptors that are benzodiazepine insensitive suggests that hispidulin may act via more than one binding site on GABA_A receptors. Interestingly, previous studies indicated that a range of natural and synthetic flavones had no affinity for recombinant $\alpha 6\beta 3\gamma$ receptors (Marder et al., 2001). Thus hispidulin appears to show a different profile of activity to apigenin at GABA_A receptor subtypes.

Luteolin (Fig. 2) is a common flavone found in many plants including celery and green pepper. It differs from apigenin by an extra hydroxyl group in the phenyl ring. Luteolin has antidepressant activity in mice and promotes chloride influx in human neuroblastoma cells that is blocked by the GABA_A receptor antagonist bicuculline (de la Pena et al., 2014). In a rat model of neuropathic pain, luteolin produced analgesia in a bicuculline-sensitive, flumazenil-insensitive manner (Hara et al., 2014). Tested as a major constituent of the essential oil lemon balm, luteolin was anxiolytic in rats; as this action was insensitive to flumazenil it was concluded that 'luteolin does not produce anxiolysis by modulation of the GABA_A receptor' (de la Pena et al., 2014). Given that we now know of flumazenil-insensitive actions of flavonoids on GABA_A receptors this is an incorrect conclusion. Luteolin showed no anticonvulsant activity in four mouse seizure models (Shaikh et al., 2013) and improves spatial memory in rats in a scopolamine-induced amnesia model (Yoo et al., 2013). In unpublished studies my colleagues, Katherine Locock and Ushma Trivedi, have shown that luteolin selectively enhances the action of GABA at human recombinant $\alpha 2\beta 2\gamma 2L$ over $\alpha 1\beta 2\gamma 2L$ GABA_A receptor expressed in

oocytes in an flumazenil-insensitive manner, consistent with a selective action on anxiety (Möhler, 2006). Thus, luteolin also appears to show a different profile of activity to apigenin at GABA_A receptor subtypes.

8. (–)-Epigallocatechin gallate, (+)-catechin, (+)-taxifolin and dihydromyricetin

(–)-Epigallocatechin gallate (Fig. 2) (EGCG) is the major polyphenol in green tea (*Camellia sinensis*). It has been extensively studied particularly for its anticancer properties (Singh et al., 2011). This flavanol demonstrates dose-dependent anxiolytic, sedative hypnotic and amnesiac activity, with evidence that these activities are mediated at least in part by GABA_A receptors (Adachi et al., 2006; Vignes et al., 2006). Studies on $\alpha 1\beta 2\gamma 2L$ GABA_A receptors showed that EGCG at low concentrations (0.1 μ M) has a potent second-order modulatory action on the first-order modulation by diazepam but inhibits the action of GABA at higher concentrations (>1 mM). EGCG was an order of magnitude more potent than apigenin in acting as a second-order modulator (Campbell et al., 2004). In addition, it has been found that EGCG, at concentrations that have no influence on the activation of GABA_A receptors by GABA, was able to reverse the negative modulation of such receptors by methyl β -carboline (Vignes et al., 2006). This indicates that EGCG may act as a second-order modulator with respect to the first-order modulation by both positive and negative modulators that act on benzodiazepine sites on GABA_A receptors.

The natural flavan-3-ol (+)-catechin (Fig. 2), an important constituent of chocolate and many other foodstuffs (Arts et al., 2000), is an allosteric agonist at recombinant $\alpha 4\beta 3\delta$ GABA_A receptors expressed in oocytes (Eghorn et al., 2014). (+)-Catechin appears to be a positive allosteric modulator for the high-affinity binding of γ -hydroxybutyric acid (GHB) on these receptors. This action is stereoselective in that (–)-catechin is much less active. Although relatively weak in activity (+)-catechin may aid in further characterization of the GHB high-affinity sites that are likely to be present on certain GABA_A receptors. (+)-Catechin has been reported to have no action on recombinant $\alpha 1\beta 2\gamma 2L$ GABA_A receptors (Campbell et al., 2004).

The structurally related (+)-taxifolin (Fig. 2), which is identical to (+)-catechin except that it has a ketone in the 4 position, was a negative modulator of GABA_A receptors (Eghorn et al., 2014). The closely related dihydromyricetin (Fig. 2, also known as ampelopsin), which has an additional hydroxyl group in the phenyl ring, acts as a positive modulator of synaptic and extrasynaptic GABA_A receptors (Shen et al., 2012). Importantly, and perhaps paradoxically, dihydromyricetin reduces the potentiation of GABA_A receptors by ethanol perhaps acting as a second-order modulator. Dihydromyricetin has similarities but also differences to benzodiazepines. It is not anxiolytic or sedative at the dose (1 mg/kg) that blocks the effect of ethanol. It is marketed as a nutraceutical for anti-alcohol intoxication.

9. Conclusion

Flavonoids in our diet have many and varied effects on the activation of ionotropic receptors for GABA, the major inhibitory neurotransmitter in our brains. There is considerable evidence that flavonoids are able to enter the brain to influence brain function. They may have a range of effects including relief of anxiety, improvement in cognition, and sedation. All of these effects are sought after in nutraceuticals.

There appear to be many sites on ionotropic GABA receptors that can be influenced by flavonoids. The sites may include ones that are insensitive to the classic benzodiazepine antagonist flumazenil and

described as low affinity benzodiazepine sites (Walters et al., 2000). Perhaps these would be more appropriately described as flavonoid sites as they appear to be activated by many naturally-occurring flavonoids. Many years ago I proposed that there were at least 11 distinct binding sites on GABA_A receptors (Johnston, 1996). This is proving to be a significant underestimate of the diversity of agents that are able to modulate these important receptors. The study of individual flavonoids in nutraceuticals will aid in future investigations of these receptors and provide information on how these flavonoids interact to produce their combined effects. From a structure-activity point of view it is very interesting that flavonoids of closely related structures can have different effects on the function of GABA_A receptors, in some cases opposite effects. Furthermore the effects of mixtures of flavonoids and other modulators on GABA_A receptors need to be more thoroughly investigated. This is important flavonoids are rarely consumed as a single flavonoid except as dietary supplements.

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