# The anxiolytic effect of *Bifidobacterium longum* NCC3001 involves vagal pathways for gut–brain communication

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## INTRODUCTION

Psychiatric co-morbidities such as anxiety and depression are common in patients with chronic gut disorders, including those with overt inflammatory conditions of the gastrointestinal (GI) tract, such as inflammatory bowel disease,[1] and those with low-grade gut inflammation as found in irritable bowel syndrome.[2] Animal models have shown that both acute and chronic GI infection and inflammation result in changes in host behavior and central nervous system (CNS) biochemistry,[3–6] but the precise mechanisms of this altered gut–brain communication are not completely understood. Possible candidate routes include neural and humoral inputs from the periphery to the CNS.

Animal studies investigating the effect of systemic inflammation have shown that pro-inflammatory cytokines can alter tryptophan metabolism, increasing levels of kynurenin, which is known to induce anxiety-like behavior in a dose-dependent manner.[7] Studies focused on the GI tract have demonstrated that the development of anxiety-like behavior during the acute phase of infection with *Campylobacter pylori* is associated with activation of vagal pathways.[4] We have recently shown that chronic colitis, caused by a noninvasive parasite *Trichuris muris*, resulted in decreased hippocampal brain derived neurotrophic factor (BDNF) levels and abnormal mouse behavior, which was independent of vagal integrity.[6] Administration of the probiotic *Bifidobacterium longum* NCC3001 normalized the altered behavior and CNS changes induced by chronic infection, although it did not affect the peripheral cytokine levels or the kynurenin/tryptophan ratio. Thus, the mechanism(s) through which this probiotic affects the gut–brain axis remain unclear.

This study explores the mechanisms of gut–brain communication underlying the effect of *B. longum* on behavior during non-infectious, chronic, low-grade gut inflammation. We assessed mouse behavior following repeated administration of low dose dextran sodium sulfate (DSS), before and after treatment with this probiotic bacterium. A non-infectious chemical model was chosen to avoid the confounding factors related to ongoing active nematode infection. To test the hypothesis that the anxiolytic effect of B. longum on behavior involves neural pathways, a group of mice was subjected to vagotomy and pyloroplasty after induction of anxiety-like behavior by chronic DSS. In separate experiments, we treated myenteric neurons in situ with *B. longum* fermented medium or non-fermented medium (control medium), to determine whether bacterial products generated during fermentation can directly alter the excitatory properties of enteric nerves. To investigate the possibility that *B. longum* can up-regulate production of neurotrophins, we incubated human SH-SY5Y neuroblastoma cells with serum of mice treated with this probiotic and assessed BDNF mRNA by real-time RT-PCR. Our results show that in this model of chronic colitis, *B. longum* gut–brain communication involves vagal pathways. Our in vitro results suggest that *B. longum* fermentation products do not directly affect BDNF production by a neural cell line but modify the functional status of enteric neurons in the gut.

# Ingestion of *Lactobacillus* strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve

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There is increasing evidence suggesting an interaction between the intestinal microbiota, the gut, and the central nervous system (CNS) in what is recognized as the microbiome–gut–brain axis (1–4). Studies in rodents have implicated dysregulation of this axis in functional bowel disorders, including irritable bowel syndrome. Indeed, visceral perception in rodents can be affected by alterations in gut microbiota (5). Moreover, it has been shown that the absence and/or modification of the gut microflora in mice affects the hypothalamic–pituitary–adrenal (HPA) axis response to stress (6, 7) and anxiety behavior (8, 9), which is important given the high comorbidity between functional gastrointestinal disorders and stress-related psychiatric disorders, such as anxiety and depression (10). In addition, pathogenic bacteria in rodents can induce anxiety-like behaviors, which are mediated via vagal afferents (9, 11).

GABA is the main inhibitory neurotransmitter of the CNS, the effects of which are mediated through two major classes of receptors—the ionotropic GABAA receptors, which exist as a number of subtypes formed by the coassembly of different subunits ($\alpha$, $\beta$, and $\gamma$ subunits; ref. 12), and the GABAB receptors, which are G protein coupled and consist of a heterodimer made up of two subunits (GABAB1 and GABAB2), both of which are necessary for GABAB receptor functionality (13). These receptors are important pharmacological targets for clinically relevant antianxiety agents (e.g., benzodiazepines acting on GABAA receptors), and alterations in the GABAergic system have important roles in the development of stress-related psychiatric conditions.

Probiotic bacteria are living organisms that can inhabit the gut and contribute to the health of the host (14). Accumulating clinical evidence suggests that probiotics can modulate the stress response and improve mood and anxiety symptoms in patients with chronic fatigue and irritable bowel syndrome (15, 16). One such organism is *Lactobacillus rhamnosus* (JB-1), which has been demonstrated to modulate the immune system because it prevents the induction of IL-8 by TNF-$\alpha$ in human colon epithelial cell lines (T84 and HT-29) (17) and modulates inflammation through the generation of regulatory T cells (18). Moreover, it inhibits the cardio–autonomic response to colorectal distension (CRD) in rats (19), reduces CRD-induced dorsal root ganglia excitability (20), and affects small intestine motility (21).

It is currently unclear whether potential probiotics such as *L. rhamnosus* (JB-1) could affect brain function, especially in normal, healthy animals. To this end, we sought to assess whether this bacteria could mediate direct effects on the GABAergic system. In parallel, behaviors relevant to GABAergic neurotransmission and the stress response were assessed subsequent to *L. rhamnosus* (JB-1) administration. Finally, the role of the vagus nerve in mediating such effects was also investigated by examining these parameters in subdiaphragmatically vagotomized mice.

# Structural basis for dual excitation and photoisomerization of the *Aequorea victoria* green fluorescent protein

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The green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* is the first known protein in which visible fluorescence is genetically encodable. The fluorophore is derived from natural residues present within the primary structure of GFP, so no exogenous cofactor or substrate is needed for fluorescence (1, 2). The tremendous potential of GFP as a reporter of gene expression, cell lineage, and protein trafficking and interactions has been extensively reviewed (3–5).

Wild-type (WT) GFP is a 238-aa protein (2). *In vitro* GFP is a particularly stable protease-resistant protein (6) and is only denatured under extreme conditions (7). The GFP chromophore, *p*-hydroxybenzylideneimidazolinone (8, 9), is formed by internal cyclization of a Ser-Tyr-Gly tripeptide and 1,2-dehydrogenation of the Tyr. This posttranslational modification is oxygen dependent, requiring ~2–4 h for the WT protein (10, 11). A mechanism for the fluorophore formation has been proposed (3) but needs to be confirmed by further studies.

GFP absorbs blue light at 395 nm, with a smaller peak at 475 nm, and emits green light at 508 nm with a quantum yield of 0.72–0.85 (12, 13). The ratio between the two absorption peaks is sensitive to factors such as pH, temperature, and ionic strength (7, 14), suggesting the presence of two different forms of the chromophore. It has been proposed that the larger 395-nm peak is due to a neutral chromophore and that the minor peak at 475 nm is caused by an ionized chromophore (10). Upon illumination with UV light, WT GFP undergoes photoisomerization, which increases the excitation absorbance at 475 nm at the expense of that at 395 nm (3). The diversion of excitation amplitude into two peaks whose ratio is dependent on past illumination history complicates the application ofWT GFP for routine fluorescence observations, though it also raises the possibility of tagging GFP by localized UV irradiation (3, 15). Mutation of Ser-65 3 Thr (S65T) completely suppresses the 395-nm peak and the photoisomerizability while amplifying the 475-nm excitation peak ~6-fold and shifting it to 489 nm (11). Therefore, this substitution is highly advantageous and has been incorporated in many of the variants of GFP (16–18).

Recently the structures of S65T as a monomer and the WT as a dimer were separately determined (19, 20). No detailed comparison of the two structures has yet appeared. We have crystallized WT GFP as a monomer and solved its structure by x-ray crystallography using the S65T model. Comparison of the two monomeric structures suggests an explanation for why WT has such unusual dual wavelength absorption and photoisomerization properties and why the replacement of a proton in WT by a methyl group in S65T causes such profound spectroscopic alterations.

# Association Between Use of Non–Vitamin K Oral Anticoagulants With and Without Concurrent Medications and Risk of Major Bleeding in Nonvalvular Atrial Fibrillation

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Atrial fibrillation is a common arrhythmia with an increasing prevalence and an association with thromboembolism and related adverse outcomes.[1] Oral anticoagulation has been proven to prevent ischemic strokes and prolong life for patients with atrial fibrillation.[2] Non–vitamin K oral anticoagulants (NOAC) are being used more frequently because of their ease of administration and comparative efficacy compared with warfarin in reducing thromboembolism and major bleeding.[3,4] However, for patients with atrial fibrillation, NOACs still pose a major bleeding risk,[5] which is particularly problematic when multiple morbidities, high-risk medications, polypharmacy, or drug-drug interactions are present.[6]

Two large clinical trials among patients with atrial fibrillation were conducted from 2006 through 2009 and approximately two-thirds of the participants (especially the elderly) took more than 5 drugs concurrently with a NOAC.[7,8] Polypharmacy among NOAC users may increase plasma levels and the risk of bleeding.[7] Current knowledge of drug-drug interactions associated with NOACs mainly comes from animal studies, case reports, and limited pharmacokinetic measurement.[9,10] Particular attention has been paid to medications (such as CYP3A4 inhibitors and P-glycoprotein competitors) that share common metabolic pathways with NOACs.[11,12] For example, ketoconazole and clarithromycin increase active NOAC levels in plasma and risk of bleeding.[10,13]

However, complex comedications and comorbidities hinder the quantification of bleeding risk associated with NOAC use in patients with atrial fibrillation.[6,14] Combining NOACs with other commonly used medications is generally avoided in clinical trials because the medications may alter NOAC levels in plasma and increase the risk of bleeding. To our knowledge, the influence of the concurrent use of CYP3A4 inhibitors or P-glycoprotein competitors on the magnitude of bleeding risk in NOAC users has not been quantified in the clinical setting. This study used a nationwide cohort of patients with nonvalvular atrial fibrillation to estimate the bleeding risk in NOAC users associated with the concurrent use of 12 commonly prescribed medications that share metabolic pathways with NOACs.

# Expression of Tryptophan 2,3-Dioxygenase and Production of Kynurenine Pathway Metabolites in Triple Transgenic Mice and Human Alzheimer's Disease Brain

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During neuroinflammation, 95% of the cerebral pool of the essential amino acid tryptophan (Trp) is catabolized through the kynurenine pathway (KP) leading to the formation of several neuroactive metabolites (Fig. 1). The downstream metabolites of the KP and especially quinolinic acid (QUIN) an agonist of NMDA receptors [1,2], have been implicated in the pathophysiology of several brain diseases.

The initial step of the KP is mainly regulated by two key enzymes: indoleamine 2,3-dioxygenase 1 (IDO-1) and tryptophan 2,3-dioxygenase (TDO), which differ in their tissue localization and regulation [3]. IDO-1 is widely expressed in all tissues and is involved in the metabolism of Trp [4,5]. IDO-1 is activated by pro-inflammatory cytokines and other molecules [6]. IDO-1 plays a key role in the regulation of the CNS immune response [7]. TDO is predominantly expressed in the liver but is also present in the brain [8]. TDO is responsible for systemic metabolism of Trp and is activated by cortisol [9] and L-Trp [10]. In mouse, TDO expression is involved in the development of the brain and nervous system [11].

AD is an age-related neurodegenerative disorder characterized by neuronal loss and dementia. The pathological mechanisms underlying this disease are still controversial, however, there is growing evidence implicating KP metabolites in the development and progression of AD. Some observations include an increased ratio of 3-hydroxykynurenine to Trp in the serum of AD patients [12] and accumulation of QUIN in the brain of AD patients [13]. We have demonstrated that IDO-1 expression and QUIN production are increased in AD hippocampus [14] and that QUIN leads to tau hyperphosphorylation in human cortical neurons [15]. Another recent study showed that IDO-1 is upregulated in the brains of AD and is associated with neurofibrillary tangles (NFT) and b-amyloid (Ab) plaques [16]. All together, these studies strongly suggest that the KP is involved in the neurodegenerative processes of AD.

The purpose of this study was to characterize the KP metabolic profile and assess TDO and IDO-1 expression in the brains of AD patients and in an AD mouse model. We use triple-transgenic AD (3xTg AD) mice, a relevant model exhibiting both Ab and tau pathologies [17]. This model provides a unique opportunity for demonstrating the importance of the KP and TDO during the progression of AD.

We studied three age groups of 3xTg AD and wild type mice. We investigated the expression of TDO and IDO-1 in different brain regions using real-time RT PCR, immunohistochemistry and Western blotting and TDO/IDO-1 activity using HPLC and gas chromatography-mass spectrometry (GC-MS). The expression of TDO and IDO-1 protein was then assessed in the hippocampus of AD patients and controls. Finally, the co-localization of TDO with QUIN, NFTs and Ab deposits was assessed to discern the roles of TDO-initiated KP in the pathological progression of AD.