Novel diagnostic method for diagnosing depression and monitoring therapy effectiveness

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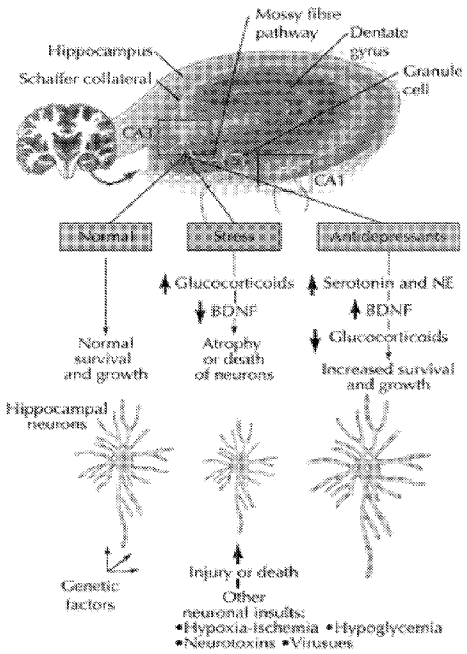
(54) Title: NOVEL DIAGNOSTIC METHOD FOR DIAGNOSING DEPRESSION AND MONITORING THERAPY EFFECTIVENESS

Fig. 1



(57) Abstract: The present invention relates to a method of diagnosing or monitoring the progression of a mood disorder, by assaying the level of lipocalin-2 in the body fluid of a subject. Preferably said mood disorder is depression, chosen from dysthymia, endogenous depression, minor depression, major depression, psychotic depression, neurotic depression, postnatal depression, burn out, overstrain, unipolar depression and bipolar depression. Also a combined detection of lipocalin-2 and BDNF for the method according to the invention is proposed.

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FIELD OF THE INVENTION

The invention relates to the field of diagnostics, more specifically diagnosis of affective disorders, more specifically diagnosis of depression, by assaying for a psychiatric disease-marker. Further, the invention relates to a method for monitoring the effect of antidepressant therapy, being medication, psychotherapy or a combination of both.

BACKGROUND

Disorders of the mood are often called affective disorders, since affect is the external display of mood or emotion which is, however, felt internally. Mood disorders are defined as mixtures of symptoms packaged into syndromes. These syndromes are consensus statements from committees writing the nosologies of psychiatric disorders for the *Diagnostic and Statistical Manual of Mental Disorders* (DSM) of the American Psychiatric Association (Table 1).

Diagnosis both in clinical practice and in clinical research studies is based on these sets of specific signs and symptoms. These criteria have helped distinguish various mood disorders that may have different causes and that certainly require different clinical management.

The most common and readily recognised mood disorder is major depression as a single episode or recurrent episodes. Dysthymia is a less severe but often longer-lasting form of depression, i.e. over two years in duration and often unremitting. Another type of mood disorder is bipolar disease, which is characterised by the occurrence of manic episodes besides depression.

There are no pathognomonic markers of depression, although this is an area of active research (Duffy A., 2000, Can. J. Psychiatr., 45:340-348).

Table 1: Diagnostic criteria for major depressive disorder*

A. The patient has depressed mood (e.g., sad or empty feeling) or loss of interest or pleasure most of the time for 2 or more weeks plus 4 or more of the following symptoms

Sleep	Insomnia or hypersomnia nearly every day
Interest	Markedly diminished interest or pleasure in nearly all activities most of the time
Guilt	Excessive or inappropriate feelings of guilt or worthlessness most of the time
Energy	Loss of energy or fatigue most of the time
Concentration	Diminished ability to think or concentrate; indecisiveness most of the time
Appetite	Increase or decrease in appetite
Psychomotor	Observed psychomotor agitation/retardation
Suicide	Recurrent thoughts of death/suicidal ideation

B. The symptoms do not meet criteria for a mixed episode (major depressive episode and manic episode)

C. The symptoms cause clinically significant distress or impairment in social, occupational, or other important areas of functioning

D. The symptoms are not due to the direct physiological effects of a substance (e.g., a drug of abuse, a medication) or a general medical condition

E. The symptoms are not better accounted for by bereavement

*Adapted from the *Diagnostic and Statistical Manual of Mental Disorders*, 4th edition.*

Depressive disorders are associated with poor work productivity, as indicated by a 3-fold increase in the number of sick days in the month preceding the illness for workers with a depressive illness compared with coworkers who did not have such an illness (Parikh, S.V. et al., 1996, J. Affect. Disord. 38:57-65; Kessler, R.C. et al., 1999, Health Aff. 18:163-171). Depressive illnesses also affect family members and caregivers (Denihan, A. et al., 1998, Int. J. Geriatr.Psychiatr. 13:691-694), and there is increasing evidence that children of women with depression have increased rates of problems in school and with behaviour, and have lower levels of social competence and self-esteem than their classmates with mothers who do not have depression (Goodman, S.H. and Gotlib, I.H., 1999, Psychol. Rev.

106:458-490). Depression is the leading cause of disability and premature death among people aged 18 to 44 years, and it is expected to be the second leading cause of disability for people of all ages by 2020 (Murray, C.J. and Lopez, A.D., 1997, The Lancet 349:1498-1504; Gredon, J.F., 2001, J. Clin. Psychiatr. 62:26-31).

Depressive illnesses have also been shown to be associated with increased rates of death and disability from cardiovascular disease (e.g. Pratt, L.A. et al., 1996, Circulation 94:3123-3129, Bush, D.E. et al., 2001, Am. J. Cardiol. 88:337-341). Among 1551 study subjects without a history of heart disease who were followed for 13 years, the odds ratio for acute myocardial infarction among the subjects who had a major depressive episode was 4.5 times higher than among those who did not have a depressive episode. Among consecutive patients admitted to hospital with an acute myocardial infarction who had their mood measured with a standard depression rating scale, even those with minimal symptoms of depression had evidence of higher subsequent risk of death following their infarction and over the next 4 months. This risk was independent of other major risk factors, including age, ventricular ejection fraction and the presence of diabetes mellitus.

Surprisingly, for such a common disease there is little agreement on the association between age and onset. This is due to the fact that research is hampered by the absence of an unambiguous and universally agreed on set of diagnostic criteria and the fact that many of the studies have included patients already in the medical care system. It is well known that many people who meet the diagnostic criteria for depression do not seek treatment. Despite its high prevalence, only one-third of all patients with depression receive adequate treatment (Judd, L.L. et al., 1996, Am. J. Psychiatry 153:1411-1417). The following are 4 common clinical errors that lead to diagnostic or treatment failures associated with depressive disorders:

· Insufficient questioning. Diagnostic failures occur when the patient is not asked questions that may elicit the symptoms of a mood disorder despite what should be a high index of suspicion based on its prevalence. The mnemonic "SIGECAPS" (*s*leep, *i*nterest, *g*uilt, *e*nergy, *c*oncentration, *a*ppetite, *p*sychomotor, *s*uicide) (Table 1) may be a useful clinical adjunct (i.e., 4 or more SIGECAPS for major depression, 2 or 3 SIGECAPS for dysthymia).

· Failure to consult a family member. Owing to the cognitive distortions associated with the disease, it is not unusual for patients to minimize or exaggerate their symptoms. Thus, in patients who are relatively new to one's practice, it is risky at best to make (or exclude) a diagnosis of depression without collateral information from a relative, such as a spouse or parent.

· Acceptance of a diagnosis of a mood disorder despite lack of diagnostic criteria (e.g., starting treatment for depression when only a "depressed mood" is present without the concomitant mental and physical symptoms [i.e., SIGECAPS]).

· Exclusion of a diagnosis or failure to start treatment for depression despite the associated symptom complex (e.g., "Of course you're depressed. Who wouldn't be depressed if these events were occurring in their life?" In other words, "explaining" the diagnosis rather than considering treatment options).

These clinical errors, coupled with the stigma associated with psychiatric conditions (Sirey, J.A. et al., 2001, Psychiatr. Serv. 52:1615-1620), result in the underdiagnosis of major mood disorders.

Another major hypothesis in the field of psychotherapy at present, is that recognition and treatment of both unipolar and bipolar depressions, causing all symptoms to remit for long periods of time, might prevent progression of the disease to more difficult states, emphasising that early recognition of mood disorder subtype is of great importance.

Taken all these data together, it is clear that there exists a major need for a reliable diagnosis of depression, or, alternatively, an assay that can confirm a diagnosis on basis of the SIGECAPS criteria.

Current theories of depression are complex. Currently at least three theories for major depressive disorder (MDD) have been postulated.

Monoamine hypothesis

Dysfunctions of monoaminergic systems (serotonin, norepinephrine and dopamine) may have a causal relation with MDD (Ruhe et al., 2007; Nutt et al., 2008). According to the monoamine theory, MDD is caused by an impaired monoaminergic neurotransmission, resulting in decreased extracellular

norepinephrine (NE) and/or serotonin (5-HT) levels (Schildkraut, 1965; Doris et al., 1999). Diminished concentrations of serotonin and its metabolites have been demonstrated in cerebrospinal fluid (Åsberg et al., 1984) and in post mortem brain tissue of depressed patients (Cheethman et al., 1989). There are also reports of altered platelet 5-HT transporter function (Nemeroff et al., 1994) and of 5-HT_2 receptor function in the brain (Arango et al., 1992) and platelets (Bakish et al., 1997). Studies investigating NE and 5-HT metabolites in cerebrospinal fluid, blood or urine of patients with MDD and post-mortem studies seem to support the monoamine hypothesis of MDD (Belmaker and Agam, 2008; Pryor and Sulser, 1991). Associations of MDD with functional polymorphisms in the gene that codes for the enzyme monoamine oxidase A (MAO-A), which degrades monoamines in the brain (Wild and Benzel, 1994Wild and Benzel, 1994) have also been reported (Chen and Ridd, 1999; Fan et al., 2010).

In the past two decades, however, investigations have revealed several limitations of the monoamine hypothesis (Hirschfeld et al, 2000). Moreover, research has failed to find convincing evidence of a primary dysfunction of a specific monoamine system in patients with MDD (Delgado et al, 2000).

Immune-inflammation hypothesis

Over the last two decades, new developments in psychiatric research have led to the hypothesis that inflammatory processes and neural-immune interactions are involved in the pathogenesis of major depression and that these might underlie some of the frequently observed serotonergic and adrenocortical correlates of MDD. This monocyte-T-lymphocyte or cytokine hypothesis of depression (Maes 1993, 1995a, 1995b, 1999; Schiepers et al., 2005) implies that pro-inflammatory cytokines, such as interleukin (IL)-1, tumour necrosis factor (TNF)-α and interferon (IFN)-γ, which act as neuromodulators, represent key factors in the (central) mediation of the

behavioural, neuroendocrine and neurochemical features of depressive disorders (Schiepers et al., 2005).

The central action of cytokines may also account for the HPA-axis hyperactivity frequently observed in depressive disorders, because pro-inflammatory cytokines may cause HPA-axis hyperactivity by disturbing the negative feedback inhibition of circulating corticosteroids on the HPA axis (van West and Maes, 1999; Leonard, 2001; Schiepers et al., 2005; Maes et al., 2008; Maes, 2010). Another link might be via TNF-α and cortisol, both influencing the phosphorylation of transcription factor cAMP response element binding protein (CREB) in T-lymphocytes (Koch et al., 2009).

Cytokines also influence monoaminergic neurotransmission, i.e. serotonin, norepinephrine and dopamine (Linthorst et al., 1995, Merali et al., 1997, Pauli et al., 1998 and Song et al., 1999, 2000; Lacosta et al., 2000). They might also reduce tryptophan (TRP) availability through activation of the TRP-metabolising enzyme indoleamine-2,3-dioxygenase (IDO). Thus, increased stimulation of IDO by cytokines may lead to depletion of serum TRP, which results in a significant reduction of 5-HT synthesis (Heyes et al., 1992; Stone and Darlington, 20021Full Text via CrossRefView Record in ScopusCited By in Scopus (177)Stone and Darlington, 2002), thus compromising 5-HT neurotransmission.

Characteristics of immune activation in depressive illness include increased serum levels of indicators of activated immune cells (e.g. neopterin, PGE2 and soluble IL-2 receptors), higher serum concentrations of C-Reactive Protein (CRP) as well as increased release of pro-inflammatory cytokines, such as IL-1, IL-2 and IL-6 by activated macrophages and IFN-γ by activated T cells (Maes et al., 1995a,b; Maes, 1999; Irwin, 1999; Nunes et al., 2002).

In line with the immune-inflammation hypothesis of depression, the increase in plasma concentrations of the pro-inflammatory cytokines IL-1 and IL-6 observed in patients suffering from depression seems to correlate with the

severity of this psychiatric disorder and with measures of HPA axis hyperactivity (Maes, 1995, 1999).

An important mediator of inflammation is prostaglandin E2 (PGE-2). An in vitro study reports increased PGE2 secretion from lymphocytes of depressed patients compared to healthy controls (Song et al., 1998). Increased PGE2 levels in saliva, serum and cerebrospinal fluid of depressed patients have previously been described (Linnoila et al., 1983; Calabrese et al., 1986; Ohishi et al., 1998; Nishino et al., 1989). Several other studies have demonstrated that an imbalance of the immune system in MDD results in an increased PGE-2 production and probably also an increased expression of cyclo-oxygenase (COX-2), which is the key enzyme in the synthesis of PGE. Moreover, these investigations reported a clinical antidepressant effect of certain COX-2 inhibitors in patients with MDD (Müller et al, 2006; Müller and Schwarz, 2008; Müller et al., 2010; Müller, 2010).

Neurogenesis and neuroplasticity hypothesis

Over the past decade, an increasing body of evidence has implicated neurotrophic factors in the pathogenesis of depression (Tanis et al., 2007)). Fig. 1 outlines some of the mechanisms underlying thishypothesis (Duman, R.S. et al., 1997, Arch. Gen. Psychiatry 54:597-608; Manji, H.K. et al., 2000, Mol. Psychiatry 5:578-593).

Stress, an important precipitant of depression, has been repeatedly shown to reduce neurogenesis and the expression of neurotrophic factor genes in the brain (Duman, 2004; Nibuya et al., 1995). Conversely, many antidepressant treatments stimulate neurogenesis and neurotrophic factor gene expression (Nibuya et al., 1995; Malberg et al., 2000).

We are now aware that "long-term" (i.e., 30 days) antidepressant treatment results in sustained activation of cyclic adenosine 3-5-monophosphate (cAMP) in specific brain regions. Protein kinase A, which is stimulated by cAMP, phosphorylates the cAMP regulatory element binding protein. This protein then regulates and activates specific target genes, including brain-derived neurotrophic factor (BDNF), a neuroprotective factor that

results in hippocampal nerve growth (Fig. 1). Furthermore, depressed patients show increased cellular atrophy in limbic and cortical areas of the brain, consistent with decreased neurotrophic activity (Duman and Monteggia, 2006). MRI scans of patients with depression have revealed a number of abnormalities in brain structures compared with healthy controls. Despite some inconsistencies, meta-analyses have shown clear evidence for smaller hippocampal volumes and an increased number of hyper-intensive lesions (Videbech et al, 1997, 2004). Furthermore, a series of brain-imaging studies consistently showed reduced neuronal activity in the dorsolateral prefrontal cortex that covaried with the severity of the depression (i.e., the more severe the depression, the larger the prefrontal deficits) (Drevets, W.C., 1998, Ann. Rev. Med. 49:341-361). Thus, an updated hypothesis on the development of a depressive disorder might posit that stress-induced vulnerability in genetically susceptible people may induce a cascade of intracellular neuronal mechanisms that increase or decrease specific neurotrophic factors necessary for the survival and function of specific brain neurons. Furthermore, not only antidepressants but also electroconvulsive therapy (Vaidya V.A. et al., 1999, Neuroscience 89:157-166) and depression-focused psychotherapy (Thase, M.E., 2001, Arch. Gen. Psychiatry 58:651-652) can affect neuronal growth and regional brain metabolism.

In 1982, brain-derived neurotrophic factor (BDNF), the second member of the "neurotrophic" family of neurotrophic factors, was shown to promote survival of a subpopulation of dorsal root ganglion neurons, and subsequently purified from pig brain (Barde, Y.A. et al., 1982, EMBO J. 1:549-533).

Among the neurotrophins, brain-derived neurotrophic factor (BDNF) has been most extensively studied in relation to depression. The results of several meta-analyses on BDNF confirm significant correlations between serum BDNF levels and depressive state as well as successful antidepressant therapy (Sen et al., 2008; Bocchio-Chiavetto et al., 2010; Brunoni et al., 2008). Recent studies clearly demonstrate that serum levels of BDNF are significantly decreased in patients with MDD and that antidepressant treatments are capable of reversing this effect, indicating that serum BDNF is a biomarker of

MDD and successful treatment (Bocchio-Chiavetto et al., 2010; Schmidt and Duman 2010; Dell'osso et al., 2010; Tadić et al., 2010).

However, BDNF levels in serum are influenced by various determinants, such as age, sex, smoking status, urbanicity, etc. (Bus, B. et al., 2011, Psychoneuroendocrinol. 36:228-239) and it appears that BDNF levels in the serum are unrelated to the clinical features of depression (Molendijk, M. et al., 2010, Mol. Psychiatr. 15:1-8). Surprisingly, it appeared that BDNF levels in the urine do provide a good indication of depression (WO 2011/002292).

Thus, there still remains need for further markers for diagnosis of mood disorders, such as depression.

SUMMARY OF THE INVENTION

Surprisingly, the present inventors now have discovered that the level of lipocalin-2, which is detectable in the body fluid of a patient, is indicative of the presence of mood disorders.

Thus, the invention relates to a method for the diagnosis of a mood disorder comprising:

a. taking a body fluid sample of a subject;
b. measuring the content of lipocalin-2 in said sample; and
c. diagnose the mood disorder if the concentration of lipocalin-2 is higher than in control healthy subjects.

Preferably the mood disorder is chosen from depression, schizophrenia, psychosis and anxiety, more preferably the mood disorder is depression, chosen from dysthymia, endogenous depression, reactive depression, minor depression, major depression, psychotic depression, neurotic depression, unipolar depression and bipolar depression, most preferably major depression.

Further, the invention comprises a method to determine the influence of antidepressant therapy, being medication, psychotherapy, or a combination of both, in a subject comprising:

a. Performing an assay method according to the invention;
b. Providing the subject with antidepressant therapy;
c. Repeat step a) with regular intervals during said treatment; and

d. Register any difference in the concentration of the measured compound in the body fluid.

Also the invention comprises a method to monitor the progress of a mood disorder in a subject comprising performing a method according to the invention. Further, the invention also relates to the use of lipocalin-2 in the diagnosis and monitoring of progression of a mood disorder.

Further part of the invention is an assay for the detection of depression in which both lipocalin-2 and BDNF are detected.

LEGENDS TO THE FIGURES

Fig. 1 A molecular and cellular model for the action of antidepressant treatments and the pathophysiology of stress-related disorders. This model of the hippocampus shows the major cell types in the hippocampus and how stress and antidepressant treatments may influence CA3 pyramidal cells. The 3 major subfields of the hippocampus (CA3 and CA1 pyramidal cells and dentate gyrus granule cells) are connected by the mossy fibre and Schaffer collateral pathways. Recent studies demonstrate that chronic stress decreases the expression of brain-derived neurotrophic factor (BDNF) in the hippocampus. This may contribute to the atrophy or death of neurons in the CA3 pyramidal cell layer of the hippocampus. Long-term elevation of glucocorticoid levels is also known to decrease the survival of these neurons. Other types of neuronal insult, such as hypoxia–ischemia, hypoglycaemia, neurotoxins and viral infections, may also cause atrophy or damage of neurons and thereby make a person vulnerable to subsequent insults. These types of interaction may underlie the observations of decreased function and volume of hippocampus in patients with affective disorders and may explain the selective vulnerability of certain people to become depressed. Long-term antidepressant treatments increase the expression of BDNF as well as tyrosine kinase receptor B (trkB) and prevent the down-regulation of BDNF elicited by stress. This may increase the growth or survival of neurons, or help repair or protect neurons from further damage. Increased expression of BDNF and trkB seems to be mediated by the sustained elevation of the serotonin and norepinephrine (NE) systems and the cyclic adenosine monophosphate cascade.

Normalization of glucocorticoid levels by long-term antidepressant treatments may also contribute to the recovery of CA3 neurons. (Adapted from Duman et al., *supra*).

DETAILED DESCRIPTION

In this application the term 'biomarker' is used for a distinctive biological or biologically derived indicator of a process, event or condition. Biomarkers can be used in methods of diagnosis, e.g. clinical screening, and prognosis assessment and in monitoring the results of therapy. They also can be used for identifying patients that are most likely to respond to a certain treatment, for drug screening and for development in medicine. Biomarkers and their uses are therefore valuable for identification of new drug treatments and for discovery of new targets for drug treatment. Further they are valuable for exploring dosage regimes and drug combinations.

Lipocalin-2 (LCN-2) is known under many names: siderocalin, α2-microglobulin-related protein, neu-related lipocalin, human neutrophil lipocalin (HNL), 24p3, SIP24 and neutrophil gelatinase-associated lipocalin (NGAL). The Lipocalin family comprises a diverse group of mostly secreted soluble proteins that bind hydrophobic ligands and act as transporters, carrying small molecules to specific cells. Lipocalins are related by possessing an 8-stranded beta-barrel structure. Lipocalin-1, also named tear lipocalin (TL), von Ebners gland protein (VEG) and tear pre-albumin, binds a large number of hydrophobic molecules and exhibits cysteine proteinase inhibitor and endonuclear activities. Lipocalin-2 is a component of granules in neutrophils from tissues that are normally exposed to microorganisms and is upregulated during inflammation. Lipocalin-2 can form homodimers and can heterodimerize with the neutrophil gelatinase MMP-9. The mature protein consists of a single protein chain of 178 amino acid residues and its molecular mass is 24-25 kDa in its glycosylated state (monomeric form). The protein

contains an intrachain disulfide bridge and the human forms contains an additional cysteinyl residue that is thought to participate in the formation of complexes with itself or with neutrophil gelatinase.

An important role of Lipocalin-2 in innate immunity is suggested by the demonstration that the protein tightly binds bacterial enterobactin siderophores, small iron-binding molecules that are synthesized by certain bacteria as a means of iron acquisition. Thus, it can act as a potent bacteriostatic agent in iron-limiting conditions. Lipocalin-2 expression further is induced upon activation of Toll-like receptors on immune cells and thus constitutes an acute phase response to infection.

Lipocalin-2 has often been suggested and/or used as marker for inflammation-related conditions or diseases. It has been suggested as marker for neutrophil activation, indicating bacterial infection (WO 95/29404). Further it has been proposed as a urinary or blood biomarker for detecting renal tubular cell injury and acute renal failure (US 2004/219603; US 2005/272101; WO 2006/066587). Lipocalin-2 has also been mentioned as biomarker for diagnosing the presence and/or development of cancer. Yang, J. et al. (2009, PNAS 106:3913-3918) mentioned the involvement of lipocalin-2 in breast cancer and evidence for the presence of the compound in lung metastases was shown by Shi, H. et al. (2008, J. Exp. Clin. Cancer Res. 27:83), while for epithelial ovarian cancer the use of lipocalin-2 as biomarker has been reported by Cho, H. et al. (2009, J. Histochem. Cytochem 57:513-521). Recently, lipocalin-2 has been named as an adjuvant biomarker for the diagnosis of cervix cancer tissues (Wang, P.H. et al., 2011, Reprod. Sci. 18:447-455).

Not yet published is the finding that cerebrospinal lipocalin-2 concentration can also serve as a biomarker for Alzheimer's disease and/or other forms of neurodegenerative diseases.

It was now surprisingly found that the levels of lipocalin-2 in body fluids are also indicative for mood disorders like depression, more specifically major

depression disorder. Especially in cases where the patient is suspected to be affected by a mood disorder a diagnosis on basis of the level of lipocalin-2 in a body fluid can be performed, e.g. to confirm the suspicion.

The body fluid in which lipocalin-2 is measured can be blood (which can be whole blood, serum or plasma), saliva or urine. Body fluid samples can be prepared in the usual way, diluted or concentrated where necessary, and stored as appropriate.

In the diagnostic assay of the invention the body fluid that is obtained from the patient preferably is peripheral blood serum or urine. Of these, urine is preferred, since this can be obtained with the least physical burden for the patient. A special advantage of an assay wherein the detection is performed on a urine sample is that no invasive techniques are necessary to obtain a sample from the patient. In principle, it would even be possible to perform the assay outside a clinical setting, i.e. at the subject's own premises.

As is shown in the experimental section, there is a difference in the lipocalin-2 level in urine between men and women. For a proper diagnosis the correct control values should be used or the level measured should be compared with a control sample of a healthy person of the same sex and preferably also a similar age. Further, to be able to compare the values measured in urine, where the concentration of the compounds can greatly differ on basis of the amount of water contained in the urine, preferably the ratio of lipocalin-2 with respect to a more or less constant reference compound is used. For measurements in urine advantageously the ratio between the compound to be analyzed (in this case lipocalin-2) and creatinine is used. This means that for men the diagnosis that the patient is suffering from a mood disorder (especially MDD) can be given when the level of lipocalin-2 in the urine corrected for creatinine (i.e. lipoclain-2/creatinine ratio) exceeds 0.1, more preferably when it exceeds 0.2, even more preferably when it exceeds 0.3, most preferably when it exceeds 0.4. For women a diagnosis can be established

when the lipocalin-2/creeatinine ratio exceeds 0.4, preferably when it exceeds 0.5, more preferably when it exceeds 0.75, most preferably when it exceeds 1.0.

Detection of lipocalin-2 can take place in any manner that would be known to a person of skill in the art. Several assay kits (for detection of lipocalin-2 in serum) are commercially available (e.g. from Abcam (Cambridge, UK), RayBiotech (Norcross, GA, USA), BioKits (Dublin, OH, USA) and many other suppliers), and these can easily be adapted for detection of lipocalin-2 in any body fluid. In principle most of these assays rely on an immunochemical reaction between the lipocalin-2 in the sample and a lipocalin-2-antibody (e.g. detectable through an ELISA assay). However, the invention is not limited to immunoassays. Analysis of lipocalin-2 in the sample of the patient may be carried out with chemical analytical methods (like mass spectrometry, MALDI-TOF, micro-Raman spectrometry), with magnetic radio imaging, flow cytometric analyses and all other quantitative analysis systems that are suitable for detecting proteins in fluids. Also receptor-based assays, using the lipocalin-2 receptor (24pR3, LRP2, see Devireddy, L.R. et al., 2005, Cell 123:1293-1305; and Hvidberg, V. et al., 2005, FEBS Lett. 579:773-777) as analytical tool may be used. The assays that are useful in the present invention are preferably quantitative assays, in which the concentration of lipocalin-2 in the sample can be determined. This can – in principle – be achieved with all of the above mentioned detection methods. For interpretation of the results of such an assay, various determinants such as sex, age, smoking status, urbanicity, food and alcohol intake should be taken into account, since these factors may affect the lipocalin-2 levels in the blood. Also it should be considered if the patient suffers from one or more of the conditions, which have been indicated to be related with changed lipocalin-2 levels, such as renal failure, cancer and Alzheimer's disease or any other neurodegenerative disease.

Most preferred is an assay based on an immunochemical reaction between the lipocalin-2 in the sample and a lipocalin-2-antibody which is made detectable and quantifiable through an ELISA assay.

Preferably, the assay, when performed on a urine sample, also includes a simultaneous assay for creatinine. Creatinine is one of the byproducts of protein metabolism. Under normal conditions it is present in the blood and is excreted as a final metabolite in the urine. Urine creatinine levels are routinely used as part of kidney function diagnosis. In particular, altered creatinine levels in urine are indicative of kidney diseases

such as acute or chronic nephritis, nephrosis, and the like. Because normative values for creatinine excretion have been established, urine creatinine levels are also useful for correction of assays for other compounds, as they document the adequacy of the urine collection for such assays. In particular, the creatinine correction can be used to correct for urine dilution, thus giving a possibility to standardize measured concentrations irrespective of the water content of the urine and/or the time of the day when the urine was produced. Further, changes in renal function, which influence rates of excretion, can be corrected by measurement of creatinine in urine.

Further, it is also preferred when the diagnosis on basis of lipocalin-2 will be affirmed with the diagnosis on basis of the presence of BDNF. As is shown in WO 2011/002292 the level of BDNF in the urine can be used to diagnose mood disorders, especially depression. Recently, it has been established that serum concentrations of BDNF can, under circumstances, also be used as a marker (Bus, B. et al., 2011, World J. Biol. Psychiatry, early online 1-9, posted online 19 January 2011).

Accordingly, the invention provides for a method for diagnosing the presence of a mood disorder in a patient by determining the levels of both lipocalin-2 and BDNF in a body fluid of the patient, preferably wherein said body fluid is urine. As is shown in WO 2011/002292, patients suffering from a mood disorder generally have a BDNF urine concentration in the range of 100 pg/ml to 1600 pg/ml, more specifically 100 pg/ml tot 600 pg/ml, whereas healthy persons have a BDNF urine concentration below 50 pg/ml. The assay for BDNF, when used in conjunction with the assay for lipocalin-2 increases the diagnostic performance of the lipocalin-2 assay. The assay for BDNF can be performed as described in the Examples of WO 2011/002292 or in any other way as will be known to the skilled person.

Preferably, detection and/or quantification of both biomarkers (lipocalin-2 and BDNF) may be performed using an immunological method, involving an antibody, or fragment thereof, capable of specific binding to the biomarker. Suitable immunological methods include sandwich immunoassays, such as

sandwich ELISA, in which the detection of the biomarkers is performed using two antibodies which recognize different epitopes on the biomarker; radioimmunoassay (RIA), direct, indirect or competitive enzyme linked immunosorbent assays (ELISA), enzyme immunoassays (EIA), fluorescence immunoassays (FIA), chemiluminescent immunoassays, western blotting, immunoprecipitation and any particle based immunoassay (e.g. using gold, silver or latex particles, magnetic particles, or Q-dots). Immunological methods may for example be performed on a microtitre plate or on test strips. It is also possible to perform the analysis with concurrent lab-on-a-chip techniques. For both markers commercial immunoassays are available. These markers can all be assayed in the same body fluid (e.g. blood or urine), but it is also possible that for some biomarkers one type of body fluid is preferred (e.g. BDNF is preferably measured in a urine sample). Thus if two or more markers are tested, it can be the case that one marker is tested in the blood and the other marker is tested in the urine. While interpreting the results of such an assay, various determinants such as sex, age, smoking status, urbanicity, food and alcohol intake should be taken into account.

Preferably, a panel of both lipocalin-2 and BDNF as markers will be provided in an assay for the diagnosis of a mood disorder, preferably depression, in a patient.

As indicated above, both markers can be used to affirm a diagnosis based on any other diagnostic tool (such as personal communications, cognitive testes, etc.).

In a further embodiment, the biomarkers lipocalin-2 and BDNF may be replaced by a molecule, or a measurable fragment of the molecule, found upstream or downstream of the biomarker in a biological pathway. The biomarkers of the present invention or their replacement molecule(s) are recognised by 'biosensors', which may comprise a ligand or ligands capable of specific binding to the biomarker. Such biosensors are useful in detecting

and/or quantifying the biomarker, preferably in quantifying. Especially useful biosensors are antibodies. The term 'antibody' as used herein may comprise polyclonal, monoclonal, bispecific, humanised or chimeric antibodies, single chain antibodies, Fab fragments and $F(ab')_2$ fragments, fragments produced by a Fab expression library, anti-idiotypic antibodies and epitope-binding fragments. The term 'antibody' also refers to immunoglobulin and T-cell receptor molecules, i.e. molecules that contain an antigen-binding site that specifically binds an antigen. The immunoglobulin molecules can be of any class (e.g. IgA, IgD, IgE, IgG and IgM) or subclasses thereof.

The identification of the biomarkers that are specific for a mood disorder, especially depression and more particularly major depressive disorder is key to integration of diagnostic procedures and therapeutic regimes. Appropriate diagnostic tools such as biosensors can be developed in methods and uses of the invention; and detection and quantification of the biomarker can be performed using a biosensor in a microanalytical system, a microengineered system, a microsepration system, an immunochromatography system or other suitable analytical devices (such as Raman or mass spectrography and the like). The biosensor may be incorporated in an immunological method for detection of the biomarker(s), or via electrical, thermal, magnetic, optical (e.g. hologram) or acoustic technologies. Using these techniques, it is possible to detect the target biomarker(s) at the anticipated concentrations found in biological samples.

Thus, according to a further aspect of the invention there is provided an apparatus for diagnosing or monitoring a mood disorder, especially depression and more particularly major depressive disorder which comprises a biosensor in a microanalytical, microengineered, microseparation and/or immunochromatography system configured to detect and/or quantify the biomarkers lipocalin-2 and/or BDNF.

The biomarker(s) of the invention can be detected using a biosensor incorporating technologies based on "smart" holograms, or high frequency

acoustic systems, such systems are particularly amenable to "bar code" or array configurations.

In smart hologram sensors (Smart Holograms Ltd, Cambridge, UK), a holographic image is stored in a thin polymer film that is sensitized to react specifically with the biomarker. On exposure, the biomarker reacts with the polymer leading to an alteration in the image displayed by the hologram. The test result read-out can be a change in the optical brightness, image, color and/or position of the image. For qualitative and semi-quantitative applications, a sensor hologram can be read by eye, thus removing the need for detection equipment. A simple color sensor can be used to read the signal when quantitative measurements are required. Opacity or color of the sample does not interfere with operation of the sensor. The format of the sensor allows multiplexing for simultaneous detection of several substances. Reversible and irreversible sensors can be designed to meet different requirements, and continuous monitoring of a particular biomarker of interest is feasible. Suitably, methods for detection the biomarkers according to the invention combine biomolecular recognition with appropriate means to convert detection of the presence or quantity of the biomarker in the sample into a signal. Biosensors to detect the biomarkers can also be detected by acoustic, plasmon resonance, holographic and microengineered sensors. Imprinted recognition elements, thin film transistor technology, magnetic acoustic resonator devices and other novel acousto-electrical systems may be employed for detection of the one or more biomarkers of the invention.

Methods involving detection and/or quantification of the biomarkers of the invention can be performed on bench-top instruments, or can be incorporated onto disposable, diagnostic or monitoring platforms that can be used in a non-laboratory environment, e.g . in the physician's office or at the patient's bedside. Suitable platforms for performing methods of the invention include "credit" cards with optical or acoustic readers. The sensor systems can be

configured to allow the data collected to be electronically transmitted to the physician for interpretation and thus can form the basis for remote diagnosis. Methods of the invention can be performed in array format, e.g. on a chip, or as a multiwell array. This enables testing for several biomarkers or for only one biomarker in multiple subjects or samples simultaneously. Methods can be adapted into platforms for single tests, or multiple identical or multiple non-identical tests, and can be performed in high throughput format. Methods of the invention may comprise performing one or more additional, different tests to confirm or exclude diagnosis, and/or to further characterize a condition.

A kit for diagnosing or monitoring a mood disorder, especially depression and more particularly major depressive disorder, or predisposition thereto is provided. Suitably a kit according to the invention may contain one or more components selected from the group: a biosensor specific for the biomarker or a molecule upstream or downstream in the biological pathway for that biomarker, where the biomarker is lipocalin-2 or BDNF; one or more controls; one or more reagents and one or more consumables; optionally together with instructions for use of the kit in accordance with any of the methods defined herein.

The identification of biomarkers for mood disorders, especially depression and more particularly major depressive disorder permits integration of diagnostic procedures and therapeutic regimes. Currently effectiveness of drug treatment or psychotherapy is difficult to test, and it has thus far not been possible to perform rapid assessment of therapy response. Traditionally, many anti-depressant therapies require treatment lasting weeks to months for a given therapeutic approach. Detection of a biomarker of the invention can be used to screen subjects prior to their participation in clinical trials. The biomarkers provide the means to indicate therapeutic response, failure to respond, unfavorable side-effect profile, and degree of medication compliance. The biomarkers may be used to stop treatment in non-responders at a very early

stage. They can also be used to fine-tune dosage, minimize the number of prescribed medications, and to reduce the delay in attaining effective therapy. Thus by monitoring a biomarker of the invention, patient care can be tailored precisely to match the needs determined by the disorder and the pharmacogenomic profile of the patient. The biomarker can thus be used to titrate the optimal dose and to identify a positive therapeutic response. Biomarker-based tests provide a first line assessment of 'new' patients, and provide objective measures for accurate and rapid diagnosis, in a time frame and with precision, not achievable using the current subjective measures. Furthermore, diagnostic biomarker tests are useful to identify family members of patients at high risk of developing a mood disorder such as depression and more particularly major depressive disorder. This permits initiation of appropriate therapy, or preventive measures, e.g. managing risk factors. These approaches are recognized to improve outcome and may prevent overt onset of the disorder. Biomarker monitoring methods, biosensors and kits are also vital as patient monitoring tools. If pharmacological treatment is assessed to be inadequate, then therapy can be reinstated or increased; a change in therapy can be given if appropriate. As the biomarkers are sensitive to the state of the disorder, they provide an indication of the impact of drug therapy.

Diagnostic kits for the diagnosis and monitoring of a mood disorder, preferably depression, most preferably major depressive disorder, are described herein. A method of diagnosis or monitoring the biomarkers may comprise quantifying the biomarker in a sample from the patient and comparing the level of the biomarker present in said sample with one or more controls. For monitoring, the control may be a test sample of the same patient at an earlier point in time.

Preferably, the diagnosis for the presence of a mood disorder in a patient according to the present invention is used to confirm a suspicion of a mood

disorder, such as depression and more particularly major depression disorder. This means that preferably the patient is already suspected of having a mood disorder at the moment that de the assay for measuring the level of lipocalin-2 is performed. This is only logical, since it has been mentioned in the introduction of the present specification that lipocalin-2 is also useful as a biomarker in other disease, such as cancer end renal failure. For interpretation of the test result, it should thus be ascertained that a change in lipocalin-2 values is not caused by these kinds of conditions. In this respect, the invention can be considered as a method for enhancing the diagnosis of depression.

This is also true, when the assay for lipocalin-2 is accompanied by an assay for BDNF.

One of the main advantages of the present invention is that it provides an easy and reliable way to monitor progress of the disease and/or effectiveness of a therapy. To this end, the lipocalin-2 values for a subject are determined at a certain moment (null-value) and after an amount of time this procedure is repeated. Over the time, several repeat measurements can be performed. In the mean time therapy can e.g. be started or changed. Change(s) in the levels of lipocalin-2 will then indicate the effectiveness of the therapy or the progress of the disease.

Suitably, the time elapsed between taking samples from a subject undergoing monitoring will be several days, a week, two weeks, a month, several months or longer. Samples may be taken prior to and/or during and/or following antidepressant therapy. Samples can be taken at intervals over the remaining life, or a part thereof, of a patient.

Of course, also in this case, the lipocalin-2 assay can be complemented with an assay for BDNF. As has been described in WO 2011/002292, especially the level of BDNF in urine can be used to monitor progress of disease and therapy.

Thus, in this way, in a non-invasive and easy manner, the progress of the disease can be monitored.

In the below examples specific embodiments of performing the invention will be explained in more detail. However, a person of skill in the art will easily find other ways of performing the assays within the scope the invention.

EXAMPLES

Example 1

Lipocalin-2 is indicative of depression.

Experimental Procedures Subjects

Urine samples were collected from both MD patients and healthy individuals. All subjects provided informed consent prior to participation. The urine samples were stored at 4°C. For quantifying lipocalin-2 in the urine samples, an ELISA test was used, which was based on a protocol provided by R&D Systems (Minneapolis, MN):

Materials, Chemicals and proteins:

- Rat anti-human Lcn2 mAb, R&D Systems, MAB 17571
- Human recombinant Lcn2, R&D systems, 1757-LC-050
- Goat anti-human biotinylated Ab, R&D Systems, BAF1757
- Avidin-HRP, Ebioscience, 18-4100-51
- O-Phenylenediamine, Sigma-Aldrich, P1526
- NUNC F96 MAXISORP-immuno plate

Plate Preparation

1. Dilute the Capture Antibody (rat anti-human Lcn2 mAb) to 2 µg/ml in PBS. Immediately coat an ELISA plate with 100 µL per well of the diluted Capture Antibody. Incubate overnight at room temperature w/o shaking.
2. Aspirate each well and wash with Wash Buffer, repeating the process two times for a total of three washes. Wash by filling each well with Wash Buffer (200 µL) using a manifold dispenser. Complete removal of liquid at each step is essential for good performance. After the last wash, remove any remaining Wash Buffer by aspirating or by inverting the plate and blotting it against clean paper towels.
3. Block plates by adding 300 µL of Reagent Diluent to each well. Incubate at room temperature for a minimum of 1 hour with shaking.
4. Repeat the aspiration/wash as in step 2. The plates are now ready for sample addition.

Assay Procedure

1. Add 100 µL of sample or standards in Reagent Diluent per well. Incubate 2 hours at room temperature with shaking. All samples and standards should be in duplex.

For making a standard curve, dilute recombinant human Lcn2 to a concentration of 5000 pg/ml. Add 200 µl to row A of colums 1 and 2. Add 100 microliter to row B-H of columns 1 and 2. Perform six 1:2 serial dilutions. Do not add Lcn2 to row H!!!

Dilution of the samples depends on expected Lcn2 levels in these samples.

2. Repeat the aspiration/wash as in step 2 of Plate Preparation.
3. Add 100 µL 0.2 µg/ml goat anti-human Lcn2, diluted in Reagent Diluent, to each well and incubate for 2 hours at room temperature with shaking.
4. Repeat the aspiration/wash as in step 2 of Plate Preparation.
5. Add 100 µL of a 1:1000 dilution of Avidin-HRP to each well and incubate for 20 minutes at room temperature with shaking. Wrap the plate in aluminium foil to avoid contact to light.
6. Repeat the aspiration/wash as in step 2.
7. Add 100 µL of Substrate Solution to each well. Incubate for 20 minutes at room temperature with shaking. Wrap the plate in aluminium foil to avoid contact to light.
8. Add 50 µL of 3N HCl to each well. Gently tap the plate to ensure thorough mixing
9. Determine the optical density of each well immediately, using a microplate reader. Read at wavelength 492 nm (ref. wavelength 620 nm). Calculate Lcn2 concentrations in each sample by comparing the sample to the standard curve.
10. Correct the calculated Lcn2 concentrations for creatinine by dividing the Lcn2 concentration with the separately measured creatinine concentration.

Buffers and Solutions:

Citric Acid Sodium Phosphate Buffer (0.05M)	890 mg $Na_2HPO_4.H_2O$
	1.05 g $C_6H_8O_7.H_2O$
	Fill up to 100 ml with UP H_2O
	pH 5.0

3N HCl	
Reagent Diluent	50 ml PBS 500 mg BSA pH 7.2 – 7.4 0.2 µm filtered
Substrate solution	10 ml 0.05M Citric Acid, 0.05M Sodium Phosphate buffer 10 mg O-phenylenediamine 10 µl 30% H_2O_2
Wash buffer (1 liter)	100 ml 10x TBS Fill up with UP H_2O 1 ml Tween20

The following results were obtained

Male/Female	Hamilton score	Lipocalin/creatinine ratio
M	30	0.3
M	30	59.3
M	26	0.9
M	27	14.2
M (control)	-	0,1
F	26	14.0
F	21	3.7
F	23	1.1
F	31	2.0
F (control)	-	0.4

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Claims

1 A method for the diagnosis of a mood disorder comprising:

a. taking a body fluid sample of a subject;

b. measuring the content of lipocalin-2 in said sample; and

c. diagnosing the mood disorder if the concentration of lipocalin-2 is higher than in control healthy subjects.

2 Method according to claim 1, wherein the body fluid is blood, saliva or urine, preferably urine.

3 Method according to claim 2, wherein the content of lipocalin-2 in the urine is given as lipocalin-2/creatinin ratio.

4 Method according to claim 1 or 2, wherein the mood disorder is chosen from depression, schizophrenia, psychosis and anxiety.

5 Method according to claim 1 or claim 2, wherein the mood disorder is depression, chosen from dysthymia, endogenous depression, reactive depression, minor depression, major depression, psychotic depression, neurotic depression, postnatal depression, burn out, overstrain, unipolar depression and bipolar depression, most preferably major depression.

6 Method according to any of the previous claims where the level of lipocalin-2 is indicative for depression if this level exceeds a lipocalin-2/creatinine ratio of 0,1 in a male subject, preferably when it exceeds said ratio of 0.2 in a male subject, or if this level exceeds said ratio of 0.4 in a female subject, preferably when it exceeds said ratio of 0.5 in a female subject.

7 Method according to any of the previous claims, wherein the method further comprises detection of the level of BDNF in a body fluid and comparing this level to the BDNF level in the body fluid of a healthy person.

8 Method according to any of the previous claims, wherein the subject is suspected of having a mood disorder, preferably depression.

9 Method for enhancing the diagnosis of a mood disorder in a subject, wherein said method comprises detection of the level of lipocalin-2 in a body fluid of a subject and comparing said level with the level of a healthy person.

10 Method according to any of the previous claims, wherein the detection of lipocalin-2 is performed via an immunoassay, preferably ELISA.

11 Method to determine the influence of antidepressant therapy in a subject comprising:

a. performing a method according to any of claims 1-7;

b. treating the subject with antidepressant(s) therapy;

c. repeating step a) with regular intervals during said treatment; and

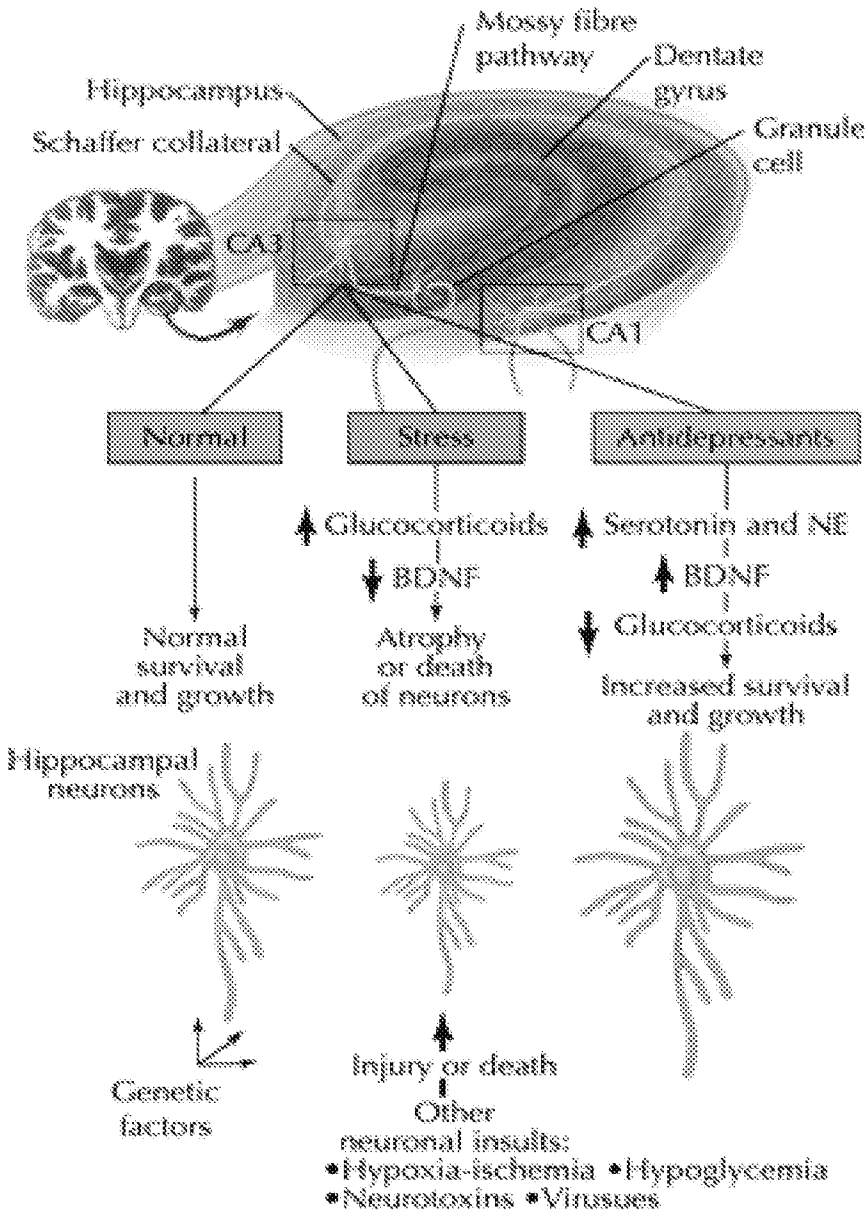
d. registering any difference in the concentration of the measured biomarker(s) in the body fluid.

12 Method to monitor the progress of a mood disorder in a subject comprising performing a method according to any of claims 1-7.

13 Use of lipocalin-2 in the diagnosis and monitoring of progression of a mood disorder.

14 Use of a combination of lipocalin-2 and BDNF in the diagnosis and monitoring of progression of a mood disorder.

Fig. 1


Mossy fibre pathway
Dentate gyrus
Hippocampus
Schaffer collateral
Granule cell
CA3
CA1
Normal
Stress
Antidepressants
Glucocorticoids
BDNF
Serotonin and NE
BDNF
Glucocorticoids
Normal survival and growth
Atrophy or death of neurons
Increased survival and growth
Hippocampal neurons
Genetic factors
Injury or death
Other neuronal insults:
•Hypoxia-ischemia •Hypoglycemia
•Neurotoxins •Viruses

INTERNATIONAL SEARCH REPORT

International application No
PCT/NL2012/050505

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/68
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, CHEM ABS Data, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2009/021521 A2 (LUNDBECK & CO AS H [DK]; EBERT BJARKE [DK]; MADSEN TORSTEN MELDGAARD [) 19 February 2009 (2009-02-19)	1-6,8-13
Y	page 1, line 14 - line 19 page 3, line 19 - line 21 page 4, line 7 - line 12 page 9, line 17 - line 27 claims 40, 43-45	7,14

Y	WO 2011/002292 A1 (BRAINLABS B V [NL]; ARNOLDUSSEN EDUARD ANTONIUS JOANNES [NL]; BOHLMEIJ) 6 January 2011 (2011-01-06) cited in the application abstract claims 1-7	7,14

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 August 2012	06/09/2012

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/NL2012/050505

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2009021521 A2	19-02-2009	AR 067903 A1	28-10-2009
		TW 200920358 A	16-05-2009
		WO 2009021521 A2	19-02-2009
WO 2011002292 A1	06-01-2011	NONE	