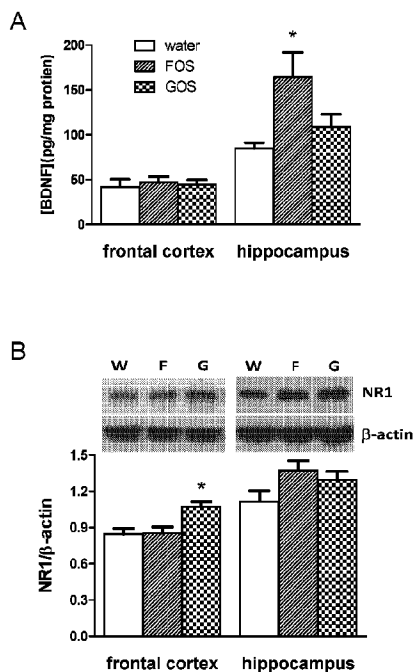




(86) Date de dépôt PCT/PCT Filing Date: 2014/03/17
(87) Date publication PCT/PCT Publication Date: 2014/10/02
(45) Date de délivrance/Issue Date: 2021/04/13
(85) Entrée phase nationale/National Entry: 2015/09/28
(86) N° demande PCT/PCT Application No.: GB 2014/050829
(87) N° publication PCT/PCT Publication No.: 2014/155056
(30) Priorité/Priority: 2013/03/28 (GB1305708.8)

(51) Cl.Int./Int.Cl. *A61K 31/7016* (2006.01),
A61K 31/702 (2006.01), *A61P 25/24* (2006.01),
A61P 25/28 (2006.01)
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(54) Titre : COMPOSITION DE GALACTO-OLIGOSACCHARIDES UTILISEE POUR PREVENIR OU TRAITER LE DYSFONCTIONNEMENT COGNITIF ET LES PERTURBATIONS EMOTIONNELLES DANS LES MALADIES NEUROPSYCHIATRIQUES OU LE VIEILLISSEMENT
(54) Title: A GALACTOOLIGOSACCHARIDE COMPOSITION FOR USE IN PREVENTING OR TREATING COGNITIVE DYSFUNCTION AND EMOTIONAL DISTURBANCES IN NEUROPSYCHIATRY ILLNESSES OR AGEING



(57) **Abrégé/Abstract:**

A galactooligosaccharide composition comprising a mixture of disaccharides, trisaccharides, a tetrasaccharide and a pentasaccharide for use in preventing or treating cognitive dysfunction and/or emotional disturbances in neuropsychiatric illnesses or ageing.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property
Organization
International Bureau



(10) International Publication Number
WO 2014/155056 A1

(43) International Publication Date
2 October 2014 (02.10.2014)

(51) International Patent Classification:

A61K 31/7016 (2006.01) A61P 25/24 (2006.01)
A61K 31/702 (2006.01) A61P 25/28 (2006.01)

(21) International Application Number:

PCT/GB2014/050829

(22) International Filing Date:

17 March 2014 (17.03.2014)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

1305708.8 28 March 2013 (28.03.2013) GB

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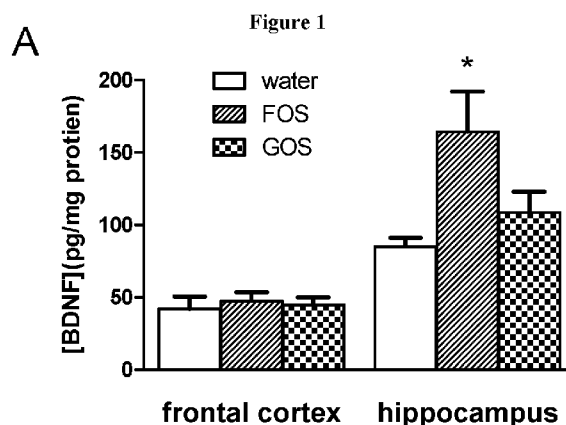
(81) Designated States (unless otherwise indicated, for every
kind of national protection available): AE, AG, AL, AM,
AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY,
BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM,
DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT,
HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR,
KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME,
MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ,
OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA,
SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM,
TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM,
ZW.

(84) Designated States (unless otherwise indicated, for every
kind of regional protection available): ARIPO (BW, GH,
GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ,
UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ,
TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK,
EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV,
MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM,
TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW,
KM, ML, MR, NE, SN, TD, TG).

Published:

— with international search report (Art. 21(3))

(54) Title: A GALACTOOLIGOSACCHARIDE COMPOSITION FOR USE IN PREVENTING OR TREATING COGNITIVE
DYSFUNCTION AND EMOTIONAL DISTURBANCES IN NEUROPSYCHIATRY ILLNESSES OR AGEING



(57) Abstract: A galactooligosaccharide composition comprising a mixture of disaccharides, trisaccharides, a tetrasaccharide and a pentasaccharide for use in preventing or treating cognitive dysfunction and/or emotional disturbances in neuropsychiatric illnesses or ageing.

**A GALACTOOLIGOSACCHARIDE COMPOSITION FOR USE IN
PREVENTING OR TREATING COGNITIVE DYSFUNCTION AND EMOTIONAL
DISTURBANCES IN NEUROPSYCHIATRY ILLNESSES OR AGEING**

The present invention relates to a composition comprising a mixture of galactooligosaccharides (GOS) for use in preventing or treating cognitive dysfunction and/or emotional disturbances occurring in neuropsychiatric illnesses or disorders, or in aging in a human. It also relates to a method of preventing or treating cognitive dysfunction and/or emotional disturbances occurring in neuropsychiatric illnesses or disorders, or in aging by orally administering to an individual an effective amount of a composition comprising a mixture of galactooligosaccharides.

10

Preventing of a disease refers to the ability of a pharmaceutical composition or treatment to not only prevent the occurrence of disease, such as risk factor reduction, but also to arrest its progress and reduce its consequences once established (Ref: adapted from Glossary of Terms used in Health for All Series. WHO, Geneva, 1984).

15

Primary prevention is directed towards preventing the initial occurrence of a disorder whereas secondary and tertiary prevention seeks to arrest or retard existing disease and reduce occurrence of relapses and establishment of chronic conditions.

20

Cognitive dysfunction refers to the loss of intellectual functions, such as thinking, remembering and reasoning, of sufficient severity to interfere with daily functioning of the individual. This can be seen in aging and dementia sufferers, especially in people suffering from Alzheimer's disease. Impairment of cognitive function can affect the ability to think, to concentrate, to formulate ideas, to reason and to remember.

25

Neuropsychiatric illnesses or disorders refers to organic cerebral disorders or neurological disorders that cause psychiatric symptoms. They include anxiety disorders and depressive disorders that commonly occur in elderly patients.

30

A review by Gryan, J F and Dinan, T G in Nature Review/Neuroscience; 13; 701-712; (2012) describes how studies in germ-free animals and in animals exposed to pathogenic bacterial infections, probiotic bacteria or antibiotic drugs suggest a role for the gut microflora in the regulation of anxiety, mood, cognition and pain.

Probiotic bacteria are defined as live bacteria that may confer a health benefit on the host if ingested.

Bravo, J A et al demonstrated antidepressant and anxiolytic-like properties of the probiotic
5 *Lactobacillus rhamnosus* when ingested by mice (see Proc Natl Acad Sci USA; 108; 16050-16055;
(2011)).

Burnet, P W J suggested that future studies using selective antimicrobials and prebiotics to
increase strains of lactobacilli and bifidobacteria indigenous to the gut may have an effect on
10 behaviours and neurophysiological outputs in animals and humans (see Proc. Natl. Acad. Sci. USA;
E175; (2012)).

Prebiotics are defined as non-digestible food ingredients that beneficially affect the host
by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the
15 colon, thereby resulting in an improvement in the health of the host. Galactooligosaccharides
(GOS) and fructooligosaccharides (FOS) are examples of prebiotics that are resistant to mammalian
gastrointestinal digestive enzymes but are fermented by specific colonic bacteria.

It has now been found that oral administration to a mammal, such as a human, of a
20 composition comprising a mixture of galactooligosaccharides can result in a direct interaction with
the neurons located in the gastrointestinal tract, that in turn may result in an unexpected increase
in the levels of N-methyl-D-aspartate receptors (NMDARs). Specifically, elevated levels of the
NMDAR NR1 protein and/or mRNA in both the cortex and the hippocampus part of the brain were
found, and also of the NMDAR NR2A protein in the hippocampus. This suggested that the
25 compositions comprising such a mixture may be beneficial in preventing or treating cognitive
dysfunction and/or emotional disturbances occurring in neuropsychiatric illnesses or disorders, or
in aging.

It has also been found that oral administration of a GOS composition lowers the secretion
30 of cortisol. Since cortisol is released in response to stress this suggests that the composition may
reduce the exaggerated cortisol secretion that is symptomatic of anxiety disorders and depressive
disorders.

The mixture of galactooligosaccharides comprised disaccharides Gal (β 1-3)-Glc; Gal (β 1-3)-Gal; Gal (β 1-6)-Gal; Gal (α 1-6)-Gal; trisaccharides Gal (β 1-6)-Gal (β 1-4)-Glc; Gal (β 1-3)-Gal (β 1-4)-Glc; tetrasaccharide Gal (β 1-6)-Gal (β 1-6)-Gal (β 1-4)-Glc and pentasaccharide Gal (β 1-6)-Gal (β 1-6)-Gal (β 1-6)-Gal (β 1-4)-Glc.

5

This mixture of galactooligosaccharides is disclosed in EP 1 644 482, which describes a novel strain of *Bifidobacterium bifidum* that produces a galactosidase enzyme activity that converts lactose to this novel mixture of galactooligosaccharides. This novel mixture has been shown to have prebiotic and anti-inflammatory properties in the gut.

10

This mixture of galactooligosaccharides is marketed commercially under the name of Bimuno (registered trade mark) and is available from Clasado Ltd (Milton Keynes, UK).

15

According to one aspect of the invention there is provided a composition comprising a mixture of galactooligosaccharides as defined above for use in preventing or treating cognitive dysfunction and/or emotional disturbances occurring in neuropsychiatric illness or disorders, or in aging.

20

According to a second aspect of the invention there is provided the use of a mixture of galactooligosaccharides as defined above in the preparation of a medicament for preventing or treating cognitive dysfunction and/or emotional disturbances occurring in neuropsychiatric illness or disorders, or in aging.

25

The cognitive dysfunction may be cognitive decline or impairment as a result of aging, dementia or schizophrenia. The neuropsychiatric illness may be depressive disorders or anxiety disorders. Anxiety disorders covers several different forms of a type of common psychiatric disorder characterised by excessive rumination, worrying, uneasiness, apprehension and fear about future uncertainties either based on real or imaged events which may effect both physical and psychological health.

30

According to yet another aspect of the invention there is provided a method of preventing or treating cognitive dysfunction and/or emotional disturbances occurring in neuropsychiatric illness or in aging comprising administering to an individual, such as a human, an effective amount

of a composition comprising a mixture of galactooligosaccharides as defined above. An effective amount of the galactooligosaccharide composition is preferably administered daily as a single dose or alternatively as two separate doses several hours apart, for example from 4 to 12 hours apart, preferably from 6 to 10 hours apart, most preferably 8 hours apart.

5

Preferably, the composition or mixture of galactooligosaccharides is administered orally on a daily basis in the form of a powder, such as a freeze-dried powder, a tablet, a capsule, a liquid formulation such as a syrup, or a soft pastille.

10

The product known as Bimuno comprises at least 49% of the dry matter as the mixture of galactooligosaccharides. The remainder of the composition may comprise non-active components such as glucose, galactose, lactose, acacia gum, maltodextrin and citric acid.

15

The powder composition preferably comprises from 1.35g to 9.6g of galactooligosaccharide in 1.65g to 20g of the powdered composition, preferably from 1.96g to 4.9g of galactooligosaccharide in 2.5g to 10g of the powder, most preferably 2.7g to 2.75g of galactooligosaccharide in 3.0g to 5.5g of composition. The composition may be added to a drink, preferably a hot drink, or sprinkled on food, for example, on breakfast cereal. The composition may also be added as an ingredient to various foodstuffs and drinks such as fruit juice, fruit drinks, water, coffee, yoghurt, cereals, bread, cakes, biscuits and the like.

20

Alternatively, the galactooligosaccharide may be presented as a syrup or pastille (dehydrated syrup) in which the non-active components may comprise glucose, galactose, lactose and citric acid. A daily dose of the syrup may comprise from 1.35g to 9.6g of the galactooligosaccharide mixture in 2.1g to 25.29g of the syrup composition, preferably from 1.96g to 4.9g of galactooligosaccharide in 3.0g to 12.9g of the syrup, most preferably 2.7g to 2.75g of galactooligosaccharide in 4.1g to 7.25g of the syrup.

25

The galactooligosaccharide composition of the invention has anxiolytic properties, reduces the activity of the hypothalamic-pituitary axis (stress hormone secretion) and reduces inflammatory responses in the brain. Thus Bimuno GOS may be beneficial in the treatment or prevention of anxiety disorders (e.g. worry, insomnia), depressive disorders, brain inflammation caused by bacterial meningitis, Herpes Simplex encephalitis or that occurs in Alzheimer's disease.

30

Bimuno GOS may also improve cognitive impairment in ageing, dementia and schizophrenia. Furthermore, the GOS composition may benefit the detrimental influence of maternal infection on the developing foetal brain.

5 The invention will be further described by way of reference to the following examples and figures.

10 Figure 1A shows the effect of FOS and GOS on levels of BDNF protein in extracts of rat frontal cortex and hippocampus.

 Figure 1B shows the effect of FOS and GOS on levels of NR1 subunits in the rat frontal cortex and the hippocampus. Western blot images of NR1 and β -actin immunoreactivity in protein extracts are shown.

15 Figure 2A shows the effect of FOS and GOS on levels of NR2A subunits in the rat frontal cortex and the hippocampus. Western blot images of NR2A subunits and β -actin immunoreactivity are shown.

20 Figure 2B shows the effect of FOS and GOS on levels of NR2B subunits in the rat frontal cortex and the hippocampus. Western blot images of NR2B and β -actin immunoreactivity are shown.

25 Figures 3A to 3D are representative auto-radiographs of BDNF (A, C, E) and NR1 subunit (B, D, F) mRNA expression in rat hippocampus following oral administration of water (A, B), FOS (C, D) or GOS (E, F). Arrows delineate increased expression and arrow head indicates reduced expression. DG = dentate gyrus, CA1 and CA3 = Cornu Ammons subfields of the hippocampus. Scale bar = 200 μ M.

30 Figures 4A to 4D show the effect of FOS and GOS on levels of BDNF, NR1, NR2A and NR2B mRNAs in the dentate gyrus (DG) of and CA1 and CA3 (Cornu Ammons) subfields of the hippocampus.

Figure 5 shows the effect on cortisol secretion in healthy adults following ingestion of FOS (Group A), GOS (Group C) and a placebo (Group B).

Figure 6A shows the effect on locomotor activity in water-fed mice following
5 lipopolysaccharide (LPS) injection.

Figure 6B shows how GOS abolished the LPS effect on locomotor activity.

Figure 7 shows the effect on natural digging and burying behaviour, as shown in the
10 marble burying test, in mice following LPS treatment.

Figure 8 shows the effect on anxiety behaviour in mice following LPS treatment. Latency (A) = time taken to move from dark (less stressful) to light (more stressful) areas. Greater latency = more stressed/reduced exploratory behaviour. Time in light (B) = time spent in light area.
15 Greater time = less anxious.

Figure 9 shows the effect on cytokine levels in the frontal cortex of mice 24 hours after LPS injection.

20 Figure 10 shows the effect on cytokine levels in the plasma of mice 24 hours after LPS injection.

Figure 11 shows the effect of BGOS on cognitive performance in healthy rats.

Example 1

25 Freeze-dried powdered composition packaged in a 'stick-pack' and containing per 5.5g final product:-

	Galactooligosaccharide (GOS) mixture	2.75g
	Lactose	1.40g
	Monosaccharides (glucose, galactose)	0.64g
30	Drying aid	0.24g
	Ash	0.23g
	Moisture	0.19g
	Protein	0.05g

Example 2

Syrup composition per 7.25g finished product:-

	Galactooligosaccharide (GOS) mixture	2.75g
5	Lactose	0.58g
	Monosaccharides (glucose, galactose)	1.69g
	Ash	0.23g
	Moisture	1.95g
	Protein	0.05g

10

Example 3

In vivo study of the effect of feeding prebiotics on central brain derived neurotrophic factor (BDNF) and N-methyl-D-aspartate (NMDA) receptor subunits.

15 MATERIALS AND METHODS*Prebiotic administration*

All rat experiments were carried out in accordance with UK Home Office guide lines and under approved licences. Male Sprague Dawley rats (225-250g) were administered a daily oral administration (gavage) or either water, FOS (fructooligosaccharide) (4g/kg) or GOS (galactooligosaccharide [Bimuno]) (4g/kg), for 5 weeks (n=8/group). This dosing regimen was based on previous studies (Anthony et al; Food Chem Toxicol.; 44 (6); 819-26 (2006)) and pilot data showing that these optimal prebiotic doses provides maximum microbiota growth (not shown). All animals were sacrificed, trunk blood collected and their brains removed, twenty-four hours after the last gavage. Blood was centrifuged to obtain plasma, and the frontal cortex and hippocampus were dissected out from half of the harvested brains. Whole brains and isolated regions were snap-frozen in isopentane on dry-ice and stored with plasmas at -80°C prior to use.

25

BDNF analysis

Cortex and hippocampus tissue from all groups were homogenized in RIPA (radio immunoprecipitation assay) buffer (1:10 w/v, Sigma Aldrich, UK) containing protease inhibitors ('Complete-Mini', Roche). Protein concentrations were determined using the Bradford reagent (Sigma, UK). Samples of protein extracts were diluted 1:5 v/v in deionised water, prior to their analysis with a commercial BDNF ELISA kit (BDNF Emax immunoassay system, Promega UK).

30

Western blotting

Equal concentrations of protein extracts of cortex, hippocampus or cerebellum (20µg) from prebiotic and control groups were mixed with loading buffer (50mM 1, 4-dithiothreitol and 0.025% bromophenol blue), and fractionated with a molecular weight marker (GE Healthcare, Buckinghamshire, UK) by electrophoresis on pre-cast 7.5% SDS/polyacrylamide gels (Biorad, UK), and trans-blotted onto polyvinyl difluoride (PVDF) membranes (Immobilon-P, Millipore, Watford, UK).

The membranes were blocked with 5% (w/v) non-fat milk in PBS (phosphate buffered saline) containing 0.1% Tween (PBST) for 45min, and then incubated for 1 h at room temperature in incubation buffer (PBST with 2% [w/v] milk) containing a primary antibody (diluted 1:1000) against one of three NMDAR subunits: NR1 (AB9864, Millipore, UK), NR2A (AB1555, Millipore, UK) and NR2B (AB15362, Millipore, UK), and b-actin (Sigma-Aldrich, UK, diluted 1:50,000). Membranes were then washed three times for ten minutes in PBST and incubated for 30min in HRP (horseradish peroxidase)-linked secondary antibody in blocking buffer. Immunoreactive bands were visualized by chemiluminescence using the ECL-Plus kit (GE Healthcare, Buckinghamshire, UK), and apposing membranes to X-ray film (Kodak BioMax AR film). All antibodies produced a single band of expected molecular weight. The optical densities (OD) of bands were measured using the Alphamager 3400, and the data expressed as OD ratios of phosphorylated: total NMDAR subunit, or total NMDAR subunit:b-actin.

In situ hybridization histochemistry (ISHH)

The frozen rat brain hemispheres were coronally sectioned (14µm) on a cryostat, thaw mounted on to Superfrost-plus slides (Fisher Scientific) and stored at -80°C. Sections containing frontal cortex were pre-treated as described (Burnet et al; Mol. Cell. Neurosci.; 46; 167-75; (2011)).

Commercially synthesized (MWG, UK) oligodeoxyribonucleotides complementary to: BDNF (bases 883-927, NM001270630.1), NR1 (bases 746-780, NM008169.1), NR2A (bases 1642-1676, NM008170.2) or NR2B (bases 1758-1792, NM010350.2) were used in an establish ISHH method (Eastwood et al.; J. Psychopharmacol.; 21; 635-644; (2007)). Oligodeoxyribonucleotide probes were 3'-end labelled with [³⁵S]-dATP using terminal deoxynucleotidyl transferase (Promega, UK). Probes were diluted in hybridization buffer, pipetted onto the tissue sections

(1×10^6 cpm/section), cover-slipped and then incubated for >16hrs at 34°C lidded Perspex trays lined with filter paper soaked with 4 x SSC (saline sodium citrate)/50% formamide.

Post-hybridization washes included: 2x SSC rinse at room temperature to remove cover-
5 slips; 0.5x SSC, 20min (x3) at 55°C; 0.5x SSC 30min (x2) at room temperature. Slides were rinsed in ddH₂O, dried and apposed to X-ray film (Kodak, Biomax MS) for 7-28 days with ¹⁴C-microscales. Average grey densities across the depth of the frontal cortex grey matter were measured for each of the mRNAs using computer-assisted image analysis, and converted to nCi/mg tissue using ¹⁴C-microscale standards.

10

HPLC analysis

Small fragments of the cortical tissue (50mg) were individually homogenized in ice-cold methanol (1:10w/v) and microfuged at 13200 rpm for 10 minutes at 4°C. Supernatants (10μl) were injected onto a Hewlett-Packard 1100 liquid chromatograph (Agilent Technologies, Palo Alto,
15 CA) and subjected to online, pre-column, derivatization as previously described (Grant et al; J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.; 844; 278-282 (2006)). Briefly, samples (10μl) were reacted with an equal volume of derivatizing reagent [o-phthalaldehyde (2 mg) and Boc-L-cysteine (2 mg) in 0.2ml of methanol and 0.8ml of 0.4M of sodium borate buffer (pH=9)], for 5 min prior to column separation. Separation was achieved using an Agilent Zorbax Eclipse XDB-C18
20 column (4.6 x 150 mm, 5μm) maintained at 30°C and a separation protocol similar to that of (Morikawa et al; J. Chromatogr. B. Biomed. Sci. Appl.; 757; 119-25 (2001)). The mobile phases consisted of acetonitrile (phase A) and 100mM Sodium acetate buffer pH=6 (phase B) and were pumped through the column at 1.4 ml/min. The following gradient system was used (min/% B): 0/91, 35/84, 65/84. Detection of derivatized amino acids was by fluorescence detection (emission:
25 443 nm; excitation 344nm). Eight point calibration curves of the D- and L-amino acids (Sigma Aldrich, UK) were constructed using authentic standards (0.5 to 1000 pmol) and in each case were found to be linear with correlation coefficients of >0.995.

Data analysis

30 All data were expressed as mean $\pm$ standard error of the mean (SEM). Statistical comparisons between groups were performed with one-way ANOVA followed by *post hoc* analysis (Tukey HSD).

RESULTS

***Bifidobacteria* in faecal pellets from control and prebiotic rats**

The numbers of *bifidobacteria* in faecal pellets (expressed as log₁₀/g) from FOS-fed rats were significantly greater than controls (9.498 ± 0.025 vs 9.354 ± 0.055 , $p < 0.05$), whereas the
 5 density of *bifidobacteria* from GOS-fed animals were significantly greater than both controls (9.624 ± 0.05 vs 9.354 ± 0.055 , $p < 0.01$) and FOS-fed rats (9.624 ± 0.05 vs 9.498 ± 0.025 , $p < 0.05$).

The effect of prebiotics on BDNF and NR1 in the rat frontal cortex and hippocampus

The levels of BDNF protein in extracts of frontal cortex did not differ between groups (Fig
 10 1A). However, BDNF in hippocampal extracts of FOS administered rats were significantly higher than those of control and GOS fed animals. Western blots revealed that GOS-fed rats contained significantly greater levels of NR1 immunoreactivity in the frontal cortex compared to control and FOS animals (Fig 1B). Analysis of the hippocampus, however, revealed that FOS rats contained
 15 significantly more NR1 subunits than the other groups, though an increased trend ($p = 0.055$) was observed in GOS animals relative to controls.

The effect of prebiotics on NR2A and NR2B subunits in the rat frontal cortex and hippocampus

On western blots hippocampal, but not cortical, extracts from GOS-fed animals, contained significantly greater NR2A immunoreactivity compared to the other two groups (Fig 2). The level of
 20 NR2B in the frontal cortex and hippocampus, was not affected by prebiotic feeding.

The effect of prebiotics on BDNF and NR subunit mRNAs in the hippocampus

Prebiotic administration increased the abundance of BDNF (Fig 3A, C, E and Fig 4A) and NR1 (Fig 3B, D, F) mRNAs in the dentate gyrus of the hippocampus, relative to controls. A
 25 reduction of BDNF mRNA in the CA3 subfield of GOS-fed rats was also observed (Fig 3C). Densitometry confirmed significantly greater BDNF and NR1 expression in the dentate gyrus of prebiotic rats (Fig 4A, B). The administration of GOS resulted in an elevation of NR2A (Fig 4C), but not NR2B (Fig 4D), mRNA in the dentate gyrus and CA1 subfield relative to controls and FOS-fed
 30 animals.

Faecal, plasma and brain amino acid concentrations after prebiotics

This study tested whether an elevation of gut bacteria increased central D-alanine concentrations by elevating the amounts of this D-amino acid in the gut and the circulation. The

concentrations of free D-alanine in faecal pellets of GOS fed rats were significantly greater than control and FOS animals, with FOS administration resulting in intermediate levels of this D-amino acid (Table 1). Both prebiotics or GOS alone elevated other amino acids including D-serine and glutamate. In plasma D-alanine levels were significantly higher in GOS-fed rats compared to control animals (Table 1), and a slight, though not significant ($p=0.086$), increase was observed in FOS-fed rats. Prebiotic administration did not alter the concentrations of other circulating amino acids (Table 1). Rats fed with GOS had a significantly higher concentration of D-serine in the frontal cortex compared to controls (Table 2), though the levels of all other amino acids in both the cortex and hippocampus did not change after prebiotic feeding. There was an overall significant correlation between the levels of cortical D-serine and NR1 protein (Pearson's $r = 0.684$, $p=0.01$). Individual group analysis revealed that this association was only significant after GOS feeding (GOS: $r = 0.96$, $p=0.04$; FOS: $r = 0.68$, $p=0.32$; water: $r = 0.01$, $p=0.989$).

15

20

25

Table 1 Amino acid concentrations in rat faecal pellets and plasma following the repeated oral administration of water or prebiotic. *p<0.05 compared to water; +p<0.05 compared to FOS

5	Amino acid	[Amino acid] (nmol/g faeces) & (nmol/ml plasma)					
		water		FOS		GOS	
	<i>Faecal pellets:</i>						
	L-alanine	7.8 ± 0.3		12.5 ± 0.8*		18.7 ± 1.8**	
10	D-alanine	4.2 ± 0.1		5.9 ± 0.2		9.0 ± 0.9**	
	L/D-alanine	1.9 ± 0.1		2.1 ± 0.1		2.1 ± 0.1	
	Glutamate	13.1 ± 1.2		20.9 ± 1.2		32.4 ± 3.4**	
	Glutamine	1.6 ± 0.1		2.8 ± 0.2*		4.2 ± 0.4**	
	Glut/Gln	8.0 ± 0.2		7.4 ± 0.2		7.8 ± 0.1	
15	L-serine	3.9 ± 0.2		7.0 ± 0.8		10.7 ± 1.1**	
	D-serine	0.1 ± 0.01		0.2 ± 0.01*		0.2 ± 0.02*	
	L/D-serine	39.0 ± 1.1		35.0 ± 2.5		53.5 ± 2.2*	
	<i>Plasma:</i>						
	L-alanine	299.9 ± 22.1		308.6 ± 21.8		310.0 ± 21.7	
20	D-alanine	4.0 ± 0.5		5.2 ± 0.4		6.1 ± 0.5*	
	L/D-alanine	79.2 ± 8.8		61.7 ± 6.9		49.3 ± 1.7*	
	Glutamate	82.6 ± 6.6		78.9 ± 7.3		68.7 ± 1.6	
	Glutamine	419.0 ± 14.1		420.9 ± 18.7		411.2 ± 17.9	
	Glut/Gln	0.2 ± 0.02		0.2 ± 0.01		0.2 ± 0.01	
25	L-serine	141.8 ± 7.8		142.9 ± 5.3		144.0 ± 6.8	
	D-serine	2.0 ± 0.1		1.9 ± 0.1		2.0 ± 0.1	
	L/D-serine	72.2 ± 2.4		74.5 ± 1.1		72.5 ± 1.6	

Table 2 Amino acid concentrations in the rat cortex and hippocampus following the repeated oral administration of water or prebiotic. . *p<0.05 compared to water

5	Amino acid	water	[Amino acid] (pmol/mg tissue)	
			FOS	GOS
	Frontal Cortex:			
	L-alanine	90.5 ± 7.9	100.9 ± 8.4	108.6 ± 6.6
10	D-alanine	2.6 ± 0.4	2.4 ± 0.2	2.3 ± 0.3
	L/D-alanine	37.7 ± 5.9	42.8 ± 3.2	42.2 ± 2.0
	Glutamate	783.9 ± 75.6	837.3 ± 48.0	822.3 ± 47.9
	Glutamine	618.9 ± 52.7	645.4 ± 44.7	700.5 ± 44.0
	Glut/Gln	1.27 ± 0.06	1.32 ± 0.1	1.18 ± 0.03
15	L-serine	141.4 ± 13.6	152.8 ± 6.0	161.8 ± 10.9
	D-serine	40.3 ± 2.9	48.7 ± 1.7	53.6 ± 3.4*
	L/D-serine	3.5 ± 0.23	3.1 ± 0.07	3.0 ± 0.14
	Hippocampus:			
	L-alanine	99.7 ± 9.5	92.0 ± 6.2	97.5 ± 9.3
20	D-alanine	2.8 ± 0.4	2.6 ± 0.2	2.3 ± 0.2
	L/D-alanine	36.9 ± 3.9	36.5 ± 3.7	42.0 ± 2.5
	Glutamate	705 ± 79.8	649 ± 59.3	643.9 ± 68.1
	Glutamine	545.1 ± 58.2	480.1 ± 52.6	504.6 ± 48.0
	Glut/Gln	1.30 ± 0.04	1.38 ± 0.09	1.27 ± 0.05
25	L-serine	121.4 ± 12.4	111.4 ± 7.8	111.9 ± 9.3
	D-serine	37.4 ± 4.1	34.6 ± 2.1	35.3 ± 3.4
	L/D-serine	3.3 ± 0.03	3.2 ± 0.04	3.2 ± 0.08

DISCUSSION

We observed 1) greater hippocampal BDNF levels in FOS fed rats compared to GOS fed rats and control animals, though BDNF mRNA was increased in the dentate gyrus of both FOS and GOS fed rats 2) elevated NR1 protein in the frontal cortex of GOS fed rats, and in the hippocampus of prebiotic-fed animals; 3) higher levels of NR2A protein and encoding mRNA in the hippocampus of GOS fed rats compared to the other groups. Based on the above pattern of effect it is clear that the effect of GOS is not based on its prebiotic properties but rather it is linked to the chemical structure of the saccharides in the GOS mixture.

10 Prebiotics increase hippocampal BDNF in the rat

The elevated expression of BDNF and encoded protein in rats fed with FOS, is consistent with the effect of a *bifidobacterium* probiotic (Bercik et al; Neurogastroenterol Motil.; 23; 1132-9 (2011b); O'Sullivan et al; Benef Microbes; 2(3); 199-207 (2011)) and the selective proliferation of these species with antimicrobials (Bercik et al; Gastroenterology; 141; 599-609 (2011a)). Thus, FOS administration may have augmented the colonization of the *B.breve*, *B.longum* and/or similar psychotropic strains, within the moderate overall increase in *bifidobacteria* densities relative to GOS fed rats (see Results). In view of these observations therefore, it was surprising that GOS did not alter the levels of hippocampal BDNF protein and, moreover, by a greater magnitude than FOS. We have demonstrated that GOS feeding led to a reciprocal change in BDNF mRNA in the dentate gyrus and CA3 region of the hippocampus. An elevation of BDNF gene expression in the dentate gyrus has been associated with antidepressant action (Kerman, I.A.; Am. J. Psychiatry; 169; 1137-40 (2012)). A similar elevation of BDNF mRNA after GOS administration is, therefore, in keeping with a potential antidepressant/anxiolytic property of gut bacteria (Bercik et al, 2011a).

25 GOS administration increases NR1 subunits in the rat cortex

The increased NR1 protein in GOS fed rats compared to control and FOS animals is consistent with or similar to an effect of the antidepressant, fluoxetine, a serotonin uptake inhibitor. Recent clinical studies suggest that blocking NMDARs has antidepressant effects (Autry et al; Nature; 475;91-5; (2011)). It is clear from the data that an elevation of cortical NR1 subunits requires a several-fold increase in *bifidobacteria*, which occurs without changes in the levels of NR2A and NR2B subunits.

Overall, GOS administration to rats appeared to have a more profound effect on NMDAR subunits than FOS. That is, GOS elevated NR1 protein and/or mRNA in both the cortex and hippocampus, and NR2A in the hippocampus, whereas FOS only elevated NR1 in the hippocampus.

5 **Relevance to brain health**

Overall, our findings have some relevance to the prevention and/or treatment of cognitive dysfunction and emotional disturbances in neuropsychiatric illness and aging. For instance, patients suffering from schizophrenia show treatment-resistant deficits in executive function including working memory, in which NMDARs are integrally involved (Coyle, J.T.; Schizophr. Bull.; 10 38; 920-6; (2012)). The augmentation of the *bifidobacteria* and *lactobacilli* by GOS, therefore, is an important adjunctive strategy to assist contemporary pharmacological and psychological therapies. Furthermore, cognitive decline during normal aging maybe prevented or hindered by the 'prophylactic' intake of GOS, since NMDAR preconditioning has neuroprotective effects (Sorriano et al; J. Neurosci.; 26; 4509-18; (2006)).

15

Example 4 – Human Study

Forty-five healthy volunteers received either one of two prebiotics (fructooligosaccharides [FOS] (Group A) or galactooligosaccharides [GOS] (Group C) or a placebo (Group B) (maltodextrin) for 3 weeks. Awakening salivary cortisol was sampled before and after treatment. On the final 20 day of treatment participants completed a computerised task battery assessing the processing of emotionally salient information (the Emotional Test Battery, ETB; Harmer et al; Am. J. Psychiatry; 161; 1256-1263; (2004)).

Awakening Salivary cortisol responses did not differ significantly between groups at 25 baseline but were significantly lower following GOS treatment compared with placebo and FOS (significant interaction between treatment group x day of sampling x sampling time point in a repeated measures ANOVA [$F(8,164)=1.20, p=.05$]). Analysis of the behavioural data revealed decreased attentional vigilance to negative vs positive information after GOS compared to placebo treatment (group x emotion x masking condition, [$F(2,41)=3.14, p=.05$]). The FOS treatment group 30 did not perform differently to the placebo group in the dot-probe task. There were no significant effects of prebiotic treatment on the remaining tasks of the ETB.

Our study demonstrates that the intake of GOS lowers cortisol secretion in healthy volunteers. In addition, GOS was shown to alter the processing of positive versus negative information as measured by attentional vigilance, which is believed to play a key role in anxiety and its modulation by anxiolytics (e.g. Browning et al; J. Psychopharmacol.; 21; 684-690; Murphy et al.; Int. J. Neuropsychopharmacol.; 12; 169-179; (2008)).

Example 5

Effect of a mixture of galactooligosaccharides on lipopolysaccharide (LPS) induced sickness behaviour, post sickness anxiety and altered cytokine levels in mice.

Material and methods

Animals, prebiotic administration and LPS injections

All experiments were carried out in accordance with UK Home Office Animals (Scientific Procedures) Act (1986) and under Home Office guide-lines. Male CD1 mice (25-30g, 6-8-week old, Harlan Orlac, UK), were housed 3 per cage (plexiglas cages 33x15x13 cms, LxWxH) and maintained under standard controlled laboratories conditions (12-h light-dark cycle, lights on at 7a.m., 21+/- 1°C, humidity 50+/-5%). After 4-5 days habituation to the animal facility, mice were fed with standard mouse chow *ad libitum*, and provided, (in a weight-match, pseudo-random fashion), with either a prebiotic solution of 1.3% w/v mixture of galactooligosaccharides marketed commercially as Bimuno, available from Clasado Ltd. (UK), hereinafter referred to as BGOS, or water alone for drinking for 3 weeks. Pilot studies confirmed this BGOS dosing regimen optimally increased *Bifidobacteria* and *Lactobacilli* in the mouse gut (Clasado Ltd, UK). To avoid a potential cross-group contamination, the 2 diet groups were kept apart from each other. After 3 weeks, all animals received drinking water alone 24h prior to LPS injections and behavioural tests. A single injection of LPS (0.75mg/Kg) in saline (0.9%), or saline alone, was administered to mice by intraperitoneal injection, 4h before behavioural tests. Four groups (n=15 mice/group, 5 different cages per treatment) were therefore tested: 1) water-fed/saline injected; 2) water-fed/LPS injected; 3) BGOS-fed/saline injected; and 4) BGOS-fed/LPS injected. This experiment was repeated to provide a total of 30 mice per test group for analysis.

Locomotor activity (LMA)

Locomotor activity is reduced by LPS treatment (Skelly et al., (2013) PLOS One8:e69123) and thus, used as a measure of sickness behaviour. This test occurred 4h following LPS or saline injections. The set up was made of transparent plexiglas boxes (48x27x21cms, LxWxH, Photo Beam Activity
 5 Hardware and Software, Open Field San Diego Instruments) covered with a transparent plexiglas top (perforated for breathing) and containing a thin layer of sawdust bedding. Lighting of boxes was of about 60 lux. Each animal was gently placed at the corner of the boxes and allowed 2-h free exploration of the arena. Locomotor activity was recorded using photo-beams across the boxes and expressed as the number of break beams made by the animals over time. The number of fecal
 10 pellets was counted by the experimenter at the end of the test and animals were returned to their home cage to rest before the next behavioural testing.

Marble burying

This test is used to screen anxiolytic and antidepressant drugs and assess anxiety and obsessive-compulsive behaviour, based on the innate behaviour of mice to bury objects in a stressful
 15 situation; it was conducted as previously described (Deacon R.M.; Nat. Protoc.; (2006); 1 (1); 122-124., Nicolas et al.; Eur.J. Pharmacol.; (2006); 547; 106-115). LPS treatment, and the related LPS-induced sickness behaviour, induce a reduction in the number of marbles buried by the mice (Njung'e & Handley; Pharmacol. Biochem. Behav.; (1991); 38 (1); 63-67). Marble burying was conducted 7h following LPS/saline injection. Twenty marbles were placed on top of 5-cm sawdust
 20 bedding in transparent plastic cages (44x28x12 cms, LxWxH), in 5 lines of 4, 2cms away from each other and 2cms away from the edge of the cages. Testing occurred under normal room lighting, (~100 lux at 1m above the floor) and as previously described (Jacobson, L. et al.; Pharmacol. Biochem. Behav.; (2007); 15 (4); 619-626) and using the recommendations from (Deacon, R., 2006). Each animal was gently placed in the cage with the marbles for 30min, after which the
 25 number of marbles buried to at least 2/3 of their surface was counted.

Light-dark box

This test is also used to assess anxiety behaviour and based on the conflict mice face between their attraction for novelty and their fear for bright open arenas (Bourin, M. and Hascoet, M.; Eur. J. Pharmacol. (2003); 463 (1-3); 55-65; O'Leary, T.P. et al; J. Neuroscience Methods; (2012); 203;
 30 315-324. doi : S0165-0270 (11) 00594-2). Mice that are less anxious spend more time in fearful areas, i.e. the light part; mice that are more anxious spend more time in the safe dark part. LPS

treatment has been shown to increase anxiety behaviour in this test (Bassi et al; Basic Clin. Pharmacol. Toxicol.; (2012); 110 (4); 359-369). This test was conducted 24h following LPS/saline injections.

The set up was made of 2 painted wood compartments, a small black one (21x16x16 cms, LxWxH, with a small opening for access to the light part, 3x2.7cms, WxH) and a bigger bright one (46.5x21x21cms, LxWxH). Testing occurred under a slightly dim light of 50 lux inside the bright compartment of the box and was conducted as previously described (Strekalova T. et al.; Neuropsychopharmacology; (2004); 29; 2007-2017). Each animal was gently placed in the dark part of the light-dark box and let free to explore the whole box for 5min. The latency to leave the dark part, number of transitions between the dark and lights parts and the time spent in the light part were measured. The criterion to enter any compartment was 4 paws in. Mice were placed back to their home cage with cage mates at the end of the procedure. The box was cleaned with a tissue slightly impregnated with 10% alcohol between each animal in order to remove odour cues without creating overt alcohol odours. There was no background noise in the room and the experimenter stayed in the room for live scoring. Animals are deemed more anxious, and thus, affected by LPS injection, if they display a higher latency to enter the light part, a lower number of transitions between compartments and a lower time in the light area.

Tissue collection

Animals were sacrificed between 12-1p.m., 3h following behavioural testing. Whole brain was immediately harvested and snap-frozen in cold isopentane on dry-ice (Sigma-Aldrich, UK) before storage at -80°C until further molecular analysis. Trunk blood was collected in potassium EDTA (Ethylene Diamine Tetra Acetic Acid) tubes and spun for 15min at 5000rpm. Plasma was isolated and stored at -80°C for further corticosterone analysis. Fecal pellets were collected from each cage throughout the study in 70% glycerol in PBS (phosphate buffered saline) and stored at -20°C for further bacterial count.

Data analysis

Data were analysed using SPSS software (version 19). Data normality was tested using a Kolmogorov-Smirnov test. Locomotor activity was assessed with a 2-way ANOVA, and all other data with one-way ANOVA (or Kruskal-Wallis for non-parametric data) followed by Tukey post-hoc test. All data are expressed as mean $\pm$ standard error of the mean (SEM) and the threshold for statistical significance was set at $p < 0.05$.

Results

Effects of BGOS on the immediate LPS-induced sickness behaviour: locomotor activity and marble burying

Water-fed animals displayed a lower locomotor activity following LPS injections, compared with saline (Fig. 6A, time effect, $F(5,260)=142.12$, $p<0.0001$; LPS injection effect ($F(1,52)=3.61$, $p=0.063$; interaction time x LPS injection $F(5,260)=5.12$, $p<0.001$). Post-hoc test revealed that water-LPS animals travelled significantly less distance than their saline counterparts at 30 and 40-min time-points (both $p<0.05$). BGOS abolished LPS effect on locomotor activity (Fig.6B) as there was still an effect of time ($F(5,260)=113.01$, $p<0.0001$), but no effect of LPS injection ($F(1,52)=1.12$, $p=0.3$) and no interaction time x LPS injection ($F(5,260)=0.12$, $p=0.99$). BGOS did not induce any difference in locomotor activity in saline animals compared with water saline group.

In the marble burying test (Fig.7), LPS had a significant effect on mice behaviour ($H(df=3)=13.79$, $p<0.01$), which was not reversed by BGOS, as both water ($p<0.05$) and BGOS ($p<0.05$)-treated animals which received LPS buried less marbles than their saline counterparts. BGOS did not induce any difference in the number of marbles buried in saline animals, compared with water saline group.

Effects of BGOS on the delayed LPS-induced anxiety behaviour: light/dark box

LPS increased anxiety behaviour in water-fed animals (Fig.8). This effect was abolished by BGOS, as assessed by the latency to light (Fig.8A, $H(df=3)=12.17$, $p<0.01$) and time in light (Fig.8B, $F(3,106)=4.71$, $p<0.01$). Indeed, post-hoc analysis revealed that water-LPS animals displayed a significantly 2-fold higher latency to light than their saline counterparts ($p<0.01$) but also than both saline- and BGOS-LPS animals (both $p<0.05$). Water-LPS animals also displayed significantly less time in the light part than all the other groups ($p<0.05$ water-LPS vs. all groups). However, there was no statistical difference between groups in the number of transitions between the dark and light parts (Fig.3C, $F(3,110)=1.7$, $p=0.17$). BGOS alone did not induce any difference in control mice, i.e. which received saline, in any of the parameters, compared with water-saline animals.

Effects of BGOS on immune parameters 24h post LPS: cytokines levels in the frontal cortex and plasma

In the frontal cortex, LPS induced changes in water, but not BGOS, animals (Fig.9) for TNF- α , IL-1 β and IL-6, but not for IL-10. Post-hoc analysis showed that water LPS animals displayed higher TNF-

α than all other groups ($p < 0.05$), higher IL-1 β ($p < 0.01$ vs. water saline and vs. BGOS saline, $p < 0.05$ vs. BGOS LPS) and higher IL-6 ($p < 0.05$ vs. water saline). Thus, cytokines levels for animals fed with BGOS, receiving either saline or LPS injection, were both similar to those of their control water saline counterparts.

- 5 In the plasma, LPS induced significant changes in water, but not BGOS, animals (Fig.10) for TNF- α , however there was no overall statistical difference between groups for IL-6 and IL-10, as well as IL-1 β , although for this latter, LPS induced a non-significant 2-fold increase in water animals compared with saline.

Discussion

- 10 The current study tested the influence of prebiotic (BGOS) intake on LPS-induced sickness behaviour, anxiety and cytokine expression in mice, and was based on the supposition that BGOS (Bimuno) affects brain function via the immune system. Our two key findings were: 1) BGOS fed mice did not manifest locomotor activity (LMA) deficits and anxiety after a single injection of LPS, compared to controls; and 2) the LPS-induced expression of pro-inflammatory mediators in the
- 15 plasma (Granulocyte colony-stimulating factor (G-CSF); chemokine (C-C motif) ligand 2 (CCL2); monokine induced by IFN γ , Chemokine (C-X-C motif) Ligand 9 (MIG)) and brain (TNF α) was suppressed by the ingestion of BGOS. Overall, our data support current notions that BGOS (Bimuno) plays an important role in the maintenance of brain health, and that a modification in the response to immune challenges, may underpin this action.

20 Example 6

Effect of BGOS on Cognitive Performance in Healthy Rats

Materials & Methods

- Normal Sprague Dawley rats were given water or a prebiotic solution of 1.3% w/v mixture of BGOS
- 25 for 3 weeks and then tested on the attentional set-shifting task (ASST) (see Bissonette, G.B. et al; Behavioural Brain Research; (2013); 250; 91-101) using standard protocols.

Results

Figure 11 shows that rats given BGOS for 3 weeks showed improved performance in the extra-
5 dimensional (ED) component of the ASST that is a measure of flexible learning. Execution of the
ED element as effortlessly as the intra-dimensional phase (ID/ED-shift) is indicative of increased
cognitive flexibility, a parameter which is impaired in the elderly. In Figure 11 #p<0.05 compared
to control ID and *p<0.05 compared to control ED.

10 Conclusion

Rats given BGOS show improved cognitive performance in a task dependent on the medical
prefrontal cortex, which is often impaired in psychiatric disorder and ageing.

CLAIMS

1. A galactooligosaccharide composition comprising disaccharides Gal (β 1-3)-Glc; Gal (β 1-3)-Gal; Gal (β 1-6)-Gal; Gal (1-6)-Gal; trisaccharides Gal (β 1-6)-Gal (β 1-4)-Glc; Gal (β 1-3)-Gal (β 1-4)-Glc; tetrasaccharide Gal (β 1-6)-Gal (β 1-6)-Gal (β 1-4)-Glc and pentasaccharide Gal (β 1-6)-Gal (β 1-6)-Gal (β 1-6)-Gal (β 1-4)-Glc for preventing or treating emotional disturbances in depressive disorders or anxiety disorders.
2. The composition according to claim 1 which is in the form of a powder, a tablet, a capsule, a liquid formulation, a syrup or a soft pastille.
3. The composition according to claim 2 wherein when in powder form it comprises from 1.35 to 9.6g of galactooligosaccharides in 1.65g to 20g of the powdered composition.
4. The composition according to claim 2 wherein when in powder form it comprises from 1.96g to 4.9g of galactooligosaccharides in 2.5g to 10g of the powder.
5. The composition according to claim 2 wherein when in powder form it comprises 2.7g to 2.75g galactooligosaccharides in 3.0g to 5.5g of the powder.
6. The composition according to claim 2 wherein when in syrup form it comprises from 1.35g to 9.6g of galactooligosaccharides in 2.1g to 25.29g of the syrup composition.
7. The composition according to claim 2 wherein when in syrup form it comprises 1.96g to 4.9g of galactooligosaccharides in 3.0g to 12.9g of the syrup.
8. The composition according to claim 2 wherein when in syrup form it comprises 2.7g to 2.75g of galactooligosaccharides in 4.1g to 7.25g of the syrup.
9. A use of a galactooligosaccharide composition comprising disaccharides Gal (β 1-3)-Glc; Gal (β 1-3)-Gal; Gal (β 1-6)-Gal; Gal (1-6)-Gal; trisaccharides Gal (β 1-6)-Gal (β 1-4)-Glc; Gal (β 1-3)-Gal (β 1-4)-Glc; tetrasaccharide Gal (β 1-6)-Gal (β 1-6)-Gal (β 1-4)-Glc and pentasaccharide Gal (β 1-6)-Gal (β 1-6)-Gal (β 1-6)-Gal (β 1-4)-Glc for preventing or treating emotional disturbances in depressive disorders or anxiety disorders.

10. A use of a galactooligosaccharide composition comprising disaccharides Gal (β 1-3)-Glc; Gal (β 1-3)-Gal; Gal (β 1-6)-Gal; Gal (1-6)-Gal; trisaccharides Gal (β 1-6)-Gal (β 1-4)-Glc; Gal (β 1-3)-Gal (β 1-4)-Glc; tetrasaccharide Gal (β 1-6)-Gal (β 1-6)-Gal (β 1-4)-Glc and pentasaccharide Gal (β 1-6)-Gal (β 1-6)-Gal (β 1-6)-Gal (β 1-4)-Glc for preparing a medicament for preventing or treating emotional disturbances in depressive disorders or anxiety disorders.

Figure 1

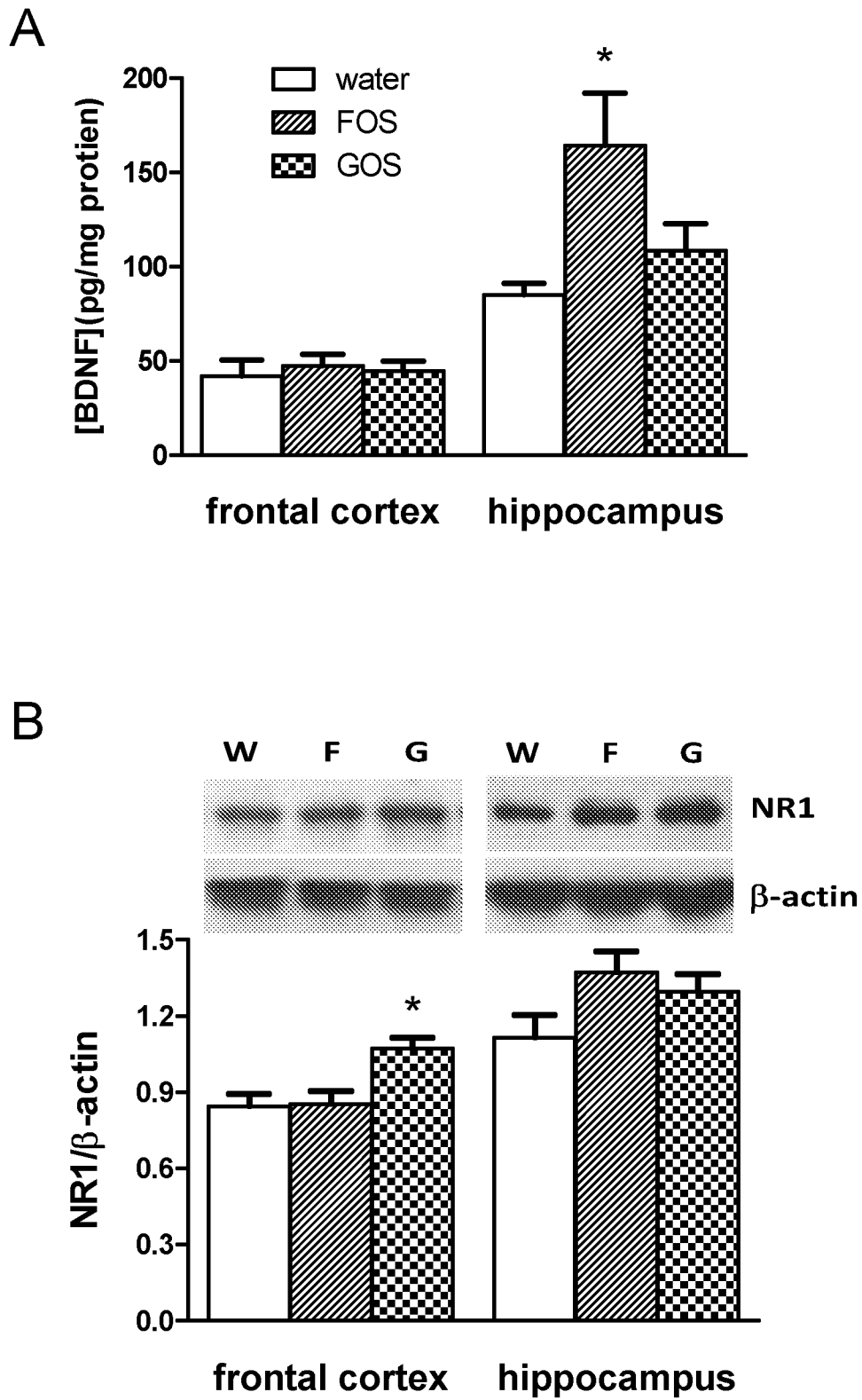


Figure 2

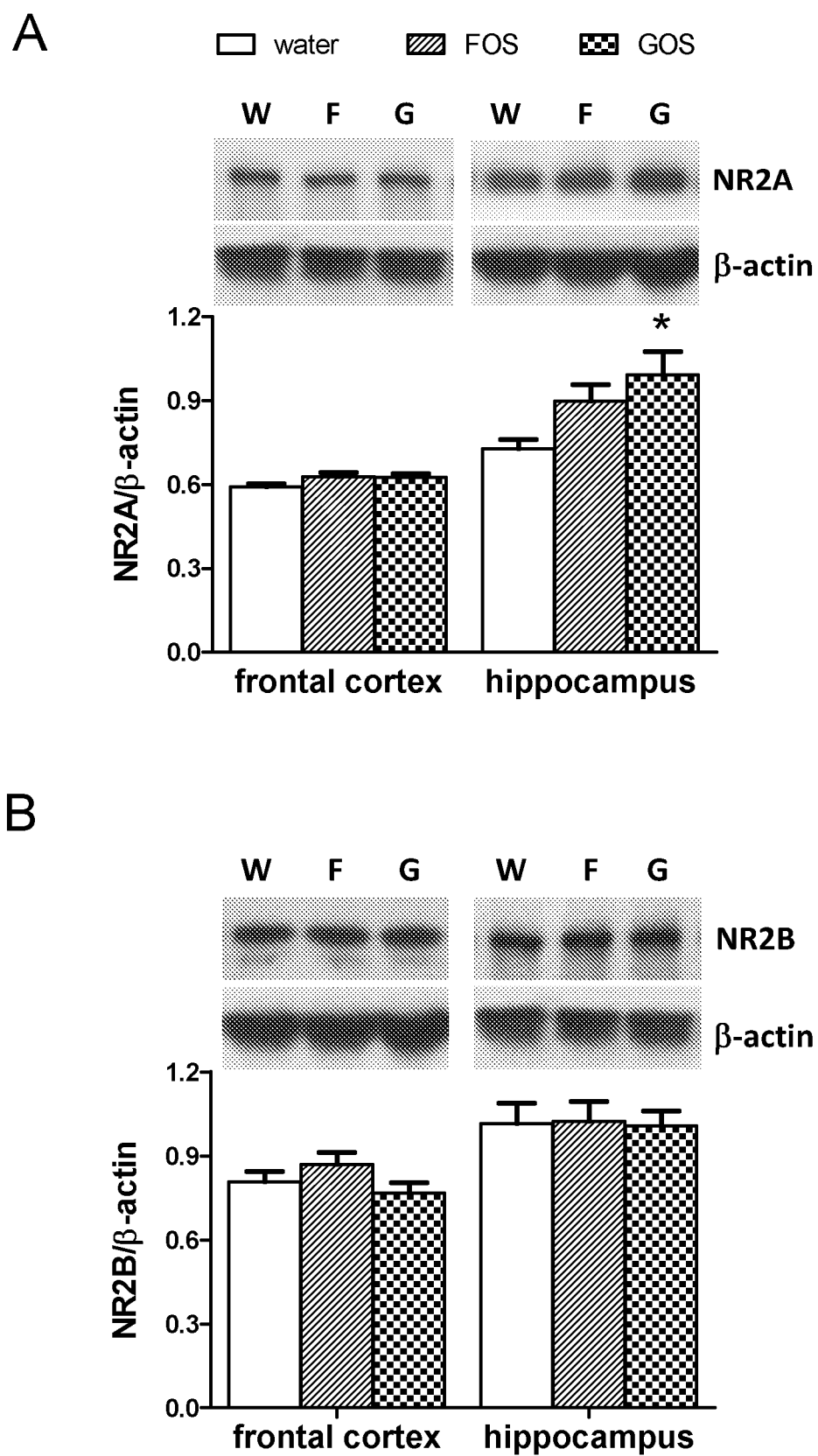


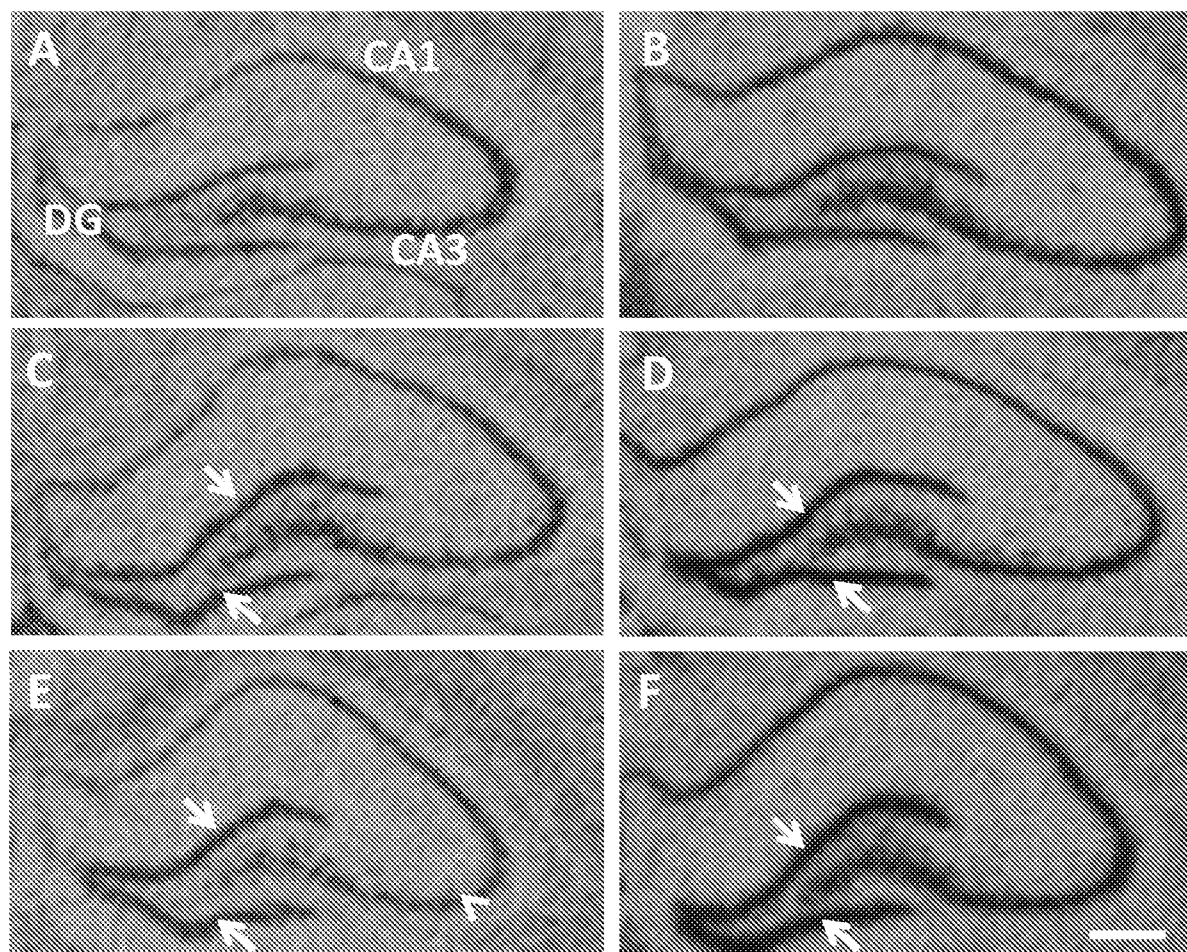
Figure 3

Figure 4

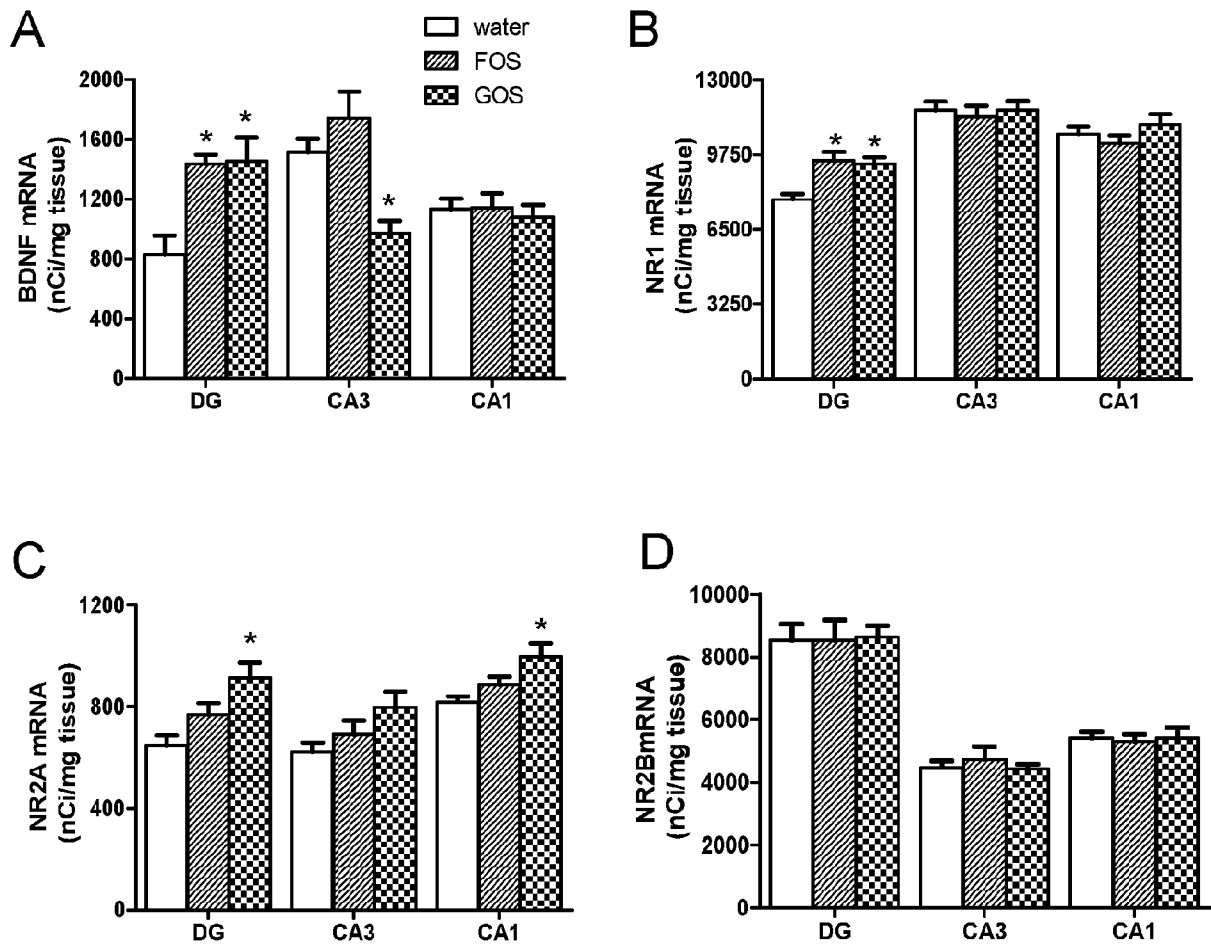
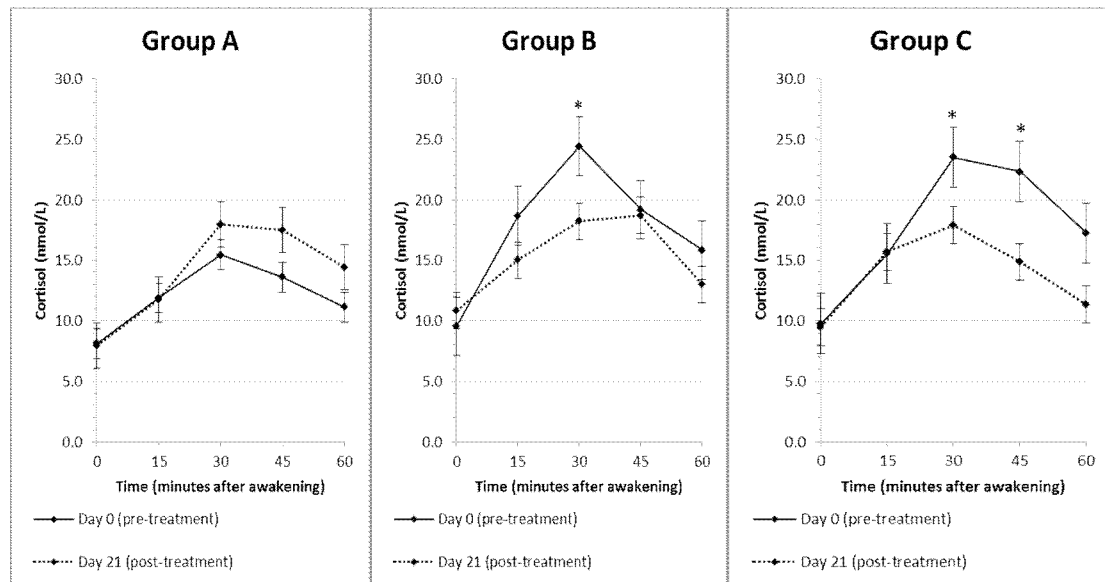


Figure 5:



*differences between Day 0 and Day 21 are significant at $p=0.05$ (for Time 30, Groups B and C), at trend at $p=0.1$ (Time 45, Group C)

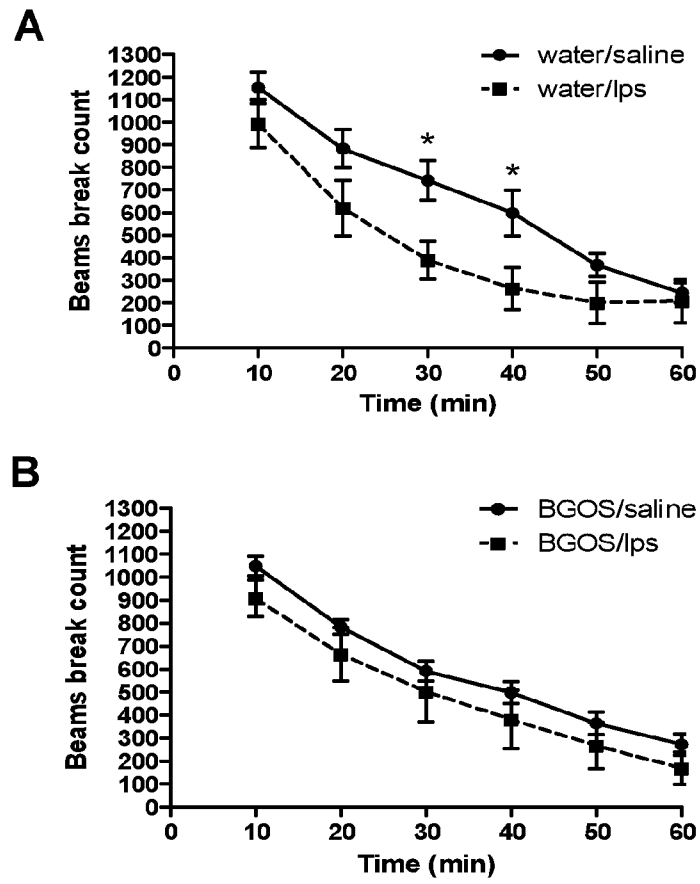
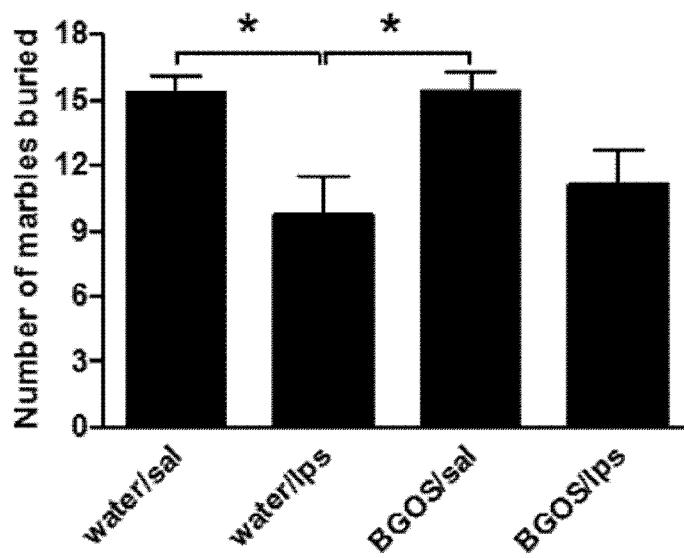
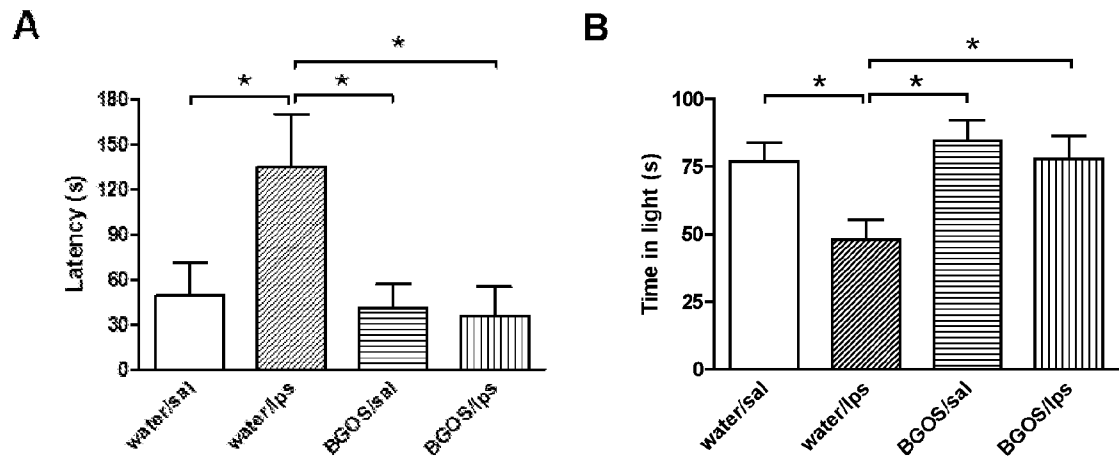
Figure 6**Figure 7**

Figure 8**Figure 9**

Frontal Cortex:

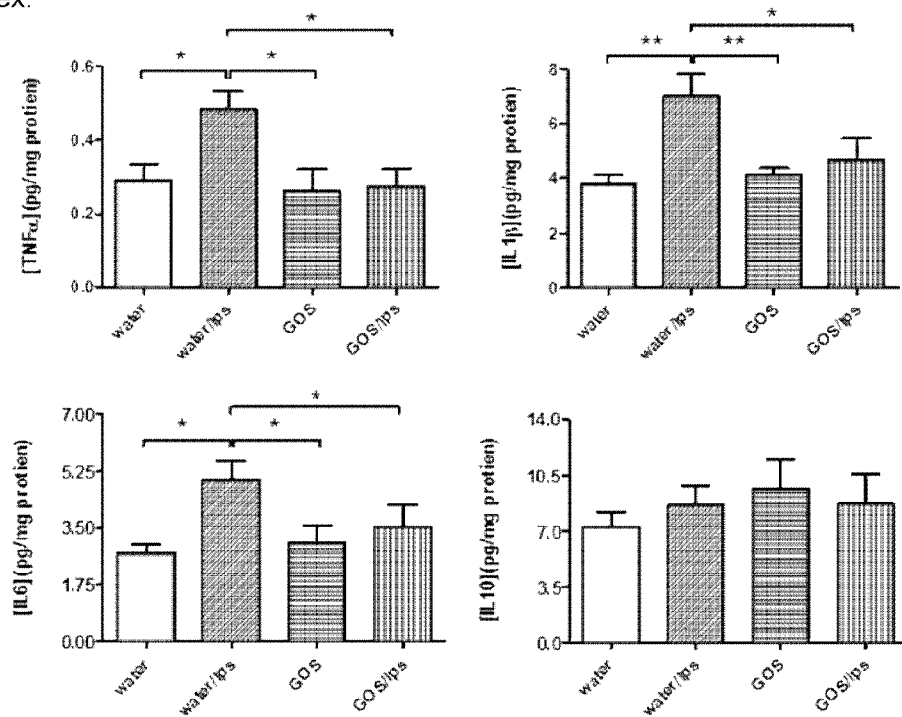
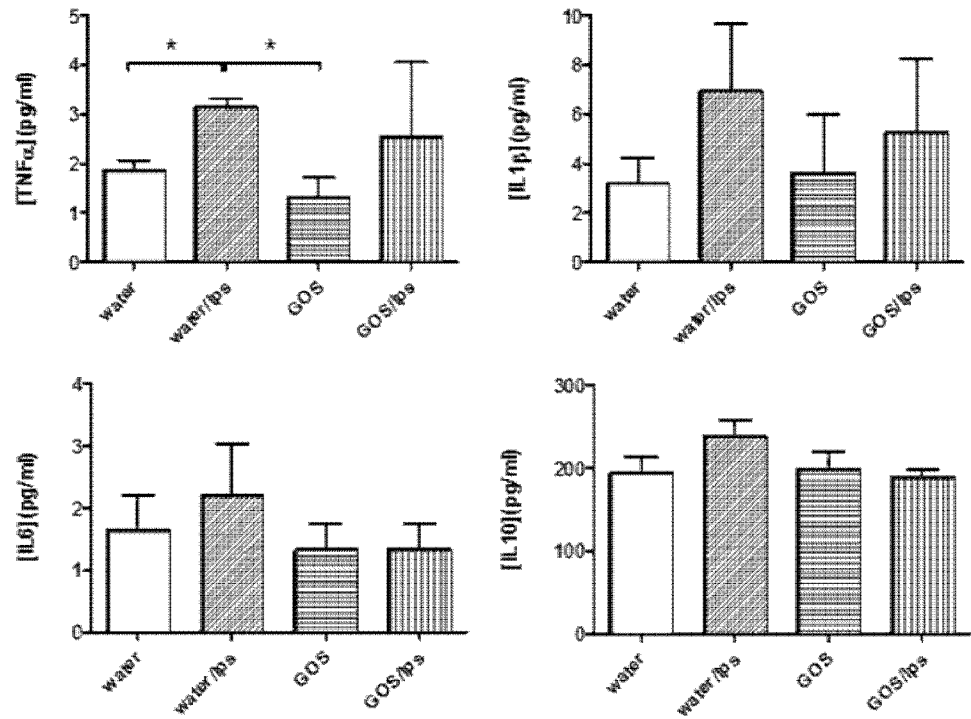
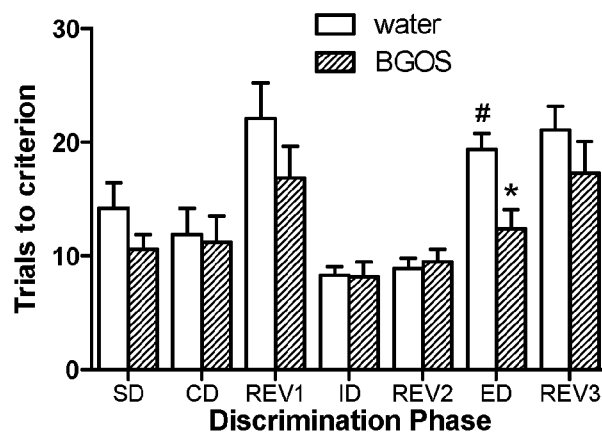
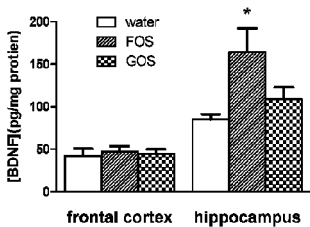


Figure 10

Plasma:

**Figure 11**

A



B

