Insulin receptor sensitization improves affective pathology in various mouse models

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Insulin receptor sensitization improves affective pathology in various mouse models

João Pedro COSTA-NUNES

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Insulin receptor sensitization improves affective pathology in various mouse models

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Prof. Dr. L. L. G Soete

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To my grandfather, Rodrigo.

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Chapter 1

Introduction

Insulin is a peptidergic hormone normally secreted by the beta cells at the pancreatic islets of Langerhans (Huang *et al.*, 2010). Discovered in 1922 by Banting and Best, it was initially assumed to be unable to transit the blood-brain barrier (BBB) and consequently have no action in the brain (Pirart, 1978). This supposition has long been proven wrong, when both insulin and its receptor were found in the brain (Havrankova *et al.*, 1978; Unger *et al.*, 1989).

Contrary to what was initially thought (Pirart, 1978), insulin originates in the peripheral tissues and migrates via the cerebrospinal fluid (CSF) into the central nervous system via a saturable insulin receptor-mediated transport process, which is hypothesized to occur through the choroid plexus (Woods *et al.*, 1985; Wallum *et al.*, 1987; Devaskar *et al.*, 1994; Craft and Watson, 2004), whereas central synthesis (*e.g.* in neurons) is still a controversial subject (reviewed in Blázquez *et al.*, 2014).

The neuronal or central insulin receptor (IR), discovered in 1978 by Havrankova and colleagues, is a heterotetrameric glycoprotein, comprised of two alpha subunits and two beta subunits of transmembrane proteins with tyrosine kinase activity, linked together by disulphide bonds (Zhao *et al.*, 1999; Huang *et al.*, 2010). Central insulin receptors differ from their peripheral counterparts by having distinctly smaller molecular weights, although reported to have similar signal transducing properties, and are distributed in particularly high concentrations in neurons and to a much lesser extent in glial cells (Heidenreich *et al.*, 1983; Heidenreich and Gilmore, 1985; Schwartz *et al.*, 1992; Zhao *et al.*, 1999).

Ubiquitously distributed in the brain, IR presence is the highest in the olfactory bulb, cerebral cortex, hippocampus, hypothalamus, and amygdala (Havrankova *et al.*, 1978), where it is thought to be associated with, but not limited to synaptic plasticity, cell differentiation, myelination and survival (Chiu *et al.*, 2008; Huang *et al.*, 2010; Lin *et al.*, 2010), metabolic processes (Govind *et al.*, 2001; Zhao and Alkon, 2001; Freude *et al.*, 2008), regulation of dopamine-mediated neurotransmission (Williams *et al.*, 2007) and extracellular levels of norepinephrine and serotonin (Daws *et al.*, 2009).

The robust density of the neuronal insulin receptor in structures such as the hippocampus and cerebral cortex (Mufson *et al.*, 1999; Sun *et al.*, 2010) together with its high structural homology in the activation loop segment with tropomyosin receptor kinase B (TrkB) suggest a prominent role in adaptive stress response (Krishnan *et al.*, 2007; Kikusui *et al.*, 2009; Spencer *et al.*, 2010). Brain insulin levels also have an impact in the modulation of neuroinflammation, both by reducing levels of proinflammatory cytokines as well as minimizing the effects of reactive oxygen species (ROS), thus maintaining tissue integrity (Abbas *et al.*, 2012).

Signalling starts when insulin binds to the extracellular subunits of the insulin receptor, inducing a conformational change that leads to autophosphorylation of the

cytosolic tyrosine residues (Kahn *et al.*, 1993), enabling the recognition and further tyrosine phosphorylation of adaptor proteins such as insulin receptor substrates (IRS) (Lizcano and Alessi, 2002). A plurality of phosphorylated IRS proteins then couples to their respective effectors and transduce the insulin signal into multiple pathways.

Fig. 1 – Transduction of signals and biological actions induced by Insulin or IGF-1

The pivotal insulin-triggered pathway is the one which, upon activation, creates binding sites for the regulatory subunit of phosphoinositol 3-kinase (PI3K), enabling downstream phosphorylation of phosphatidylinositol 4,5-bisphosphate (PIP₂) and assembly of the protein kinase B (PKB/AKT) activator, phosphatidylinositol (3,4,5)-trisphosphate (PIP₃). Active AKT phosphorylates and inactivates cytosolic forms of glycogen synthase kinase 3 (GSK3), pro-apoptotic regulators, and also modulates

downstream regulation of transcription and translation. In neurons, the balance between these intricate pathways are critical for survival signalling (Cole and Frautschy, 2007) (See **Fig.1**).

Insulin resistance

Insulin resistance is a pathological condition, normally attributed to genetics, obesity and aging (Folli *et al.*, 1993; Saad *et al.*, 1993; Carvalho *et al.*, 1996; Anai *et al.*, 1998, 1999), that may also be precipitated by a so-called Western diet, enriched with high-cholesterol, -fat, -sugar or -salt content foods (Ogihara *et al.*, 2001; Pipatpiboon *et al.*, 2012), a growing cause for concern given its synergy with the stressful and modern lifestyle.

A chronic inflammation stage usually precedes the development of peripheral insulin resistance in key metabolic tissues, including the liver (Schenk *et al.*, 2008). Systemic and local cytokines trigger kinases capable of inhibiting key elements of the insulin signalling pathway (Hotamisligil, 2006), leading to an increase in lipid peroxidation, altered high (HDL) and low density lipid (LDL) levels, liver steatosis and hyperinsulinemia (Abbas *et al.*, 2012; Janczyk and Socha, 2012).

Peripheral insulin resistance reduces the transport of insulin into the brain across the BBB. Reduced neuronal insulin receptor signalling either by substrate deficiency or receptor desensitization compromises neuronal survival, plasticity, growth and remodelling, microtubule assembly, energy production, gene expression, white matter integrity through aberrant kinase activity, induces changes in protein levels and structure, endoplasmic reticulum stress, promotes the generation of reactive oxygen and nitrogen species that damage proteins, nucleic acids, and lipids, mitochondrial dysfunction and triggers signalling through pro-inflammatory and pro-apoptosis cascades (Krogh-Madsen *et al.*, 2004a,b; Watson and Craft, 2006; Hu *et al.*, 2014 – **Fig. 2**).

Fig. 2 – Normal insulin signalling (left) vs. insulin resistance (right)

Initially thought to be a cause of insulin resistance (Petersen *et al.*, 2003), mitochondrial dysfunction is considered to be a consequence of this phenomena. In the central nervous system, brain mitochondria play an important role in energy-demanding neurotransmission, in controlling calcium homeostasis (Wang *et al.*, 2010) and regulating adenosine triphosphate (ATP) production via oxidative phosphorylation. Insulin stimulates mitochondrial functions at multiple steps including oxidative phosphorylation, gene transcription and protein expression (Stump *et al.*, 2003).

Mitochondrial recycling is an essential process, regulated by autophagy, where damaged mitochondria are degraded and used for energy production through recycling membrane lipids (Zhang and Ye, 2012). Disruption of electron transport chains can lead to decreased ATP with increased reactive oxygen species (ROS) production (Pipatpiboon *et al.*, 2012), normally converted into H₂O and CO₂ by

functional mitochondria, deleterious to the processes of lipid oxidation, and responsible for protein and DNA damage.

N-methyl-D-aspartate receptors and insulin signalling

The N-methyl-D-aspartic acid receptors (NMDAr) are hetero-oligomeric complexes comprised of two NR1 subunits co-assembled with two subunits of the NR2 (NR2A-D) subfamily and, at times, an NR3 subunit (Das et al. 1998; Laube et al. 1998; Schorge and Colquhoun 2003). The different NR2 subunits are differentially expressed throughout the brain and during development (Watanabe et al. 1993; Monyer et al. 1994), and they confer distinct electrophysiological and pharmacological properties to the receptors (Riedel et al. 2003).

These are an important subtype of ionotropic glutamate receptors that function as a ligand-gated ion channel which initiate cation influx upon activation (McBain *et al.*, 1994; Sucher *et al.*, 1996). Then, permeability to Ca^{2+} rises tenfold, which will act as a second messenger in multiple pathways. NMDA receptors also contains regulatory sites for other ions such as K^+ , Mg^{2+} , Zn^{2+} , for glycine, polyamines, and phencyclidine (Zhang *et al.*, 1998; Salehi-Sadaghiani *et al.*, 2012).

In the central nervous system (CNS), modulation of the NMDA receptors occurs particularly in the hippocampus (Trudeau et al. 2004) and has also been shown to have a role in learning and memory (Zhao and Alkon 2001; Huerta *et al.*, 2000; Nakazawa *et al.*, 2002), including acquisition, consolidation, and reconsolidation (Bank *et al.*, 1988; Olds *et al.*, 1989; Alkon, *et al.*, 1998; Bonini *et al.*, 2007).

The insulin-driven potentiation of NMDA receptor activity is not direct. Instead, potentiation occurs via recruiting new channel molecules to the cell surface by regulated exocytosis (Skeberdis *et al.*, 2001). At post-synaptic sites, the activation of insulin receptors results in tyrosine phosphorylation of insulin receptor substrates 1

and 2 (IRS-1 and IRS-2), which bind to effector molecules that activate protein kinases (Zhao and Alkon, 2001), ultimately leading to a positive-feedback phosphorylation of NR2A/NR2B subunits of the NMDA receptor (Christie *et al.*, 1999) thus increasing the opening rate of functional channels (Lin *et al.*, 2006).

NMDA receptor dysfunction has been reported in various CNS disorders including dementia (Zekry and Gold 2010), epilepsy (Metzler 2011), schizophrenia (Kantrowitz and Javitt, 2010), stroke (Cho *et al.* 2010), Parkinson's disease (Gardoni *et al.* 2010), and Huntington's disease (Milnerwood and Raymond, 2010), and also depression (Berman *et al.*, 2000; Zarate *et al.*, 2006; Machado-Vieira *et al.*, 2008; Sanacora *et al.*, 2008; Hashimoto, 2009; Salehi-sadaghiani *et al.*, 2012). Insulin receptor sensitizers were demonstrated to have inhibitory effects over NMDA-mediated calcium currents, protecting neurons from excitotoxicity (Zhao *et al.*, 2006).

Glycogen synthase kinase 3, insulin signalling and brain functions

The glycogen synthase kinase 3 (GSK-3) was originally identified in 1984 as a key regulator in glycogen synthesis (Woodgett and Cohen, 1984), presenting itself in two isoforms, α and β very similar to one another in terms of sequence homology and biochemical characteristics (Woodgett and Cohen 1984; Woodgett, 1990, 1991), each exhibiting a N-terminal inhibitory Ser phosphorylation site and a facilitative Tyr site in their catalytic loop.

GSK3 has several unique features compared with other kinases: (1) it is constitutively activated, (2) phosphorylation at Ser21 (α) and Ser9 (β) inhibits kinase activity; (3) in general, 'primed' phosphorylation of its substrates by other kinases is required, with the exception of b-catenin and Axin; (4) plays a part in a number of physiological processes, such as glycogen metabolism (Welsh and Proud, 1993), gene transcription (Troussard *et al.*, 1999) and apoptosis (Turenne and Price, 2001). GSK3 activity is modulated by insulin and WNT signalling, both pathways act in a negative regulatory

manner (Lizcano and Alessi, 2002; Saltiel and Kahn, 2001), in contrast with its action on the NF κ B signalling pathway (Lee and Kim, 2007).

The beta isoform is ubiquitous in neuronal tissue (Bhat *et al.*, 2004). Under normal conditions, GSK3 inhibits glycogen synthesis by suppressing glycogen synthase (GS) via phosphorylation. In turn, insulin (and IGF-I) keep GSK-3 kinase activity suppressed through IRS-1 and PI3K dependent mechanism via Akt-mediated phosphorylation of the enzyme isoforms at the serine residues. (Sutherland *et al.*, 1993; Yuan *et al.*, 2001; Lee and Kim, 2007; Bhat and Thirumangalakudi, 2013). This neuroprotective effect of GSK-3 inhibition increases cell survival, decreases death signals, inflammatory signals (COX-2), and neutrophil infiltration, reduces brain swelling, and decreases post-ischemic serum glucose levels related to neuronal cell death (Koh *et al.*, 2008).

High fat and high cholesterol diets have been shown to significantly induce insulin resistance, reduce hippocampal AKT activity and conversely enhancing GSK-3 β activity and tau hyperphosphorylation (Hu *et al.*, 2013; Bhat and Thirumangalakudi, 2013). Tau is a microtubule-associated protein, hyperphosphorylated at specific serine residues by abnormal activated state of several proline-directed kinases, such as GSK-3 (Sengupta *et al.*, 2006; de la monte *et al.*, 2012). As a result, tau protein folds in erroneous conformations and self-aggregates into insoluble fibrillary structures (paired helical filaments and straight filaments) that form neurofibrillary tangles, dystrophic neurites, and neuropil threads (Iqbal *et al.*, 2009).

Intraneuronal accumulations of fibrillary tau disrupt cytoskeletal networks and axonal transport, leading to synaptic disconnection, progressive neurodegeneration and necrosis (Iqbal *et al.*, 2009). Besides fibrillary tau, pre-fibrillary tau can also aggregate (Takashima, 2010). The accumulation of insoluble fibrillary tau is also responsible for oxidative stress and ROS generation which promote apoptosis, mitochondrial dysfunction, and neuronal death, present in Alzheimer's disease

(Mandelkow *et al.*, 2003) recently considered to be a neuronal form of diabetes (Steen *et al.*, 2005).

Studies have implicated GSK-3 β in the mechanism of action of mood stabilizers (Chen *et al.*, 1999; Li *et al.*, 2002) and antidepressants (Rosa *et al.*, 2008). Insulin sensitizers were shown to ameliorate intracerebral insulin resistance, and reduce tau-protein hyperphosphorylation via GSK-3 kinase activity inhibition (Rosa *et al.*, 2008; Hu *et al.*, 2013).

Toll-like receptors, inflammatory response and insulin resistance

Toll-like receptors (TLRs) are a family of transmembrane glycoproteins, located either in the plasma membrane (Lucas *et al.*, 2006; Nataf, 2009; Hanamsagar *et al.*, 2012; Ghasemi *et al.*, 2013; Najjar *et al.*, 2013; Obulesu and Jhansilakshmi, 2013) or in intracellular compartments (Hanke *et al.*, 2011; Konner and Bruning, 2011; Shatz *et al.*, 2012; More *et al.*, 2013), and are widely expressed in the brain (Kim and Sears, 2010, Tanti *et al.*, 2013, Hemmati *et al.*, 2014), liver, adipose tissue, skeletal muscle, vasculature and pancreatic β cells (Kim and Sears, 2010; Tanti *et al.*, 2013).

This receptor family recognizes pattern-associated molecular patterns (PAMPs, such as lipids, proteins, lipoproteins, and nucleic acids) derived from bacteria, viruses, fungi, and parasites (McGettrick and O'Neill, 2010; Wang *et al.*, 2011) as well as endogenous danger signals (damage-associated molecular patterns or DAMPs (Liu *et al.*, 2012).

Toll-like receptors and insulin signalling cross paths in numerous ways. TLR activation modulates the PI3K/Akt pathway, which, in turn, limits TLR overactivity (Fukao and Koyasu, 2003; Gelman *et al.*, 2006; Hazeki *et al.*, 2007). Whereas normal activation is needed to repair mitochondrial damage (Bauerfeld *et al.*, 2012), abnormal signalling

is also shown to increase inflammation (Androulidaki *et al.*, 2009; Chaurasia *et al.*, 2010) and link to insulin resistance.

Perturbation in PI3K/Akt pathway by TLR drives a GSK3 β overactivation, associated with insulin resistance (Liu *et al.*, 2010) by the hyperphosphorylation of tau, shown to have an effect on learning and memory deficits (Gong and Iqbal, 2008; Hooper *et al.*, 2008; King *et al.*, 2013) and being related to psychiatric disorders like depression or schizophrenia (Emamian *et al.*, 2004; Wilkinson *et al.*, 2011).

Pathological activation of toll-like receptors triggers two myeloid differentiation primary response gene, 88 (MyD88)-dependent and MyD88-independent pathways, both converging in the activation of the IKK/NF- κ B pathway, with release of NF κ B and its translocation into the nucleus, promoting inflammation and downstream insulin resistance (Gao *et al.*, 2002; Zuani-Amorim *et al.*, 2002; Kawai and Akira, 2007; Kim and Sears, 2010; Velloso *et al.*, 2015, Zhang *et al.*, 2015], but also via ceramide-induced inhibition of Akt phosphorylation (Holland *et al.*, 2011; Chavez and Summers, 2012).

Cellular stress caused by TLR activation causes ROS production, triggers downstream kinases and promotes interferon-driven inflammation (Mogensen, 2009; Kim and Sears, 2010; Hemmati *et al.*, 2014). Lastly, together with cytokines, TLR also triggers suppressor of cytokine signaling 3 (SOCS-3)-mediated insulin resistance, achieved via targeting of IRS-1 and IRS-2 for proteasomal degradation (Howard and Flier, 2006), and a downregulation of Akt signalling (Zhang *et al.*, 2008).

This large receptor family has been used as sentinel molecules for detection of metabolic disorders such as insulin resistance, obesity and diabetes (Shi *et al.*, 2006; Tsukumo *et al.*, 2007; Jin *et al.*, 2013), where Increased expression is detected in the liver, muscle, brain, adipose tissue, vasculature and pancreatic β -cells of obese

subjects (Kim and Sears, 2010), accompanied by increased NFB activity (Reyna *et al.*, 2008; Poulin-Godefroy *et al.*, 2010; Hardy *et al.*, 2013).

Consumption of fat-rich foods is responsible for two mechanisms of insulin resistance. Firstly, there is induction of endoplasmic reticulum (ER) stress by ROS in a TLR-independent pathway, where lipid excess causes a disequilibrium between folding capacity and amount of proteins, generation of IL-1 β and inflammasomes (Tsiotra and Tsigos, 2006; Jager *et al.*, 2007; Zhang and Kaufman, 2008; Boden, 2009; Franchi *et al.*, 2009; Deldicque *et al.*, 2010; Wen *et al.*, 2011). Secondly, TLR-4 activation by saturated and free fatty acids elicits metabolic endotoxemia, where alterations in the composition of gut microbiota and an increase in gut permeability (Cani and Delzenne, 2009; Burcelin *et al.*, 2011) promote live Gram-negative enterobacteria translocation mediated by Fetuin-A stimulation of macrophage migration and activation from the gut to the adipose tissue, inducing inflammation (Neal *et al.*, 2006; Amar *et al.*, 2011; Jin *et al.*, 2013) via NF κ B/JNK pathways, which also contributes to ER stress (Anghel and Wahli, 2007; Zhang *et al.*, 2008; Raso *et al.*, 2013; Enos *et al.*, 2014).

Notwithstanding the effects in obesity, diabetes and metabolic syndrome, TLRs have also been linked to both Alzheimer (Chen *et al.*, 2006) and Parkinson (Hirsch and Hunot, 2009), where recurrent expression promotes microglial activation, with concomitant release in pro-inflammatory cytokines, leading to neurodegenerative inflammation. Moreover, higher levels of TLRs and inflammatory cytokines have been linked to schizophrenic and bipolar patients and induced behavioural deficits, social withdrawal was reported in (Najjar *et al.*, 2003; DeMiranda *et al.*, 2010; McKernan *et al.*, 2011; Forrest *et al.*, 2012).

Studies show that downregulation, suppression or mutation of TLRs elicit a weak effect on insulin resistance-mediated inflammation (Radin *et al.*, 2008; Saberi *et al.*,

2009; Amyot *et al.*, 2012; Liang *et al.*, 2013; Jia *et al.*, 2014), preventing activation of IKK (JNK in mice fed a high fat diet (Tsukumo *et al.*, 2008). In the CNS, normal values of TLRs restore normal plasticity, activity and insulin sensitivity (Hennige *et al.*, 2009; Benarroch, 2010; Heni *et al.*, 2011; Sartorius *et al.*, 2012; and **Chapter 6**).

The use of insulin sensitizers such as pioglitazone (Gurley *et al.*, 2008; Dasu *et al.*, 2009; Takagi *et al.*, 2009), rosiglitazone (Wu *et al.*, 2010, 2011; Pan *et al.*, 2014), troglitazone (Appel *et al.*, 2005; Gurley *et al.*, 2008; Zhao *et al.*, 2011) and dicholine succinate (**Chapter 6**) have shown to be linked to a reduction of TLR expression and associated inflammatory response.

Cyclooxygenase-mediated aberrations in the CNS functions

Inflammation induces the expression of an array of proteins, among which are the two isoforms of cyclooxygenases (COX), inflammatory response regulators and main target for all non-steroidal anti-inflammatory drugs (NSAID). Whereas COX-1 is constitutively expressed in most tissues and mediates the synthesis of prostaglandin and thromboxane from arachidonic acid (Smith and DeWitt 1996), COX-2 is expressed in inflamed tissues in response to proinflammatory stimuli, responsible for systemic PGE2 production and the generation of reactive oxygen species (ROS) (Diaz *et al.*, 1998; Lipsky, 1999; McAdam *et al.*, 1999; Whelton *et al.*, 2000; Dannenberg *et al.*, 2001; Huang *et al.*, 2005; Lee *et al.*, 2006; Tian *et al.*, 2011), mainly under pathological conditions (Smith and DeWitt, 1996). In the adipose tissue, COX-2 activation crucially up-regulates the expression of MCP-1 in inflamed fat which causes macrophage infiltration and leads to endotoxemia (Hsieh *et al.*, 2010).

Non-steroidal anti-inflammatory drugs target inhibition of both COX isoforms, through blocking the formation of prostaglandins in normal and inflamed tissues. Celecoxib, a selective COX-2 inhibitor has been shown to have neuroprotective effects (Hunter *et al.*, 2007), reduce the risk and delay the onset of various age-

related diseases, including cancers (Thun *et al.*, 1991; Smalley and DuBois, 1997; Thompson *et al.*, 1997; Fukutake *et al.*, 1998; Hida *et al.*, 1998; Kismet *et al.*, 2004), Alzheimer's disease, and other neurodegenerative diseases (t' Veld *et al.*, 2001; Aisen, 2002; Etminan *et al.*, 2003; Asanuma *et al.*, 2004). Similarly, thiazolidinediones have been shown to relieve the inflammatory response, restore mitochondrial function, and reduce cell loss (Hunter *et al.*, 2007).

In high-fat and high-fructose enriched diet models of inflammation, COX-2 inhibition has been shown to attenuate whole body insulin resistance (Hsieh *et al.*, 2008, 2010), suppress fat inflammation and macrophage recruitment (Hsieh *et al.*, 2010), decrease circulating levels of free fatty acids and liver lipid accumulation due to a concomitant reduction in TNF- α expression, diminishes adipocyte hypertrophy and reverses the suppression of differentiation markers of adipocytes (Hsieh *et al.*, 2010), consequently preventing insulin resistance.

Disruption of normal sleep patterns and the impact on insulin sensitivity

Normal sleep is composed of rapid eye-movement (REM) sleep and stages N1-3 of non-REM (NREM) sleep, with most profound and restorative being N3, also known as slow-wave sleep (SWS). Fluctuations between REM and NREM stages take place every 90 min and repeat four to six times during the night for a 6-9h total duration (Reutrakul and van Cauter, 2014).

Reductions in self-reported sleep to fewer than 6 hours per night over the last 30 years has overlapped with a marked increase in the prevalence of metabolic disorders over the same period (Knutson and van Cauter, 2008; Buxton *et al.*, 2010), attributable to a decrease in insulin sensitivity rather than to impairments in insulin secretion or glucose effectiveness (Buxton *et al.*, 2010).

Putative mechanisms by which insulin sensitivity is altered are increased sympathetic activity (Spiegel *et al.*, 1999; Stamatakis and Punjabi, 2010), decreased cerebral glucose utilization, elevated evening cortisol levels, increased growth hormone secretion, increased norepinephrine and epinephrine (Irwin *et al.*, 1999; Nedeltcheva *et al.*, 2009; Buxton *et al.*, 2010), through a dysregulation of the neuroendocrine control of appetite (Sakurai *et al.*, 1998), increased monocyte counts (Boyum *et al.*, 1996; Boudjeltia *et al.*, 2008; Faraut *et al.*, 2011), and of proinflammatory cytokines (Shearer *et al.*, 2001; Vgontzas *et al.*, 2004; Irwin *et al.*, 2006; van Leeuwen *et al.*, 2009), abnormal adipocyte function, stimulation of lipolysis and increased free fatty acid (Hucking *et al.*, 2003).

Sleep curtailment has a similar physiological effect to the administration of lipopolysaccharide (LPS), promptly increasing endotoxin levels (Everson, 2005). LPS acts as a TLR4 agonist, stimulating the innate immune response via activation of macrophages and monocytes, triggering the NF κ B pathway and synthesis of pro-inflammatory cytokines (Shearer *et al.*, 2001; Meier-Ewert *et al.*, 2004; Irwin *et al.*, 2006, 2008; Henning *et al.*, 2014; Venâncio and Suchecki, 2014; *see previous sections*). Also disrupted is the PI3K-Akt pathway in adipose tissue, with associated perturbation in leptin secretion and lipid metabolism (Broussard *et al.*, 2012).

Suppression of sleep aggravates the phenotype with an approximate decrease of insulin sensitivity by 25%, similar to what is reported in older adults and in populations at high risk for diabetes (van Cauter *et al.*, 2001; Tasali *et al.*, 2009; Tasali *et al.*, 2008). Additionally, sleep deprivation decreases IRS-1 phosphorylation and correlated IL-10 secretion (Kooijman and Coppens, 2004), compromises hippocampus dependent memory and synaptic plasticity by affecting the levels of target of rapamycin (TOR)(Vecsey *et al.*, 2012).

Chronic sleep perturbations increase the risk for obesity (Ayas *et al.*, 2003; Hasler *et al.*, 2004; Cizza *et al.*, 2005; Gangwisch *et al.*, 2005; Vorona *et al.*, 2005), diabetes (Ayas *et al.*, 2003; Gottlieb *et al.*, 2005; Meisinger *et al.*, 2005; Yaggi *et al.*, 2006; Gangwisch *et al.*, 2007), and metabolic syndrome (Jennings *et al.*, 2007) but also for age-related pathologies hypertension (Gottlieb *et al.*, 2006), cardiovascular disease (Spiegel *et al.*, 1999; Mallon *et al.*, 2002) and early mortality (Hammond, 1964; Kripke *et al.*, 2002; Mallon *et al.*, 2002; Patel *et al.*, 2004; Ferrie *et al.*, 2007; Hublin *et al.*, 2007; Meisinger *et al.*, 2007). Primary sleep disturbance is also observed in depression, with shortened REM latency periods and NREM instabilities (Jones *et al.*, 1987).

Ageing, insulin resistance and mental disorders

With a progression to old age, decrease in brain mass, atrophy and re-structuration of morphological and chemical balance are associated with impaired memory, a decline in regeneration, plasticity and cognitive ability (Golomb *et al.*, 1994; Smith *et al.*, 2000; Uylings and de Brabander, 2002; Rusinek *et al.*, 2003; Jack Jr. *et al.*, 2005; Wilson *et al.*, 2006).

Premature CNS aging (Biessels *et al.*, 2002) and increased risk of neurodegenerative disorders (Arvanitakis *et al.*, 2004) are associated to long-term insulin resistance, which usually is attributed to an impairment of HPA-axis feedback mechanism (Dallman *et al.*, 1993; McEwen, 1998), COX-mediated inflammation or oxidative stress (Helmersson *et al.*, 2004).

Studies propose the implication of insulin/insulin growth factor 1 (IGF1) receptor signalling as an important factor in invertebrate and vertebrate development, nutrient sensing, growth and aging (Hafen, 2004; Bartke, 2006). IGF-1 promotes cell survival through ERK and PI3-K > AKT (Wei *et al.*, 2002), where Insulin-mediated AKT activation acts as an upstream inhibitory kinase for GSK3 β via serine 9, limiting the

ability to phosphorylate tau (Rickle *et al.*, 2004) and concomitant neurodegeneration (Kahn and Suzuki, 2010).

The Akt signalling pathway likewise downregulates the activity of FOXO (forkhead box) transcription factors (Chen *et al.*, 1989; Chen *et al.*, 2003; Calabrese *et al.*, 2004), involved in the processes of autophagy and proteolysis (Denne *et al.*, 1991; Donati *et al.*, 2001; DiRaison *et al.*, 2003; del Roso *et al.*, 2003).

Reversion of insulin resistance by new therapies with insulin receptor sensitizers

Reversion of insulin resistance can be achieved through the use of sensitizer molecules, *i.e.*, compounds that increase the bioavailability of insulin in the tissues. Although other compounds were used in the past (Bailey, 1992), the thiazolidinediones (TZD), are the standard therapeutic agents, interacting with cells via peroxisome proliferator-activator receptor (PPAR), where they act as agonists, or directly by binding to the mitochondria and modulating respiratory function (Feinstein, 2004).

Represented by the rosiglitazone (high-affinity to PPAR γ – Form *et al.*, 1975; Sakamoto *et al.*, 2000) and pioglitazone (partial PPAR α and high affinity to PPAR γ – Shinkai *et al.*, 1927; Sakamoto *et al.*, 2000), TZD are responsible for decreasing a release of free fatty acids in peripheral tissues with concomitant increase in the number of adipocytes, subcutaneous adipose tissue mass, and triglyceride storage (Goldstein, 2002; Czaja, 2009; Jain *et al.*, 2013). Simultaneously with the inhibition of pro-inflammatory cytokines, production of adiponectin (Czaja 2009) and increase in glycolysis, thiazolidinediones promote glucose oxidation and a more efficient uptake (Jafari *et al.*, 2007). Taken together, these PPAR agonists protect pancreatic β cell integrity, liver and skeletal muscle from toxicity while at the same time increasing insulin sensitivity (Jain *et al.*, 2013).

In the brain, thiazolidinediones promote insulin sensitivity while reducing both dopaminergic neuronal loss and microglial, astrocytical activation (McGuire *et al.*, 2001; Hong *et al.*, 2012), suppresses phosphorylation of p38 mitogen-activated protein kinase (Ghisletti *et al.*, 2007; Straus and Glass, 2007) and neurotrophic factor kappa B (He *et al.*, 2012), reduces neuroinflammation and enhances endogenous peroxisomal proliferation mitochondrial biogenesis (Balfour and Plosker, 1999; Strum *et al.*, 2007). Curiously, these PPAR agonists show no effect in healthy individuals (Miles *et al.*, 2000; Kiek-Wilk *et al.*, 2005 – Fig.3).

Fig. 3 – Known effects of PPAR γ activation.

Due to their anti-inflammatory and neuroprotective profile, some authors have used these insulin sensitizers in a variety of medical conditions such as inflammatory bowel disease (Saubermann *et al.*, 2002), psoriasis (Mittal *et al.*, 2009), and atherosclerosis

(Igarashi *et al.*, 2008), but also in depression (Rosa *et al.*, 2008; Eissa Ahmed *et al.*, 2009; Kemp *et al.*, 2009, 2012; Salehi-Sadaghiani *et al.*, 2012; Sepanjnia *et al.*, 2012), autism (Boris *et al.*, 2007), Alzheimer's Disease (Pershadsingh *et al.*, 2004), and multiple sclerosis (Kaiser *et al.*, 2009), where its ability to reduce microglial and astrocytical activation, decrease neuronal damage, and increase neuronal mitochondrial biogenesis holds promise for treating neuropsychiatric disorders (Heneka *et al.*, 2005; Zhao *et al.*, 2006; Strum *et al.*, 2007).

A mitochondrial complex II substrate from a subfamily of the TZD, dicholine succinate (DS), was found to intervene in a pivotal regulatory event of neuronal insulin receptor function, where it plays a role as an important endogenous sensitizer (Pomytkin, 2008; Cline *et al.*, 2012). This compound, in presence of other respiratory substrates, dose-dependently stimulates insulin-dependent H_2O_2 production at the mitochondrial respiratory chain in cerebellar neurons, promoting an enhancement of the central insulin receptor autophosphorylation at the tyrosine residues (Storozhevykh *et al.*, 2007; Storozheva *et al.*, 2008; Shomaker *et al.*, 2010; Persiyantseva *et al.*, 2013 – Fig.4).

Fig. 4 – Mitochondrial central insulin receptor activation.

Previous studies have shown antidepressant, anxiolytic and memory-preserving effects of DS in CD1 mice (Cline *et al.*, 2012; Costa-Nunes *et al.*, 2012), paralleled with increased hippocampal expression of Insulin-like Growth Factor 2 (IGF-2), a member of the insulin gene family with neurotrophic properties (Chen *et al.*, 2012; Bracko *et al.*, 2012; Basta-Kaim *et al.*, 2014). Moreover, DS has rescued normal neuronal function and viability with maintenance of hippocampus-dependent tasks in rat models (Storozheva *et al.*, 2008).

Objectives

In this work, we sought to study the role of neuronal insulin receptor signaling in the development of affective changes evoked by different ethopathological origins, in mice. We combined a mixed approach of already established models and validated new methodologies.

First, we validated the use of ethological stressors as an accurate methodology to mimic cognitive and emotional abnormalities associated with human phenotypes, and investigated the hippocampal relative-fold mRNA expression of NMDAr subunits as it mimics clinically relevant situations of stress-related disturbances in learning and emotionality (**Chapter 2**). This methodology would be one pillar for subsequent studies to evaluate insulin receptor sensitization properties.

Then, we have used chronic stress, elderly and naïve models of induction of depression to assess the effects of a neuronal insulin receptor sensitizer on sleep, behaviour, hippocampal GSK-3 β and mRNA expression levels of NMDA receptor subunits (**Chapter 3**). This approach was coupled with Illumina gene expression analysis.

Later, for the first time, we validated the administration of high amounts of dietary cholesterol to naïve mice as a model of affective pathology (**Chapter 4**). It was shown

to have an impact in anxiety-, depression-like and impulsive behaviours, levels of hepatic steatosis and inflammation, both central and peripheral.

We were also able to validate the use of an oral route, with food, for the delivery of antidepressant treatment, and the efficacy of a low dose (**Chapter 5**). These successes are highly important for their translational validity to the human clinic and use as a potential food supplement, as they are to animal welfare, allowing to circumvent invasive techniques.

Lastly, in **Chapter 6**, we demonstrated the use of a previously established oral delivery of a neuronal insulin receptor sensitizer in successfully abolishing *Tlr4* overexpression both in brain and liver, improving mouse behaviour in anxiogenic and depressogenic paradigms without effecting hepatic lipid metabolism or dystrophy. In parallel it was also found to normalize the expression of a marker for mitochondrial activity.

Altogether, we validated new methodologies of inducing affective pathologies in mice, sensitive to neuronal insulin sensitization, shown to have ameliorative behavioural and physiological effects, and paralleled with a positive balance in gene expression changes. The use of three different paradigms of depressive-like syndrome: stress-, aging and high cholesterol-diet related conditions, suggested the importance of insulin receptor signalling in their pathogenesis.

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Figure references

Fig. 1. – Adapted from Blásquez, E. *et al.*, 2014, *see above*

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Chapter 2

Altered emotionality, hippocampus-dependent performance and expression of NMDA receptor subunit mRNAs in chronically stressed mice.

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Abstract

N-methyl-D-aspartate receptor (NMDAR)-mediated neurotransmission in the hippocampus is implicated in cognitive and emotional disturbances during stress-related disorders. Here, using quantitative RT-PCR, we investigated the hippocampal expression of NR2A, NR2B and NR1 subunit mRNAs in a mouse stress paradigm that mimics clinically relevant conditions of simultaneously affected emotionality and hippocampus-dependent functions. A two-week stress procedure, which comprised ethologically valid stressors, exposure to a rat and social defeat, was applied to male C57BL/6J mice. For predation stress, mice were introduced into transparent containers that were placed in a rat home cage during the night; social defeat was applied during the daytime using aggressive CD1 mice. This treatment impaired hippocampus-dependent performance during contextual fear conditioning. A correlation between this behaviour and food displacement performance was demonstrated, suggesting that burrowing behavior is affected by the stress procedure and is hippocampus-dependent. Stressed mice (n=22) showed behavioural invigoration and anomalous anxiolytic-like profiles in the O-maze and brightly illuminated open field, unaltered short-term memory in the step-down avoidance task and enhanced aggressive traits, as compared to non-stressed mice (n=10). Stressed mice showed increased basal serum corticosterone concentrations, hippocampal mRNA expression for the NR2A subunit of the NMDAR and in the NR2A/NR2B ratio; mRNA expression of NR2B and NR1 was unchanged. Thus, stress-induced aberrations in both hippocampal-dependent performance and emotional abnormalities are associated with alterations in hippocampal mRNA NR2A levels and the NR2A/NR2B ratio and not with mRNA expression of NR2B or NR1.

Key words: burrowing behavior; contextual fear memory; hippocampus; N-methyl-D-aspartate receptor; predator stress; social defeat

Introduction

N-methyl-D-aspartate receptor (NMDAR)-mediated neurotransmission is involved in the aetiology of stress-related cognitive deficits and behavioural abnormalities (Cull-Candy *et al.*, 2001). This particularly implicates altered expression of the two NR1 (GluN1) and two NR2 (GluN2) subunits (NR2A and NR2B) of NMDAR tetramers, which are the most abundant in the hippocampal formation, a brain structure that mediates stress-induced aberrations in both learning and emotional behaviour (Li and Ju, 2012). Both NR2A and NR2B were shown to have distinct pharmacology and a role in the regulation of NMDAR, and have been suggested to be differentially involved in the mechanisms of learning and emotionality (Fleischman *et al.*, 2003; Li and Tsien, 2009). A body of evidence has demonstrated the involvement of NR2A, NR2B and NR1 subunits in the neurobiology of neuropsychiatric conditions such as anxiety, psychosis, impulsivity, Alzheimer's disease and major depression (Tsang *et al.*, 2008; Geissler and Lesch, 2011; Davies *et al.*, 2012).

Most experiments investigating the roles of NMDAR subunits in neuropsychiatric symptoms target selective subunits of this receptor using pharmacological and genetic manipulations that are frequently applied *in vitro* (Boyce-Rustay and Holmes, 2006; Longordo *et al.*, 2009; Cui *et al.*, 2013). However, fewer studies have addressed the changes in NMDAR subunit expression in disease-modeling conditions. Understanding changes in relative-fold mRNA expression of NMDAR subunits in animal models of cognitive and emotional disturbances related to chronic stress is important for the identification of new pharmacological interventions and therapies that effectively alter glutamatergic transmission (Vignisse *et al.*, 2013). Previous work on gene expression (Strekalova *et al.*, 2011) has highlighted abnormal hippocampal expression of NMDAR subunits in mice subjected to repeated stressors. We have hypothesized that in male C57BL/6J mice, a novel two-week stress procedure, comprised of ethologically valid stressors, exposure to a rat and social defeat, results

in concomitant cognitive, emotional and NMDA receptor expression abnormalities in the hippocampus. Thus, the current study specifically investigated the hippocampal relative-fold mRNA expression of NR2A, NR2B and NR1 subunits in the above-mentioned chronic stress procedure in mice that mimics clinically relevant situations of stress-related disturbances in learning and emotionality (Strekalova and Steinbusch, 2010; Cline *et al.*, 2012).

Materials and methods

Animals and housing

Male C57BL/6J and CD1 mice were 3 months-old, Wistar rats of 3-5 months age were used for predator stress; mice and rats were provided by Harlan, the Netherlands and Charles River, France, respectively. 14 days before the behavioural experiments, mice were single housed under a reverse 12h:12h light-dark cycle (lights on: 21:00 h) in standard laboratory conditions ($22 \pm 1^\circ\text{C}$, 55% humidity, food and water *ad libitum*). All experiments were carried out in accordance with the European Committees Council Directives and had been approved by the ethics committee of Maastricht University for animal research (CPV, DEC-UM 2009-109).

Experimental conditions and study outline

This study applied only ethological stressors to male C57BL/6J mice, for 14 days, based on previous work (adapted from Cline *et al.*, 2012; Couch *et al.*, 2013). The stress procedure consisted of dark-cycle rat exposure between the hours of 09:00 h and 18:00 h and light-cycle application of a social defeat paradigm, combined with exposure to an aggressive CD1 mouse. Body weight and parameters of social behaviour were determined one week before the chronic stress procedure in a social interaction test as described elsewhere (Strekalova *et al.*, 2004). The experimental and control groups were balanced for these parameters. Ethological stressors were applied to a stress group for 14 days, as described below. The control group received

daily handling only. At the end of the stress experiment, 12 h after the application of the last stressor, short-term memory in the step-down avoidance task (Vignisse *et al.*, 2011) and aggressive behaviour in a resident-intruder test (Strekalova *et al.*, 2004) were investigated in stressed and control mice. At this time, body weight was also assessed. The next day (Day 1), mice were tested in the elevated O-maze and food displacement tube tests (Strekalova and Steinbusch, 2010). On Days 2 and 3, respectively, contextual fear conditioning training and testing for recall (Vignisse *et al.*, 2013) were performed. The open field testing was carried out on Day 4 (under red light) and on day 5 (under white light). All behavioural tests were recorded on video.

We used a battery of behavioural tests based on previous literature (Calabrese *et al.*, 2012). Although it is possible that the different behavioural tests may affect subsequent tests, previously published test batteries with similarly employed paradigms have revealed an absence of any testing effects in C57Bl/6 mice (Strekalova and Steinbusch, 2009, 2010; Vignisse *et al.*, 2011, 2013; Malatynska *et al.*, 2012). Hence, we considered possible interfering effects of multiple behavioural test to be minimal in the current study.

A separate cohort of mice from both groups was pre-exposed to a mixture of CO₂ and O₂ and euthanized via cervical dislocation (according to Dutch law), for gene expression analysis 24 hours after the termination of the stress procedure.

Chronic stress procedure

Rat exposure while in a small container. Mice were introduced into cylindrical containers, which were placed into a rat home cage for 15 h (over-night, from 18:00 h to 9:00 h). Containers (15 cm x Ø 8 cm) were made from customized transparent plastic with holes in the covers (Ø < 0.5 cm). This ensured protection of the mouse

from the rat, but allowed visual and odour contact. During the weekends, mice were kept in their home cages, situated on top of the rat cages.

Social defeat stress. Social defeat procedures took place during the dark phase of the light cycle (between 12:00 h and 16:00 h). To enable visual control by the experimenter over the resident-intruder confrontation, the test was carried out under red light. In a preliminary test, aggressive CD1 mice that were able to attack the counter-partners in less than 60 sec, without injuring them, were selected for this procedure; these mice were introduced into the home cages of mice from the stress group during social defeat sessions for 5 min. During social defeat stress, test mice typically showed flight responses, submissive postures and vocalizations. Pairs of mice were carefully observed in order to prevent physical harm. In rare cases, aggressive mice were immediately removed from the cage of resident mice. After a 5-min period of social defeat, C57BL/6 mice were placed into small containers and put inside a CD1 mouse cage, where they stayed for a 3 h-period. After the 3 h period, the 5-min social defeat procedure was repeated. In order to randomize the procedure, the same pairs of C57BL/6 and CD1 mice were never put together.

Behavioural procedures

Step-down passive avoidance model. The step-down passive avoidance test was used as described elsewhere (Strekalova *et al.*, 2001; Vignisse *et al.*, 2011, 2013). The step-down apparatus (Evolocus LLC Tarrytown, NY, USA and Technosmart, Rome, Italy) was a transparent plastic cubicle (25 cm x 25 cm x 48 cm) with a stainless-steel grid floor (33 rods 2 mm in diameter), onto which a square wooden platform (7 cm x 7 cm x 1.5 cm) was placed. The illumination strength was 25 Lux. A shocker was used to deliver an alternating electric current (AC, 50 Hz). In this paradigm, mice are trained to avoid an electric shock by staying on the platform above the grid floor. During the training session, mice were placed on the platform inside a transparent cylinder for 30 s to prevent them from stepping down immediately. After removal of

the cylinder, the time until the mouse left the platform, with all four paws, was measured as baseline latency of step-down. Immediately after step-down, mice received a single electric foot shock (0.5 mA, 2 sec) and were returned to their home cages. One hour later, during the recall trial session, mice were exposed to the apparatus again by being handled in the same way as in the training session; no foot shock was delivered. Latency of step-down with all four paws was measured until 180 s had elapsed.

Elevated O-maze. Testing on the elevated O-maze was carried out as described elsewhere (Strekalova *et al.*, 2005). The O-maze consisted of a black circular path (runway width 5.5cm, $\varnothing = 46$ cm) with two opposing compartments protected by walls made of polyvinyl-chloride (height = 10 cm) and two open sectors of equal size. The maze was elevated 20 cm above the ground and illuminated from the top with red light. At the start of the testing session, mice were placed inside one of the two closed compartments. The test was recorded with a web camera. The latency to the first entry into the anxiety-related open arms of the maze, total number of entries into the open arms and total duration of time spent in open arms were scored for 5 min.

Food pellet displacement (burrowing) behaviour in a tube test. In order to further assess hippocampal function, all experimental groups were tested for burrowing behaviour. Burrowing behaviour, a tendency to displace small objects, e.g., small stones or food pellets, from a tube inside the home cage, is species-specific in mice and has been demonstrated to depend on an intact hippocampal formation. Using a paper tube (internal diameter 4 cm, length 10 cm), filled with 20 food pellets and placed in the middle of a mouse home cage, the latency to displacement of the first food pellet, time required to empty the tube, number of pellets removed after 1h and 1h 30 min were assessed in stressed and control mice during the dark phase

(Strekalova and Steinbusch, 2009, 2010). Time elapsed was 90 min.

Contextual fear-conditioning paradigm. The contextual fear-conditioning test procedure was adapted from previously described protocols (Strekalova *et al.*, 2003; Vignisse *et al.*, 2013). The apparatus (Evolocus LLC Tarrytown, NY, USA and Technosmart, Rome, Italy) consisted of a transparent plastic cubicle (25 cm x 25 cm x 50 cm) with a stainless-steel grid floor (33 rods 2 mm in diameter). A shocker was used to deliver an alternating electric current (AC, 50 Hz; 0.7 mA, 2 sec) after a 2-min acclimatization of a mouse to the chamber. After delivery of the current, the mouse was immediately placed back in the home cage. Freezing behaviour was scored by visual observation during a test of memory recall that was carried out 24 h later. The occurrence of freezing behaviour in the chamber was assessed every 10 sec for 180 sec; each 10-sec score was assigned to a freezing or non-freezing period, and the percentage of time spent in freezing was calculated.

Open field. The open-field apparatus consisted of four square arenas (25cm x 25cm x 40cm), made of wood covered with white resopal. Mice were put in the center of one of the four square open field arenas, and their behaviour was video recorded for 10 min. The open field was illuminated with white light (25 Lux) or red light. Behaviour was analyzed off-line using the Any-maze software (Stoelting Co, Wood Dale, IL, USA). Among other parameters, time spent immobile was analyzed in the central (area 20 x 20 cm) and peripheral (remaining part of the apparatus) zones.

Resident-intruder test. The resident-intruder test procedure was performed as described elsewhere (Strekalova *et al.*, 2004). In this paradigm, the C57BL/6J mice were placed individually in an observation cage (30 cm x 60 cm x 30 cm) for 30 min. Thereafter, a male CD1 mouse, which was group housed before the test, was introduced as an intruder to the same cage and left with the resident mouse for 8 min. During the observation period, resident and intruder mice were scored for

aggressive social behaviours. Latency of the first attack and number of attacks were scored.

Brain dissection and blood collection. On the day following the termination of the stress procedure, a cohort of mice from both groups was euthanized as described above, their hippocampi were dissected and trunk blood was collected for corticosterone evaluation.

Corticosterone concentration. To assay serum corticosterone, trunk blood was stored at 4°C overnight and centrifuged at 10 x g for 10 minutes; the assay was performed as previously described (Pawluski *et al.*, 2012). Serum was collected and stored at -80°C until use. All samples were run in duplicate. For total serum corticosterone concentrations, a commercially available RIA kit for rat corticosterone from MP Biomedicals (Corticosterone I25 for rats and mice, MP Biomedicals, LLC, Orangeburg, NY, USA) was used. Average intra- and inter-assay coefficients of variation for all corticosterone assays were below 10%. Assay sensitivity was 7.7 ng/mL.

RNA isolation and RT-PCR. RNA was extracted as previously described (Couch *et al.*, 2013) using the RNeasy RNA extraction kit (Qiagen, Hilden, Germany) and first strand cDNA synthesis was performed using random primers and Superscript III transcriptase (Invitrogen, Darmstadt, Germany); 1 µg total RNA was converted into cDNA. Quantitative RT-PCR (qPCR) for NR2A, NR2B, NR1 genes and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed using TaqMan probes and the CFX96 Real-time System (BioRad, Hercules, CA, USA). Cycling conditions and sequences of primers used are indicated in **Table 1** of *Supplementary data*. Results were normalised to GAPDH mRNA expression and calculated as relative-fold changes compared to control mice as described elsewhere (Couch *et al.*, 2013). Results of the qPCR measurements were expressed as Ct values, where Ct is defined as the threshold cycle of PCR at which amplified product was 0.05 % of normalized

maximal signal. We used the comparative Ct method and computed the difference between the expression of the gene of interest and GAPDH expression in each cDNA sample ($2^{-\Delta\Delta}$ Ct method). Results are given as expression-folds compared to the mean expression values in non-stressed control mice (Couch *et al.*, 2013, adapted from Livak and Schmittgen 2001).

Statistical analysis

GraphPad Prism 5.00 (San Diego, CA, USA) was used for analyses. A comparison of normally distributed independent variables was carried out using unpaired two-tailed *t*-tests. Independent measurements that were not normally distributed were analysed via the non-parametric Mann-Whitney test, and repeated measurements were compared by the Wilcoxon non-parametric test. The Pearson test was applied for correlation analysis. The level of confidence was set at 95% ($p < 0.05$).

Results

Stressed mice showed a significant loss of body weight compared to the control mice ($p < 0.0001$, $t = 6.801$, $df = 21$; unpaired *t*-test; **Fig.1A**) and a significant elevation of circulating corticosterone concentration ($p = 0.030$, $U = 39$; Mann-Whitney test, **Fig.1B**), indicating a profound impact of the two-week stress procedure. In comparison to control mice, the open-field activity of the stressed mice was significantly higher both in the peripheral ($p = 0.040$, $U = 61.00$, Mann-Whitney test) and even more notably, in the central zones ($p = 0.011$; $U = 73.50$) of the apparatus, when white lighting was employed (**Fig.1C**), but not when activity was scored under red light ($p = 0.15$, $U = 68.00$ and $p = 0.64$, $U = 97.50$, respectively). There was a significantly lower latency to the first exit in the elevated O-maze ($p = 0.010$, $U = 49.50$, Mann-Whitney test), increased time spent in the open arms ($p = 0.020$, $U = 55.50$) and number of entries into the open arms ($p = 0.028$, $U = 58.50$; **Fig.1D**) in stressed versus control mice. Together, these data indicate differences in the brightly lit open field induced by stress: behavioural

invigoration and an anomalous “anxiolytic-like” profile. In addition, chronically stressed mice displayed a significant increase in the number of attacks and no change in the latency to the first attack in the resident-intruder test ($p=0.049$, $t=1.698$, $df=30$ and $p=0.12$, $t=1.78$, $df=23$; unpaired t -test; **Fig.1E**).

Both stressed and control mice showed a significant increase in the latency of step down 1 h after training, in comparison to baseline latencies ($p=0.033$, $W=-37.00$ and $p=0.05$, $W=-51.00$, respectively, Wilcoxon test). There was no significant difference between stressed and control mice in the latencies for step-down evaluated at baseline conditions ($p=1.0$, $U=71.50$) or 1 h after training ($p=0.98$, $U=71.00$; **Fig.2A**), indicating similar scores of anxiety-like behaviour and short-term memory in these groups. In the contextual fear-conditioning paradigm, stressed mice spent a significantly shorter time freezing during a recall session than the control group ($p=0.021$, $U=50.00$, Mann-Whitney test; **Fig.2B**), which demonstrates impaired hippocampus-dependent contextual memory in this group. Stressed mice showed no significant inhibition of burrowing behaviour in comparison to the control group, as assessed by latency for the first pellet displacement ($p=0.18$, $U=73.00$; Figure 2C) and number of pellets displaced over time intervals of 0–60 min ($p=0.14$, $U=53.50$) and 60–90 min ($p=0.34$, $U=47.50$, Mann-Whitney test). Nonetheless, there was a significant correlation between the two latter measures and contextual freezing ($r = 0.36$, $p=0.046$ and $r=0.36$, $p=0.048$, respectively). There was no significant correlation between the duration of freezing and the latency for pellet displacement ($r = -0.16$ $p = 0.39$, Pearson correlation).

Concerning mRNA levels of NMDA receptor subunits in the hippocampus, in comparison to control mice, the stressed group had a significant increase in expression of NR2A ($p=0.029$, $t=2.050$, $df=15$, unpaired t -test; **Fig.3A**) and no significant change in the expression of NR2B ($p=0.27$, $t=0.6431$, $df=15$, **Fig.3B**). The

ratio of NR2A/NR2B was significantly increased ($p=0.015$, $t=2.452$, $df=13$; **Fig.3C**) but the expression of the NR1 subunit was unaltered ($p=0.64$, $t=0.4665$, $df=14$; **Fig.3D**).

Discussion

In accordance with our hypothesis and in line with the literature (Cull-Candy *et al.*, 2001; Fleischman *et al.*, 2003; Li and Ju, 2012; Calabrese *et al.*, 2013), the current study implicates altered expression of NMDAR subunits of the hippocampus in stress-induced deficits in both cognitive and emotional traits. The present study showed that in C57BL/6J mice, a two-week ethological stress paradigm, comprised of exposure to a rat and social defeat, resulted in the impairment of contextual fear conditioning, as well as behavioural disinhibition in the open field test and increased aggressive behaviour. These cognitive and emotional abnormalities were accompanied by increases in the hippocampal mRNA expression of the NR2A subunit of the NMDAR and in the NR2A/NR2B ratio, while the mRNA expression of NR2B and NR1 was unchanged.

A significant reduction in body weight and increase in basal serum corticosterone concentration of stressed mice demonstrated the impact of the two-week stress procedure (**Fig.1A,1B**). Stressed mice displayed behavioural hyperarousal under stressful testing conditions, as shown by a significant decrease in the time spent immobile in the central and peripheral parts of the brightly illuminated open field, but a lack of these changes during stress-free open field testing under red light (**Fig.1C**). Behavioural alterations of the stressed group in the open field illuminated with white light are in agreement with behavioural changes in these mice in the elevated O-maze. In the O-maze test, stressed mice showed a significant shortening of the latency to enter the open arms, an increase in the time spent and the number of entries into the open arms (**Fig.1D**). Together, these findings indicate that the stress procedure evoked anomalous “anxiolytic-like” traits and behavioural

invigoration in mice. In our experiments, these changes were accompanied by a significant increase in the number of attacks by stressed mice in the resident-intruder paradigm, which is an indicator of enhanced aggressive traits (**Fig.1E**). This is in agreement with previous work showing that increased anxiety levels typically correlate with increased scores of aggressive behaviour in C57BL/6J male mice (Willner, 2005).

In line with our findings, different chronic stress procedures have been reported to cause an increase in time spent in anxiety-related compartments of the elevated plus maze and the dark/light box in rodents, which was interpreted as a sign of “anxiolytic-like” effects (D’Aquila *et al.*, 1994; Cancela *et al.*, 1995; Sanchez, 1997; Hata *et al.*, 1999). Such phenomena, also considered as manifestations of impulsivity and disinhibition, are well-known consequences of chronic stress in rodents (Strekalova *et al.*, 2005; Willner, 2005; Belujon and Grace, 2011). It is commonly accepted that the limbic system, in general, and the hippocampus, in particular, have a central role in the behavioural inhibition system (Gray and McNaughton, 1983; Rawlins *et al.*, 1985; McNaughton *et al.*, 1997; McNaughton and Gray, 2000; Geissler and Lesch, 2011; Abela and Chudasama, 2013), while the crucial role of the hippocampus for memory was established much earlier (Squire, 1992). Behavioural invigoration, such as during stress, is likely to be associated with alterations of other hippocampal functions, which may include learning processes and molecular changes related to gene expression regulating glutamatergic neurotransmission (Geissler and Lesch, 2011; Belujon and Grace, 2011; Calabrese *et al.*, 2012). Thus, these potential changes were investigated in the present study.

Stressed mice had a normal acquisition of the short-term step-down avoidance task, a form of cortex-dependent learning, which was shown by their unaltered latencies to step-down (**Fig.2A**). However, a lower percentage of freezing during the recall session of the fear-conditioning test in stressed mice indicates a deficiency in their

long-term hippocampus-dependent memory (**Fig.2B**). The possibility that a hyperactivity of chronically stressed mice in our study interferes with scoring of freezing was largely ruled out by a lack of differences in locomotor activity during a two-minute acclimatization pre-training period between stressed and control mice (Strekalova *et al.*, 2003). Also, both groups had similar values for the baseline latencies of step down behaviour (**Fig.2A**) suggesting their comparable scores of anxiety-like traits that was assessed under testing conditions which were used to assess contextual learning. Baseline step-down behaviour was previously reported as a highly sensitive measure of subtle changes in anxiety and locomotion in C57BL/6J mice (Strekalova and Steinbusch, 2009, 2010; Vignisse *et al.*, 2011, 2013).

The changes in contextual freezing significantly correlated with decreased burrowing behaviour, although burrowing parameters were not altered significantly (Figure 2C). A tendency to displace food pellets is often regarded as not a fully specific indicator of hippocampal dysfunction in rodents (Tarr *et al.*, 2012; Hart *et al.*, 2012; Kaczmarczyk *et al.*, 2013) and its neurobiology is debatable. Hence the finding of a correlation between this behaviour and hippocampus-dependent learning in the present study might be potentially important in relating the burrowing behaviour to the dorsal hippocampus, as originally proposed (Deacon *et al.*, 2002).

This study revealed stress-induced increases in the hippocampal expression of NR2A and the NR2A/NR2B ratio (Figure 3A,C), which were previously shown to accompany elevated anxiety (Boyce-Rustay and Holmes, 2006; Gao *et al.*, 2010; Calabrese *et al.*, 2013), impulsivity and aggression (Meyer *et al.*, 2004; Bortolato *et al.*, 2012), home cage hyperactivity and a stress-induced increase in peripheral concentrations of corticosterone (Longordo *et al.*, 2009, 2011; Huang *et al.*, 2010) in various conditions. A limitation of our study, however, is the need for confirmation that the mRNA changes result in corresponding changes in subunit protein levels and in altered synaptic function in the hippocampus. In agreement with our data, separate studies

have reported that molecular changes such as we found are associated with a disruption of long-term memory, but not short-term learning (Huang *et al.*, 2010; Cui *et al.*, 2013; Calabrese *et al.*, 2013). In the present experiment, mRNA expression of NR2B and the NR1 in the hippocampus was not significantly changed by stress (**Fig.3**). However, previous work has reported a significant decrease in NR2B expression in several brain areas during stress (Cull-Candy *et al.*, 2001; Huang *et al.*, 2010), aging (Dere *et al.*, 2003) and compromised plasticity (Bortolato *et al.*, 2012). Previous work has also reported a decrease in NR1 mRNA after stress (Cull-Candy *et al.*, 2001; Schenberg *et al.*, 2006). Discrepancies between our findings and others may be due to the different stress paradigms employed. However, previous work we have done in a gene expression profiling Illumina study (Integrage, Evry, France and Northwestern Chicago University, USA and Ingenuity Systems, Redwood city, CA, USA), using hippocampi obtained in a similar chronic stress model (Strekalova *et al.*, 2011), suggests diminished expression of NR1 receptor subunit mRNA in stressed mice in particular, and speaks for systemic differences in the hippocampal glutamatergic receptors expression in these mice in general. In addition to NR1 expression results, these microarray data pointed to statistically significant changes in several elements of the glutamatergic system in the hippocampal formation of chronically stressed mice: NR2B receptor, AMPA receptor, glutamate metabotropic receptor 5, and in NR2A/NR2B ratio, which changes are overall in line with the outcome from mRNA evaluation in the current study. Remarkably, a segregation of stress-susceptible and stress-resilient individuals in the microarray study revealed differential expression of the above genes between the sub-groups that additionally supports functional importance of molecular changes in response to stress, obtained both in the microarray and mRNA experiments

In summary, this study demonstrates that elevated hippocampal expression in stressed mice of mRNAs for NR2A and of the NR2A/NR2B ratio, but not for NR2B and

NR1, is associated with concomitant abnormalities in both cognitive and emotional elements that mimic one of the most characteristic consequences of experiencing chronic stress in humans. These data suggest that a paradigm comprising ethological stressors evokes behavioural disinhibition and molecular changes that likely mimic epidemiologically spread human syndromes associated with stress-related emotional and cognitive deficits. Thus, the stress paradigm employed in the present study can be useful for translational studies in the search for pharmacological compensation of these combined symptoms of stress-related pathologies.

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Conflict of interests declaration

All other authors declare that they have no conflicts of interest.

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Figure legends

Figure 1. Exposure of mice to stressors for two weeks affects body weight, serum corticosterone and parameters of emotionality. (A) Weight loss and (B) increased serum corticosterone concentration in the stress group. (C) Stressed mice showed a reduced total time spent immobile at the periphery and in the centre of the open field lit with white light. There were no significant differences in locomotor behaviour between groups tested under red light. (D) Stressed mice displayed decreased latency of entries into the open arms, an increased time spent therein and increased number of entries. (E) Elevated aggressive behavior (number of attacks) in stressed mice. * $p < 0.05$ vs. control (A,E: unpaired t -test, B–D: Mann-Whitney test). Control group, $n=10$; stress group, $n=22$. All data are means $\pm$ standard error of the mean (SEM).

Figure 2. Hippocampus-dependent performance in stressed mice. (A) Stressed and control mice showed a significant increase in the latency for step down 1 h after training, in comparison to baseline latencies; * $p > 0.05$ vs. baseline, Wilcoxon test; there were no differences between groups. (B) Stressed mice spent a significantly shorter time freezing, during a recall session in the fear-conditioning paradigm; (C) there were no significant differences for latency to food displacement or number of pellets displaced at 0–60 min and 60–90 min in the tube test. * $p < 0.05$ vs. control; Mann-Whitney test. Control group, $n=10$; stress group, $n=22$. All data are means $\pm$ the standard error of the mean (SEM).

Figure 3. Hippocampal expression of mRNAs for N-methyl-D-aspartate receptor (NMDAR) subunits in stressed mice. In the stressed group, relative to controls: (A) mRNA expression of the NR2A subunit of the NMDAR was significantly greater; (B) mRNA expression of NR2B was not altered; (C) the ratio of mRNAs for NR2A/NR2B was significantly increased; (D) mRNA expression of the NR1 subunit of NMDAR was not altered; * $p < 0.05$ vs. control; unpaired t -test. Control group, $n=9$; stress group, $n=12$. Data are means $\pm$ the standard error of the mean (SEM).

Figure 1

A STEP-DOWN AVOIDANCE

B FEAR CONDITIONING

C TUBE TEST

Figure 2

A NR2A

B NR2B

C NR2A/NR2B

D NR1

Figure 3

Supplementary material

Table 1 – Cycling conditions and sequences of primers used in the RT PCR assay.

mRNA target	Primer sequence (5' → 3')		Annealing Temperature (C°)
NR1	Forward	GTTCTTCCGCTCCGGCTTTG	58
	Reverse	AGGGACACATTTTGCTTCCA	
	TaqManProbe	6-FAM CGGCATGCGCAAGGACAGCC BHQ-1	
NR2A	Forward	GCTACACACTCTGCACCAATT	64
	Reverse	CACCTGATAGCCTTCCTCAGTGA	
	TaqManProbe	6-FAM TGGTCAATGTGACTTGGGATGGCAABHQ-1	
NR2B	Forward	CTGCATGCGGAATACAGTC	56
	Reverse	TTGATGTAGCCTGGTTCCT	
	TaqManProbe	6-FAM CAGAAGCGCATCATCTCTGAGAATBHQ-1	
GAPDH	Forward	TGCACCACCAACTGCTTAG	54
	Reverse	GGATGCAGGGATGATGTTT	
	TaqManProbe	HEX ATCAGCCACAGCTTTCCAGA BHQ-1	

The qPCR was performed in a 25 µl reaction volume containing a 10xPCR Buffer (2.5 µl), 25 mmol MgCl₂ (4 µl), 10 mmol dNTPs (2 µl), specific forward and reverse primers at 20 pmol/l concentration (1 µl), 5pmol/l Taq Man probe (1.5 µl), cDNA (2 µl), 5 u/l Taq DNA polymerase (1 µl) (Beagle, st. Petersburg, Russia), and ddH₂O (10 l). All samples were run in duplicate. Cycling was performed at 95 C° for 5 min followed by a 45-cycle amplification at 95°C for 10s, then at the annealing temperature for 15 s and at the temperature 72°C for 20 s.

Chapter 3

Dicholine succinate, the neuronal insulin sensitizer, normalizes behaviour, REM sleep, hippocampal pGSK3 β and mRNAs of NMDA receptor subunits in mouse models of depression

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Abstract

Central insulin receptor-mediated signalling is attracting the growing attention of researchers because of rapidly accumulating evidence implicating it in the mechanisms of plasticity, stress response and neuropsychiatric disorders including depression. Dicholine succinate (DS), a mitochondrial complex II substrate, was shown to enhance insulin-receptor mediated signaling in neurons and is regarded as a sensitizer of the neuronal insulin receptor. Compounds enhancing neuronal insulin receptor-mediated transmission exert an antidepressant-like effect in several pre-clinical paradigms of depression; similarly, such properties for DS were found with a stress-induced anhedonia model. Here, we additionally studied the effects of DS on several variables which were ameliorated by other insulin receptor sensitizers in mice. Pre-treatment with DS of chronically stressed C57BL6 mice rescued normal contextual fear conditioning, hippocampal gene expression of NMDA receptor subunit NR2A, the NR2A/NR2B ratio and increased REM sleep rebound after acute predation. In 18-month-old C57BL6 mice, a model of elderly depression, DS restored normal sucrose preference and activated the expression of neural plasticity factors in the hippocampus as shown by Illumina microarray. Finally, young naïve DS-treated C57BL6 mice had reduced depressive- and anxiety-like behaviours and, similarly to imipramine-treated mice, preserved hippocampal levels of the phosphorylated (inactive) form of GSK-3 β that was lowered by forced swimming in pharmacologically naïve animals. Thus, DS can ameliorate behavioural and molecular outcomes under a variety of stress- and depression-related conditions. This further highlights neuronal insulin signalling as a new factor of pathogenesis and a potential pharmacotherapy of affective pathologies.

Key words: chronic stress; insulin receptor; dicholine succinate; phosphorylated glycogen synthase kinase-3 β (p GSK-3 β); NMDA receptor subunits NR2A and NR2B; sleep EEG; aging; hippocampal plasticity.

Introduction

Central insulin receptor signalling is important in brain function/dysfunction including cognitive disorders, stress response and depression. As a member of a subfamily of receptor tyrosine kinases, the neuronal insulin receptor has been shown to be involved in synaptic plasticity, cell differentiation, myelination and survival (Chiu *et al.*, 2008; Huang *et al.*, 2010a, Lin *et al.*, 2010) and metabolic processes (Govind *et al.*, 2001; Zhao and Alkon, 2001; Freude *et al.*, 2008). Insulin signalling has been found to regulate dopamine-mediated neurotransmission (Williams *et al.*, 2007) and extracellular levels of norepinephrine and serotonin (Daws *et al.*, 2009). The robust density of the neuronal insulin receptor in the hippocampus and cerebral cortex (Mufson *et al.*, 1999; Sun *et al.*, 2010) and its high structural homology in the activation loop segment with TrkB suggest its role in stress response (Krishnan *et al.*, 2007; Kikusui *et al.*, 2009; Spencer *et al.*, 2010). Compromised insulin signalling can result in cognitive deficits (van der Heide *et al.*, 2006; Kuhad *et al.*, 2009), neurodegeneration (Pomytkin, 2012) and depressive-like syndrome (Banks *et al.*, 2012; Gold *et al.*, 2013; Pan *et al.*, 2013).

The latest clinical and translational studies have revealed antidepressant-like effects, increased neuronal mitochondrial biogenesis, decreased neuronal damage and anti-inflammatory properties for compounds that potentiate the binding of insulin to its receptor or its immediate molecular consequences via various mechanisms and are therefore called “sensitizers of the neuronal insulin receptor” (Igarashi *et al.*, 2008; Storozheva *et al.*, 2008; Storozhevych *et al.*, 2008; Eissa Ahmed *et al.*, 2009; Mittal *et al.*, 2009; Rasgon *et al.*, 2010; Kemp *et al.*, 2011). Such effects were reported for the thiazolidinediones rosiglitazone and pioglitazone (Saubermann *et al.*, 2002; Ali *et al.*, 2006; Zhao *et al.*, 2006; Asghar *et al.*, 2007; Strum *et al.*, 2007; Eissa Ahmed *et al.*, 2009; Mittal *et al.*, 2009; Rasgon *et al.*, 2010; Kemp *et al.*, 2011). For instance, rosiglitazone, one of the insulin sensitizers of the thiazolidinedione class, induces an

antidepressant-like effect in the tail suspension and forced swim tests in mice, reducing immobilization and floating behaviour (Eissa-Ahmed *et al.*, 2009). Similar effects were found for pioglitazone, another insulin receptor sensitizer, which were shown to be NMDA receptor-dependent (Sharma *et al.*, 2012; Salehi-Sadaghiani *et al.*, 2012). Rosiglitazone and pioglitazone were reported to be effective in the treatment of major depressive disorder that was refractory to standard antidepressant treatment and accompanied by insulin resistance (Rasgon *et al.*, 2010; Kemp *et al.*, 2011).

The antidepressant-like effects were also reported for a mitochondrial complex II substrate, Dicholine Succinate (DS) (Cline *et al.*, 2012; Costa-Nunes *et al.*, 2012, 2015). DS was found to dose-dependently stimulate insulin-dependent H₂O₂ production of the mitochondrial respiratory chain in cerebellar neurons leading to an enhancement of the insulin receptor via insulin-stimulated autophosphorylation of the insulin receptor kinase at tyrosine residues in neurons, which is a key regulatory event of the insulin receptor function. The effect of DS is dependent on the presence of insulin (Storozheva *et al.*, 2008; Storozhevyykh *et al.*, 2008; Shomaker *et al.*, 2010; Persiyantseva *et al.*, 2013).

Our previous studies utilizing a mouse depression model where a depressive-like state is induced by chronic stress and defined by a reduction in reward sensitivity, anhedonia, showed the antidepressant- and anti-anxiety effects of DS in CD1 mice (Cline *et al.*, 2012). As for instance, chronic intraperitoneal administration of DS for seven days, at 25 mg/kg/day before the onset of a 10-day stress, rescued normal sucrose preference, floating and step-down avoidance learning, as well as hippocampal expression of Insulin-like Growth Factor 2 (IGF-2), a member of the insulin gene family with neurotrophic properties (Chen *et al.*, 2011; Bracko *et al.*, 2012; Basta-Kaim *et al.*, 2014). In other experiments, administration of DS for seven days in mice at similar doses rescued aging-related decreases of brain N-

acetylaspartate/creatine, a marker of neuronal function and viability and the acquisition of hippocampus-dependent tasks in rat models of chronic cerebral hypoperfusion and beta-amyloid peptide-(25–35)-induced toxicity (Storozheva *et al.*, 2008).

Meanwhile, the antidepressant effects of DS were not assessed in other than chronic stress depression model, e.g., in models that mimic a state of learned helplessness which is distinct from hedonic deficit and an important feature of depression (Porsolt and Papp, 1998). Moreover, the possibility might exist that the antidepressant effects of DS could be limited by the conditions induced by stress and will not preclude other origins / manifestations of a depressive-like syndrome. However, the above mentioned efficacy of other insulin receptor sensitizers with regard to measures of helpless behaviour, e.g., in the tail suspension and forced swim test, and the ameliorative effects of DS in aged rodents suggest the efficacy of DS in a variety of experimental conditions. Based on this, the current study's objectives were to examine the effects of DS on several behavioural, molecular and EEG variables that were previously characterized as biological correlates of depressive state and adaptive response to stress in mice.

In the first experiment, using a model of stress-induced anhedonia (Cline *et al.*, 2014; Costa-Nunes *et al.*, 2014) we investigated whether a pre-treatment in C57BL6J mice with DS, at the dose of 25 mg/kg/day intraperitoneally for seven days, would improve normal sleep rebound (augmentation) following acute stress, a sign of adaptive stress response (Marinesco *et al.*, 1999; Suchecki *et al.*, 2012; Keshavarzy *et al.*, 2014; Albu *et al.*, 2014), as well as contextual fear conditioning learning that is regarded to be related to the adaptive sleep function (Rolls *et al.*, 2013; Barnes and Wilson, 2014). Also, we studied hippocampal gene expression of NMDA receptor subunit NR2A and the ratio of NR2A/NR2B, whose increases were previously demonstrated to accompany a development of stress-induced anhedonia in the here applied chronic

stress model (Costa-Nunes *et al.*, 2014). Notably, changes in the NMDA-receptor mediated transmission were shown to underlie the antidepressant effects of the neuronal insulin sensitizer pioglitazone (Salehi-Sadaghiani *et al.*, 2012).

Next, we have examined the potential antidepressant-like effects of DS in a recently validated model of elderly depression, where naïve 18-month-old C57BL6 exhibit hedonic deficit in a sucrose test, which is reversible by drugs with antidepressant and neuroprotective activity (Malatynska *et al.*, 2012). The effects of 7-day intraperitoneal injections of DS at the dose of 25 mg/kg/day to aged mice were assessed in the sucrose test and Illumina assay of gene expression profiling of the hippocampus and prefrontal cortex.

Finally, we applied a two-week administration of DS via drinking water at two doses of 25 and 75 mg/kg/day, in young naïve C57BL6J mice, and tested them in two-day forced swim test. The latter treatment group of mice was also investigated in the novel cage and elevated O-maze, to assess potential changes in their depressive- and anxiety-like behaviours, as well as locomotion / exploration. A two-week dosing with imipramine via drinking water (2.5 and 15 mg/kg/day) was used as a reference antidepressant treatment in the forced swim test. Additionally, hippocampal levels of the phosphorylated (inactive) form of Glycogen synthase kinase-3 β (pGSK-3 β), a previously determined marker of depressive-like behaviour and antidepressant activity in the forced swim test (Markova *et al.*, 2013a, 2014), were evaluated after the exposure of mice to forced swimming and treatment with DS at the dose of 75 mg/kg/day or imipramine at the dose of 15 mg/kg/day.

Materials and Methods

Animals

Studies were performed using 3.5-month-old male C57BL/6J mice. 3.5-month-old male CD1 mice were used for resident-intruder for social defeat paradigms and 2-5-

month-old Wistar rats were used for predator stress. All animals were from the Gulbenkian Institute of Science, Oeiras, Portugal. C57BL/6J mice were housed individually for fourteen days before the start of experiments; CD1 mice and rats were housed in groups of five before the experiment and then individually. All animals were under a reversed 12-hour light–dark cycle (lights on: 21:00 h) starting from the day of animals' transportation in the laboratory, with food and water *ad libitum*, under controllable laboratory conditions ($22 \pm 1^\circ\text{C}$, 55% humidity).

All studies were carried out in accordance with the European Communities Council Directive for the care and use of laboratory animals. A licence BH-2007 had been issued by the Ethics Committee on Animal Experimentation of Claude Bernard University of Lyon, in compliance with the decree No.: 03-505-2008 of the French Agriculture Ministry; permission 0421/000/000/2013 was issued by General Directory of Ethical Committee of the New University of Lisbon, in accordance with Portuguese Law-Decrees DL129/92 (July 6th), DL197/96 (October 16th) and Ordinance Port.131/97 (November 7th). This study had been also approved by the ethics committee of Maastricht University for animal research: CPV, DEC-UM 2009-109.

Study design with chronic stress depression model and EEG analysis of sleep

Chronic Stress Procedure and Behavioural Testing: Chronic stress and behavioural tests associated with its analysis were performed as described previously (Strekalova *et al.*, 2011, 2014; Couch *et al.*, 2013). Mice assigned to the stress group were injected with DS ($n = 20$; 25 mg/kg/day, *i.p.*) or vehicle ($n = 19$; see below) during 1 week prior the stress procedure as described elsewhere (Cline *et al.*, 2012). Control mice ($n = 7$) were not treated. Animals were assigned to three experimental conditions and had similar body weight and baseline sucrose preference (**Fig.2A**). Briefly, animals were exposed to 10 days of chronic unpredictable stress followed by behavioural testing using a two-bottle sucrose preference test (performed on Day 11; see below),

as well as a contextual fear conditioning learning task (performed on Days 12 and 13) that was carried out as described previously (Strekalova *et al.*, 2003; Vignisse *et al.*, 2014; see below). 24 hrs after the termination of behavioural testing, on Day 14, a fraction of stressed vehicle- (n=8), DS-treated (n=6) and control (n=7) mice, were sacrificed for brain dissection and subsequent RT PCR analysis of NMDA receptor subunits NR2A and NR2B. Another fraction of stressed vehicle- (n=7), DS-treated (n=6) mice were subjected to a sleep rebound paradigm and EEG recording (see below, **Fig.1A**). Remaining mice were used for other assays not reported in the current work. In addition, non-stressed mice that either received DS or not (n=15 in each group) were studied in a sucrose preference test before and after ten days following the dosing with DS was performed as described above.

The chronic stress procedure employed in this study comprised night time rat exposure and day time application of two of three stressors: a social defeat, restraint stress and tail suspension, a combination of which was applied in a semi-random manner (**Fig.1B**; Couch *et al.*, 2013). Briefly, between the hours of 09:00 and 18:00 two stressors per day were employed in the following sequence: social defeat for 30 minutes, restraint stress for 2 hours and tail suspension for 40 minutes with an inter-session interval of at least 4 hours. This procedure induces anhedonia in a considerably shorter time than previously validated models by increasing the daytime stress load. Details of rat exposure and chronic stressors can be found in *supplementary materials*.

Sleep rebound paradigm and EEG recording: One week after the termination of stress procedure, another fraction of vehicle-injected control and chronically stressed animals received surgically implanted electrodes. Animals were anaesthetised using a ketamine-xylazine mixture (respectively, 4 and 75 mg/kg, i.p.), placed in a stereotaxic frame and body temperature was maintained at 36.5-37°C by use of a homoeothermic blanket. Two electrodes (length, 2mm; diameter, 500 µm; both

stainless steel and connected to Teflon-insulated wires) were placed into the left and right frontal cortices (2mm lateral and anterior to Bregma (Cespuglio *et al.*, 1999). Two additive electrodes were placed into the left and right parietal cortices (2mm lateral to the midline at the midpoint between Bregma and lambda (Cespuglio *et al.*, 1999) for electroencephalographic recordings (EEGs). To obtain electromyograms (EMGs), three electrodes (active length, 1 mm; diameter, 500 μ m, all stainless steel and connected to Teflon-insulated iron wires) were inserted between two neck muscle layers. After placement, all electrodes were soldered to two miniature five-pin connectors (Sei 3D, Lyon, France) and the entire assembly anchored to the skull using Super-Bond glue (Sun Medical, Co., Shiga, Japan) and dental acrylic resin (Ivoclar, Lyon, France). Together, four electrodes were implanted within the frontal and parietal cortex, and one electrode in the neck muscle.

After one week of recovery that was combined with an acclimatization procedure to the EEG recording chambers, where mice were connected to recording cables and placed individually in plastic cages in a sound-insulated room (ambient temperature, $22\pm1^{\circ}\text{C}$; light-dark cycle 12h-12h, water and food ad libitum). Thereafter, starting at 16.00, 48-h EEG polysomnographic recordings (Embla, Medcare, Iceland) were performed in these mice during baseline conditions and immediately following the 6-h rat exposure stress (from 10.00 to 16.00) as previously described (Couch *et al.*, under revision).

For acute predation stress, mice were introduced into specialized cylindrical containers allowing visual and odour contact (Costa-Nunes *et al.*, 2014), which were placed into the rat home cage. Visual scoring of digitized EEG and EMG traces (EEG filtering 0.5-49.9 Hz and EMG filtering 15-49.9 Hz) was performed over 10 second bins to quantify the number and duration of sleep-wake episodes and the circadian scheduling of sleep-wake states as described elsewhere (Cespuglio *et al.*, 2012; Strekalova *et al.*, 2014). EEG power spectra (Somnologica software, Medcare, Iceland)

were also characterized. To this end, EEG traces sampled at 100Hz were subjected to fast-Fourier transformation (256 points, computational window 2.56s, and 50% overlap). Spectra were averaged over 10 second bins and divided into five adjacent bands: delta, 0.5-4Hz; theta, 4-8Hz, alpha, 8-11.5Hz, sigma, 11.5-14.5Hz; beta-1, 14.5-18.6Hz, and beta-2, 18.6-30Hz, and expressed as percentages of total band power (0-49.9Hz).

The duration of slow wave sleep (SWS) and Rapid Eye Movement (REM) sleep was averaged for 48-h baseline and 48-h after-challenge periods for each animal. Because of well-known inter-individual variability in sleep parameters of rodents, to evaluate the effects of a predation stress, the EEG data for that period were expressed in percent from the averaged baseline for each mouse, as described earlier (Cespuglio *et al.*, 2012; Strekalova *et al.*, 2014).

Study design with model of elderly depression

In this experiment, we examined the potential effects of DS on the consumption of palatable 1%-sucrose solution by old mice using a two-bottle sucrose preference test. A decrease in sucrose intake and preference over water is generally taken as a putative sign of anhedonia in rodents (Willner, 2005; Harro *et al.*, 2011) and was shown to be decreased in 18-month-old C57BL6 mice; imipramine and the neuroprotective drug dimebon were shown to reverse this deficit (Malatynska *et al.*, 2012). It was investigated whether DS administered to 18-month-old mice (n=9) for 1 week at the dose 25 mg/kg/day would affect the parameters of the sucrose preference test, in comparison with a group of mice of the same age that did not receive such a treatment (n=8). The dose of DS was based on previous studies with CD1 mice, in which its administration, with the above-indicated dosing scheme, effectively reduced the stress-induced decrease in sucrose intake and preference, floating behaviour and alteration of hippocampal gene expression typical of the subgroup of mice susceptible to anhedonia (Cline *et al.*, 2012). 24 h after the

termination of the sucrose test, mice were sacrificed and their hippocampal formation and prefrontal cortex were isolated for subsequent gene expression profiling using Illumina assay as described elsewhere (Markova *et al.*, 2013b; see also below and *Supplementary Material*; **Fig.1C**).

Study design with tests for anxiety- and depressive like behaviour in naïve mice

Three-months-old male C57BL/6J mice received normal water (control group), imipramine (2.5 or 15 mg/kg/day) or dicholine succinate (DS, 25 or 75 mg/kg/day; Buddha Biopharma Ltd, Helsinki, Finland; both compounds were dissolved in drinking water) for two weeks (n=15 in each group), and were tested for a depressive-like behaviour in a two-day forced swim test (Malatynska *et al.*, 2012; Markova *et al.* 2013a, 2014; Costa-Nunes *et al.*, 2015). Prior this testing, mice that received DS at the dose 75 mg/kg/day were additionally compared with control animals in a novel cage test (Strekalova *et al.* 2004) and elevated O-maze (Malatynska *et al.*, 2012; Cline *et al.* 2012), in order, in particular, to rule out potential confounds in the assessment of floating behaviour (n=10 from each group was tested; **Fig.1D**). Because other studies on mice revealed no effects at the dose of 25 mg/kg/day of DS on the parameters of anxiety and locomotion (Cline *et al.*, 2012), animals from the current experiment treated with this dose were not examined in additional assays.

Since previous studies revealed a decrease of hippocampal *p*GSK-3 β levels to be a marker of depressive-like behaviour in a two-day forced swim test that was preserved by an antidepressant treatment including imipramine (Markova *et al.*, 2014), we have chosen to study whether this variable is sensitive to the effects of DS treatment as well. Therefore, mice subjected to a two-day FST test and received DS at the dose 75 mg/kg/day, or imipramine at the dose 15 mg/kg/day or remaining untreated, were sacrificed 10 min after the second swimming session for subsequent isolation of the hippocampus and ELISA assay (see below and *Supplementary Material*, **Fig.1D**). An

additional group of naïve control animals that were not subjected to FST, was sacrificed and analyzed as well.

Behavioural tests

Sucrose test. In order to assess the hedonic state of mice, they were given a free choice for 8 h (between 9.00 – 17.00 h) of two drinking bottles; one with 1%-sucrose solution, and another with tap water, as described elsewhere (Strekalova *et al.*, 2011, 2014). To prevent possible effects of side-preference in drinking behaviour, the position of the bottles in the cage was switched after 4 h. Special precautions have been made in order to minimize the spillage of liquids and error of measurement during sucrose test. The consumption of water, sucrose solution and total intake of liquids was estimated simultaneously in the control and experimental groups by weighing the bottles. Percentage preference for sucrose was calculated using the following formula:

$$\textbf{Sucrose Preference} = \left(\frac{V_{\text{Sucrose solution}}}{(V_{\text{Sucrose solution}} + V_{\text{Water}})} \right) \times 100\%$$

Fear Conditioning test. The test procedure was adapted from a previously described protocol (Strekalova *et al.*, 2003; Vignisse *et al.*, 2014). The apparatus (Evolocus LLC Tarrytown, NY, USA and Technosmart, Rome, Italy) consisted of a transparent plastic cubicle (25 cm x 25 cm x 50 cm) with a stainless-steel grid floor (33 rods 2 mm in diameter). A single alternating electric current (AC, 50 Hz; 0.7 mA) was delivered after a 2-min acclimatization period. Freezing behaviour was scored by visual observation during an acclimatization phase and a test of memory recall that was carried out 24 h later. The freezing episode was defined by a complete lack of movement other than respiration accompanied by the occurrence of a specific posture of tension with the tail in a straight and tense position, as described previously (Strekalova *et al.*, 2003; Fleischmann *et al.*, 2003; Vignisse *et al.*, 2014). The occurrence of freezing behaviour was assessed every 10 sec for 180 sec; each 10-sec score was assigned to a freezing

or non-freezing period, and the percentage of time spent in freezing was calculated. During delivery of foot shocks, the reaction of the animals was closely observed and rated using a 3-grade score system as maximal (jumping and squeaking), intermediate (jumping only), or modest (running). After delivery of the current, the mouse was immediately placed back in the home cage.

Forced swim test. The Porsolt forced swim test has been used as described elsewhere (Malatynska *et al.*, 2012; Couch *et al.*, 2013). Mice were subjected to two 6-min swimming sessions spaced 24 h apart in a transparent cylinder ($\varnothing$ 17 cm) filled with water (+23 °C, water height 13 cm, height of cylinder 20 cm, illumination intensity 25 Lux). Floating was defined by the absence of any directed movements of the animals' head and body and was scored manually; criteria of scoring were previously validated using Noldus EthoVision XT 8.5 (Noldus Information Technology, Wageningen, The Netherlands) and CleverSys (CleverSys, Reston, VA, USA). Using this method, the latency of the first episode of floating and the duration of floating behaviour were recorded during the 6-min swimming session on the second day of the test. Latency to begin floating was scored as time between introduction of the animal into the pool and the first moment of complete immobility of the entire body for a duration of >3 seconds. The total time spent floating was scored for the entire duration of the test using post-test video footage.

Elevated O-maze. The apparatus (Technosmart, Rome, Italy), which consisted of a circular path (runway width 5.5 cm, diameter 46 cm), was placed 20 cm above the floor. Two opposing arms were protected by walls (height 10 cm), and the illumination strength was 5 Lux. The apparatus was placed on a dark surface in order to reduce reflection and maintain control over lighting conditions during testing. Anxiety-like behaviour was assessed using previously validated parameters that were scored manually as described elsewhere (Cline *et al.*, 2014; Costa-Nunes *et al.*, 2014). Mice were placed in one of the closed-arm compartments of the apparatus. The

latency of the first exit to the anxiety-related open compartments of the maze, the total duration of time spent therein, the number of risk assessment exploratory events and the number of exits to the open arms were scored during a 5-min observation period. The risk assessment exploratory events were defined by the stretching of the head and a body out of the area protected by the walls towards open arm zone, combined with exploratory pose and movements, directed to the edges of the maze. Half of the body and back limbs of a mouse stayed in the close arm area during these events.

Novel cage test. The novel cage test was performed to assess vertical activity in a new environment (Couch *et al.*, 2013). Mice were introduced into a standard plastic cage (21 cm x 21 cm x 15 cm) filled with fresh sawdust. The number of exploratory rears per each minute was counted under red light during a 5-minute period.

Dosing

The current reference antidepressant treatment was selected because of its effects in lowering the rate of stress-induced anhedonia over other methods of delivery and doses of antidepressants (Cline *et al.*, 2014; Costa-Nunes *et al.*, 2012, 2014). Previous experiments revealed an antidepressant-like effect of one-week pre-treatment with daily i.p. injections of DS (25 mg/kg/day) in CD1 mice for stress-induced depressive-like changes (Cline *et al.*, 2012). Likewise, here DS was administrated during 7 consecutive days to young mice preceding chronic stress or to 18-months-old mice preceding sucrose test, at the above-indicated dose. DS, provided by Buddha Biopharma Ltd (Helsinki, Finland), was dissolved in water for injections. The volume of DS and vehicle injections was 0.01 ml / g body weight 0.01 ml/kg.

In a study of young non-stressed mice exposed to a battery of tests for emotionality, DS was applied via drinking water at the doses of 25 and 75 mg/kg/day. In this study, imipramine (Sigma-Aldrich, St. Louis, MO, US) was administrated via drinking as well.

It was dissolved in tap water; the solution was freshly prepared every 2-3 days. Dosage for imipramine was set at 2.5 or 15 mg/kg/day. Since imipramine is light sensitive, bottles were protected by aluminium covers. The calculation of the concentration of DS and imipramine in drinking water was based on the previously evaluated mean volume of daily water consumption in C57BL6J mice that was about 3.0 ml and on the dosage of treatment.

Brain dissection and quantitative RT-PCR (qPCR)

Mice were killed by cervical dislocation and their brains were dissected. RNA extraction was performed from microdissected snap-frozen hippocampi using RNeasy RNA extraction kit with DNase I treatment, as previously described (Qiagen, Hilden, Germany; Couch *et al.*, 2013; Costa-Nunes, 2014). Using random primers and Superscript III transcriptase (Invitrogen, Darmstadt, Germany), 1 µg total RNA was converted into cDNA. The expression levels of NR2A and NR2B as well as the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH), that was used as a reference gene for quantification, were evaluated with TaqMan probes and the CFX96 Real-time System (BioRad, Hercules, CA, USA). Cycling conditions and sequences of primers used are indicated in the *Supplementary data*.

Illumina assay

Gene expression profiling was performed using Illumina technology (Northwestern Chicago University, USA) with the hippocampi obtained from 18-months old mice (drug-naïve or treated with DS); five animals per group were analysed. Total RNA samples were hybridized to IlluminaBeadChips (MouseRef-8 v2 Expression BeadChip; Illumina, Inc. San Diego, CA, USA) which were prepared using the IlluminaTotalPrep RNA Amplification kit (Applied Biosystems/Ambion, Carlsbad, CA, USA); the samples were assigned to the chips in random order with the constraint that no two samples from the same group were assigned to the same chip, to avoid confounding of experimental groups with the chips. Microarray data were analysed using standard

analysis procedures, which included assessment of the overall quality of array data and statistical evaluation of differentially expressed genes. Once the quality of array data was confirmed, the Gene Chip Operating System (Illumina, Inc. San Diego, CA, USA) was used to calculate signal intensities, detection calls, and their associated P values for each transcript on the array. Gene expression was normalized to the expression of the housekeeping gene, beta-actin, due to its stable expression, and calculated as percent mean of the control group of young mice. Differences in gene expression between groups were evaluated using unpaired two-tailed t-test.

Illumina data were imported into Partek Genomics Suite and quantile-normalized. Arrays that appeared as outliers on PCA were removed from the dataset. Comparisons between experimental groups were carried out in Partek-GS using ANOVA with appropriate contrasts. P values were adjusted for multiple testing using step-up False Discovery Rate (FDR). The following criteria were used to select differentially expressed genes at different stringency levels: Strict: FDR < 0.05 and |fold change| > 2; Medium: FDR < 0.1 and |fold change| > 1.5; Loose: unadjusted p value < 0.001 and |fold change| > 1.3, Very loose: unadjusted p values < 0.01 and no fold change threshold (only used when more stringent selection criteria yielded zero or very few hits). In the current analysis, “medium” criteria were applied.

ELISA of pGSK-3 β

Hippocampus was homogenized in buffer containing 10 mM Tris (pH7,4), 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM Na₄P₂O₇, 10% glycerol, 2 mM Na₃VO₄ in the presence of a protease inhibitor cocktail (Sigma, USA). The GSK-3 β [pS9] ELISA kit (Invitrogen Corporation, USA) was used for detection and to quantify the level of GSK-3 β protein phosphorylated at serine residue 9. After three incubations according the instruction manual, a signal intensity provided by monoclonal capture antibody specific for GSK-3 β that has been coated onto the wells, was evaluated at 450 nm using a plate reader (Wallac 1420 VICTOR, USA). The

results were normalized to total protein level in tissues homogenates, which was determined by the biuret assay; bovine serum albumin was used as a standard (for further details, see *Supplementary data*).

Statistics

Data were analysed with GraphPad Prism version 5.0 for Windows (San Diego, CA). Unpaired two-tailed test was used to compare two groups; one-way ANOVA was used followed by Tukey's, or Dunnett's *post-hoc* comparison tests was applied to compare three or more groups. Repeated measurements with non-parametric data were evaluated with Wilcoxon test. The level of confidence was set at 95% ($p < 0.05$) and data are shown as mean $\pm$ SEM.

Results

Dosing with Dicholine Succinate preserves normal hedonic status and fear conditioning in a chronic stress paradigm

At the baseline, there was no difference in sucrose preference between the groups ($p > 0.05$, $q = 0.25$, Tukey, **Fig.2A**). Following a chronic stress paradigm, ANOVA revealed a significant difference for sucrose preference ($F(5, 87) = 8.608$, $p < 0.0001$). *Post-hoc* analysis showed that only the vehicle-treated stressed group had a significant reduction in sucrose preference compared to controls ($p < 0.001$, $q = 5.53$, Tukey) as well as to their DS-treated stressed counterparts ($p < 0.05$, $q = 4.55$, Tukey, **Fig.2A**), indicating that treatment with DS was able to preclude a hedonic deficit. Sucrose preference was similar in non-treated non-stressed mice (76.22 ± 2.84 %) and DS-treated non-stressed mice (82.01 ± 3.1 ; $p = 0.193$; $t(12) = 1.381$; unpaired two-tailed *t*-test).

During training in the fear conditioning model, control, vehicle-treated stressed and DS-treated stressed groups had a similar percent of mice expressing responses to foot shock: maximal (45, 50 and 55%, respectively), intermediate (30, 25 and 25%,

respectively), and a modest response to the foot-shock (25, 25 and 20%, respectively; $p > 0.05$, exact Fischer test). Baseline rates of freezing behaviour measured during training were minimal and did not differ between the three groups either (control vs vehicle: $p > 0.05$ $q = 0.25$, control vs DS: $p > 0.05$, $q = 2.09$; data not shown, Tukey); together, suggesting their similar behaviour under untrained conditions.

Analysis of freezing behaviour during a recall session using ANOVA and Tukey *post-hoc* test revealed a significant difference between the groups ($F(5,68) = 4.724$, $p = 0.0009$) and showed that the vehicle-treated stressed group had significantly less freezing as compared to their counter parts control ($p < 0.01$, $q = 5.56$, **Fig.2B**) and DS-treated stressed mice ($p < 0.01$, $q = 5.27$, **Fig.2B**).

Effects of dosing with Dicholine Succinate on sleep parameters of chronically stressed mice in acute-stress sleep rebound paradigm

A fraction of mice exposed to chronic stress, was implanted with electrodes and, after a recovery period, was habituated to the recording chamber and connection to the cables and then subjected to a forty-eight hour EEG registration. In order to assess the effects of acute stress on chronically stressed mice that were either treated with DS, or remained pharmacologically naïve, the recording procedure was interrupted for a 6-h rat exposure stress and then re-started for another 48 h. The duration of SWS and REM sleep was averaged for 48-h baseline and after-predation periods for each animal. Because of well-known inter-individual variability in sleep parameters of rodents, EEG data that were obtained after the predation period, were expressed as percent from the averaged baseline values.

Both groups had an increase of the duration of SWS and REM sleep after acute predation stress in comparison to baseline values (stressed non-treated group: $p = 0.0158$, $W = 28.0$ and $p = 0.0469$, $W = 24.0$, stressed DS-treated group: $p = 0.0313$, $W = 21.0$ and $p = 0.0255$, $W = 28.0$, Wilcoxon). The duration of REM sleep, normalized

to baseline, was significantly longer in the DS-treated stressed group compared with the pharmacologically naive stressed group ($t(10)=2.478$, $p=0.0327$, unpaired two-tailed t -test); however, no differences were seen for SW sleep ($t(11)=0.3451$, $p=0.7366$, unpaired two-tailed t -test, **Fig.2C, 2D**). Thus, DS-treated stressed mice demonstrated enhanced REM sleep rebound following acute stress, a sign of adaptive stress response, in comparison with vehicle-treated stressed animals. Power spectra analysis revealed no changes in comparison to baseline measures in both challenged groups ($p>0.05$, Wilcoxon) and no differences between the groups challenged with a predator stress ($p>0.05$, unpaired two-tailed t -test), during SWS nor during REM sleep stages, as expressed in percent from initial baseline values for these animals (**Fig.2C, 2D**; power spectra data for baseline and after-predation conditions can be found in *Supplementary Table 2*).

Dosing with Dicholine Succinate prevents stress-induced increases of mRNA of NMDA receptor subunits in the hippocampus of chronically stressed mice

Since changes in the NMDA-receptor mediated transmission were shown to underlie the antidepressant effects of other neuronal insulin sensitizers, we studied hippocampal gene expression of NMDA receptor subunit NR2A and the ratio of NR2A/NR2B, whose increases were previously demonstrated to accompany a susceptibility to stress-induced anhedonia in the here applied chronic stress model (Costa-Nunes *et al.*, 2014). Twenty-four hours after the last behavioural test, i.e., on the 5th day after the termination of chronic stress, in accordance with previously established protocols (Strekalova *et al.*, 2011; Cline *et al.*, 2012), animals were sacrificed for the study of hippocampal gene expression of NMDA receptor subunits. The mRNA levels of NR2A were significantly increased in chronically stressed animals which were not treated with DS following chronic stress ($p<0.05$, $q=379$, Tukey, $F(2,17)=4.010$, $p=0.0375$, ANOVA, **Fig.2E**). There was no overall significant changes in the NR2B mRNA expression levels between the groups following chronic stress

($F(2,21)=0.8881$, $p=0.4264$, ANOVA, **Fig.2F**). Untreated stressed mice had an increased ratio of NR2A/NR2B as compared to both controls ($p<0.05$, $q=4.70$, Tukey) and the DS stressed groups ($p<0.05$, $q=4.62$, Tukey, $F(2, 17) = 7.625$, $p=0.0043$, ANOVA **Fig.2G**).

Effect of dosing with Dicholine Succinate on sucrose preference of old mice

At the baseline, there was no difference in sucrose preference between the groups ($t(7)=0.4509$, $p=0.6657$, unpaired t -test). Animals, which did not receive treatment, aged 18 months showed no differences in preference for sucrose ($t(7)=0.4509$, $p=0.6657$, paired two-tail t -test **Fig.3A**) or in sucrose intake ($t(7)=0.8845$, $p=0.4058$, paired two-tailed t -test, **Fig.3B**) between two repeated assays of sucrose test. However, sucrose preference and intake of sucrose was significantly increased after dosing with DS ($t(7)=2.656$, $p=0.0327$, **Fig.3A** and $t(8)=2.359$, $p=0.0461$, paired two-tailed t -test, **Fig.3B**; respectively). None of the groups showed any differences for water intake ($t(8)=1.099$, $p=0.3038$, control; $t(8) = 1.850$, $p= 0.1015$, DS, paired two-tailed t -test, **Fig.3C**). Total liquid consumption was also not changed in any of the groups ($t(8)=0.8135$, $p=0.4395$, control; $t(8) = 0.7358$, $p=0.2414$, DS, paired two-tailed t -test, **Fig.3D**).

Gene expression profiling of the hippocampus and prefrontal cortex of old mice treated with Dicholine Succinate

Gene expression profiling of the hippocampus of DS-treated 18-month-old mice revealed expression changes in 27 genes, in comparison to a control group, for over 1.5 fold, and FDR was <0.1 . Among these genes are those involved in neuronal synaptic plasticity: Arc and Nptx2, SGK1, Taf15, Vgf, Egr1, Gatad2b; all of them were up regulated (**Fig.4A**, *Supplementary Table 3*). Apart from them, genes encoding ascorbate transporter Slc23a3, regulator of axonal transport Dctn1, serine protease Htra1, serine proteases inhibitor: Slpi were up-regulated as well. The functions of the proteins encoded by 6430548M08Rik and 6030419C18Rik genes were not described

in the literature. Functional categories of down-regulated genes in DS-treated old animals constitute genes that regulate sleep and circadian rhythm: Gm129, Cirbp and Dbp, as well as ascorbate transporter Slc23a2, fatty acids transporter Slc27a1.

As for the prefrontal cortex, four genes whose expressions were significantly changed according to the criterion described above were detected. One gene was up regulated (Inhba) while three were down regulated (**Fig.4B**, *Supplementary Table 3*).

Effects of dosing with Dicholine Succinate of naïve mice: changes in depressive-, anxiety-like behaviours and hippocampal levels of phosphorylated GSK-3 β

In the forced swim test (FST), a one-way ANOVA revealed no changes between the groups in the latency to float and a significant effect over the total time spent floating ($F(4,70)=1,371$, $p=0.2528$; $F(4,70)=6.36$, $p=0.0002$, respectively; **Fig.5A**). A *post-hoc* Dunnett's test showed no significant differences between treated animals and a control group for the latency to swim, whereas the duration of immobility was significantly decreased in animals receiving higher doses of imipramine (15 mg/kg/day) and dicholine succinate (75 mg/kg/day) in comparison with controls ($p<0.01$, $q=3.79$; $p<0.05$, $q=2.81$, respectively; **Fig.4A**). In the elevated O-maze test mice treated with a dose of 75 mg/kg/day of dicholine succinate, displayed significantly longer duration in the open arms, with no significant changes to a latency to exit, total number of exits, or risk assessment behaviour in comparison with control animals ($p=0.038$, $t(18)=1.88$; $p=0.28$, $t(18)=0.59$; $p=0.15$, $t(18)=1.07$; $p=0.34$, $t(18)=0.99$, respectively; unpaired two tailed t -test; **Fig.5B**). In the novel cage test for locomotion / exploration, animals treated with dicholine succinate exhibited unchanged number of rearings in comparison to a control group (1st min: $p=0.61$, $t(17)=0.52$; 2nd min: $p=0.40$, $t(17)=0.86$; 3rd min: $p=0.89$, $t(17)=0.13$; 4th min: $p=0.20$, $t(17)=1.34$; 5th min: $p=0.49$, $t(17)=0.71$; total rearings: $p=0.27$, $t(17)=1.13$; unpaired two-tailed t -test; **Fig.5C**).

A one-way ANOVA reveals significant group differences in the levels of phosphorylated GSK-3 β in the hippocampus of mice subjected to the forced swim test ($p=0.0145$; $F=4.130$). A *post-hoc* Tukey test showed a significant reduction in GSK-3 β in untreated animals as compared with to intact control mice ($p<0.05$, $q=3.85$). No such decrease was found in animals tested in the forced swim test that were treated with either imipramine or dicholine succinate (imipramine: $p>0.05$, $q=0.21$ and dicholine succinate: $p>0.05$, $q=0.43$; **Fig.5D**). Untreated animals subjected to the forced swim test had significantly lower levels of the hippocampal pGSK-3 β levels in comparison to imipramine- and DS-treated animals ($p<0.05$, $q=3.95$ and $p<0.05$, $q=4.03$, respectively; Tukey *post-hoc* test; **Fig.5D**).

Discussion

Effects of Dicholine Succinate in a chronic stress model

In the current work, stress exposure lowered sucrose preference in agreement with other reports (Willner *et al.*, 1987; Harro *et al.*, 2001; Krishnan *et al.*, 2007). Stressed mice treated with DS showed no significant change in sucrose preference measured after the 10th day of stress as compared to control animals (**Fig.2A**), similarly to the effects of classical antidepressants (Cline *et al.*, 2014; Costa-Nunes *et al.*, 2014; Strekalova *et al.*, 2014). Earlier, we have shown in a model of stress-induced anhedonia that the decrease in sucrose preference is paralleled by a reduction in sucrose intake (Strekalova *et al.*, 2004, 2006). Importantly, administration of DS did not alter sucrose test parameters in non-stressed animals ruling out any possible confounding artefacts for sucrose test measurements which could be related to treatment. Thus, the partial preclusion of the stress-induced reduction for sucrose preference by treatment with DS manifests their antidepressant-like activity in our study that is in line with previous findings obtained in a similar model on CD1 mice (Cline *et al.*, 2012).

Treatment with DS prevented stress-induced memory impairment in the fear conditioning task (**Fig.2B**). Similar freezing scores at baseline and the responses to foot shock in all experimental groups suggest that the deficits, revealed here in the contextual memory performance in mice subjected to stress and their rescue in the DS-treated stressed animals, are unlikely to be due to any distinct sensitivity to the foot-shock or to basal differences in the emotionality in the groups. While a similarity in these parameters in tested mice is a prerequisite of their unaltered acquisition of fear conditioning, the here employed study design does not exclude the ameliorative effect of DS on either or both learning phase(s), acquisition or / and consolidation of contextual memories. Of interest, the stimulation of neuronal insulin receptor is implicated both in memory acquisition and consolidation (Moosavi *et al.*, 2007) suggesting that DS can be involved in two of these processes.

The effects of DS in the mouse fear conditioning paradigm, as previously validated in our model studies of hippocampus-dependent performance in mice (Strekalova *et al.*, 2003, Vignisse *et al.*, 2014), are in line with the ameliorative effects of DS on hippocampus- and cortex-dependent learning in step-through, step-down and Morris water maze paradigms which this drug exerted under pathological conditions of diverse origins (Storozheva *et al.*, 2008; Storozhevykh *et al.*, 2008). Previously reported effects of DS on increased levels of hippocampal IGF-2, brain N-acetylaspartate/creatine, choline acetyltransferase activity (Storozheva *et al.*, 2008; Cline *et al.*, 2012) can attest for the here observed memory preserving effects of DS. Interestingly, choline acetyltransferase activity in the brain was shown to be regulated by neuronal insulin receptor-mediated mechanisms (Hoyer, 2003). Recent evidence for a critical role of IGF2 in inhibitory avoidance learning and adult neurogenesis as shown in the fear conditioning paradigm (Agis-Balboa *et al.*, 2011; Chen *et al.*, 2011; Bracko *et al.*, 2012) can additionally explain the beneficial effects of DS on memory performance in chronically stressed mice. Finally, recently shown

activation of insulin receptor-mediated transmission a newly discovered mechanism of augmented neurogenesis (Ziegler *et al.*, 2014), can per se result in pro-neurogenetic and neuroprotective activities that are characteristic for the antidepressants of various classes (Duman and Li, 2012), and, thus, can underlie pro-cognitive and antidepressant effects of DS.

In the present study, we found significantly longer duration of REM but not SWS sleep in chronically stressed DS-treated mice subjected to acute stress of predation (**Fig.2C**). It is well established that acute stress of various natures, as for instance, immobilization or predation, induces an adaptive effect of sleep rebound, consisting in an increase of the REM stage of sleep and to a lesser extent SWS, this is regarded as one of the important anti-stress mechanisms (Cespuglio *et al.*, 1995; Marinesco *et al.*, 1999; Koehl *et al.*, 2002; Tang *et al.*, 2007; Tiba *et al.*, 2008; Couch *et al.*, under revision). It was shown that stress-induced sleep rebound is decreased during aging (Descamps and Cespuglio, 2010, Clement *et al.*, 2003), development of anhedonia during stress (Couch *et al.*, under revision) and various neurochemical abnormalities associated with neuropsychiatric conditions (Bonnet *et al.*, 2000; Boutrel *et al.*, 2002; Meerlo *et al.*, 2002; Vázquez-Palacios *et al.*, 2004; Albu *et al.*, 2014). While the exact functions of each of the stages of sleep are, as yet unclear, it is claimed that normal REM sleep is a crucial factor of memory consolidation (Rolls *et al.*, 2013; Barnes and Wilson, 2014). Additionally, insulin receptor mediated signalling is regarded as one of the regulatory mechanisms of sleep (Valatx *et al.*, 1999; Kashyap and Defronzo, 2007).

While recent studies suggest that challenging insulin receptor mediated transmission in the brain might have long-term effects lasting for weeks (Hoyer, 2003), we trust that the ameliorative action of DS reported here on sleep rebound is likely to be related to reduced manifestations of depressive-like changes and stress response in chronically stressed mice that was found to be elevated for weeks in the model

applied here when no antidepressant therapies are used (Cline *et al.*, 2014). At the same time, power spectra activity was not changed in DS-treated mice (**Fig.2C, Table 2**) ruling out non-specific general changes in EEG activity of the treatment and suggesting preserved cerebral homeostasis in DS-treated mice that can be compromised by some antidepressants or aging (Cespuglio *et al.*, 1995, Clement *et al.*, 2003).

Our study evidenced preventive effects of DS on stress-induced increases of hippocampal gene expression of NMDA receptor subunits NR2A and the NR2A/NR2B ratio (**Fig.2E-2G**). The increases of these measures were previously shown to accompany elevated anxiety and occurrence of hedonic deficit during stress (Boyce-Rustay and Holmes, 2006; Gao *et al.*, 2010; Pochwat *et al.*, 2013; Calabrese *et al.*, 2013; Costa-Nunes *et al.*, 2014), impulsivity and aggression (Meyer *et al.*, 2004; Bortolato *et al.*, 2012), home cage hyperactivity and a stress-induced elevation in peripheral concentrations of corticosterone (Longordo *et al.*, 2009, 2011; Huang *et al.*, 2010b). A number of findings evidence that hippocampal NR2A and NR2B subunits of the NMDA receptor display fast kinetics in response to CORT and adverse experiences, where changes in gene expression parallel rapid alterations in total and surface protein levels as well as receptor trafficking (Zhang *et al.*, 1997; Tse *et al.*, 2011; Pochwat *et al.*, 2013) suggesting that the changes reported in this study for mRNA levels of the NMDA-receptor subunits reflect its functional alterations.

Other studies demonstrate the importance of NMDA-receptor mediated currents in the antidepressant effects of pioglitazone, as it was discussed above (Salehi-Sadaghiani *et al.*, 2012), which allows speculation that amelioration of depressive-like conditions via enhancement of insulin receptor mediated signalling by different drugs might commonly implicate glutamatergic neurotransmission via this receptor.

Effects of Dicholine Succinate in a model of elderly depression

DS-treated 18-month old mice displayed higher sucrose intake and preference than pharmacologically naïve mice of this age, suggesting a normalization of hedonic state by the treatment. Comparable to these changes, similar effects were also demonstrated for treatment with imipramine or the neuroprotective drug dimebon (Malatynska *et al.*, 2012). There was a non-significant reduction of water intake in the DS-treated group that was obviously accounted for compensatory changes in drinking behaviour, while total liquid intake was not altered by the treatment (Figs. 3A-D). Together, the current findings may be interpreted as a manifestation of an antidepressant-like activity of DS in a model of elderly depression that is in line with previous reports showing that chronic administration of DS counteracts the development of aging-related neurochemical and cognitive deficits in mice (Storozheva *et al.*, 2008) and preserves normal sucrose preference in chronically stressed mice.

Interestingly, Illumina gene expression study showed that among 27 significantly changed genes in accordance to the criterion applied here, 17 were up-regulated: 7 genes from this cohort (41.2%) constituted genes encoding factors of synaptic plasticity (**Fig.4A** and *Supplementary Table 3A*). These functions are well established for most of them, such as for immediate early gene *Arc*, whose activity is regulated by stimulation of insulin receptor in neurons (Kremerskothen *et al.*, 2002; Chen *et al.*, 2014), suppressed by chronic stress (Elizalde *et al.*, 2008, 2010) and increased by antidepressants (Molteni *et al.*, 2008; Alme *et al.*, 2007), immediate-early gene *Nptx2* encoding neuronal activity-regulated pentraxin (Narp) that modulates AMPA-receptor functions (O'Brien, *et al.*, 1999; Chang *et al.*, 2010), *SGK1*, which regulates hippocampal postsynaptic density-95 and dendritic growth (Ma 2006; Yang 2006). Also, *TAF15* was shown to be implicated in the trafficking of NMDA glutamate receptor (Ibrahim F, 2013). *VGF* and *Egr1* were found to enhance hippocampal

synaptic plasticity and neurogenesis (Thakker-Varia and Alder, 2009). GATAD2B was shown to be required for normal cognitive performance and synapse development (Willemsen, 2013).

Another cohort of altered genes in DS-treated mice whose function is well established constitute genes that are involved in the regulation of sleep and circadian activity. These genes include Gm129, a novel regulator of the feedback loop that involves activators and suppressors of circadian regulation (Annayev *et al.*, 2014), Cirbp, a factor of cytokine-regulated expression of clock genes (Lopez *et al.*, 2014) and Dbp, a putative clock-controlled transcription factor, which is increased under sleep deprivation (Wisor *et al.*, 2002).

Remarkably, many of these altered genes are functionally associated with insulin receptor signalling. As for instance, activation of Arc is regulated by insulin receptor in neurons through IRS/Grb2/Raf/Mek/Erk pathway (Kremerskothen *et al.*, 2002); Sgk1 is encoding a kinase that is activated by insulin via PI3-kinase (Lang F, 2010). Vgf is encoding a neuropeptide, which expression is up regulated by BDNF and insulin (Salton *et al.*, 2000; Busse *et al.*, 2012); Rsg4 plays a role as a negative regulator of insulin-stimulated GLUT4 translocation in adipocytes (Kanzaki *et al.*, 2000). Finally, Htra1 is encoding a protease that regulates the availability of insulin-like growth factors (IGFs) by cleaving IGF-binding proteins (Zumbrunn *et al.*, 1996); FosB is encoding a transcription factor, which its periphery expression is up-regulated by insulin (Coletta *et al.*, 2008).

Notably, DS evoked limited changes in gene expression in the prefrontal cortex. Among four genes, whose expression was significantly changed in this study is at least one factor that was shown to be implicated in the morphological plasticity of the brain and antidepressant response. Inhba encodes a beta A subunit that is shared by glycoprotein families Activins and Inhibins, that were shown to have opposite

functions concerning antidepressant mechanisms (Ganea *et al.*, 2012). Activin A, the homodimer of beta A, was shown to exert an acute antidepressant-like effect and increase the formation of synaptic contacts by modulating the dynamics of actin in the neuronal spines (Shoji-Kasai *et al.*, 2007; Ganea *et al.*, 2012). Expression of other genes belonging to various classes of regulators whose functions in the CNS are not well defined are associated with autophagy (Gabarapl1), other structural functions (Opalin) and myelin organization (Stmn4) were also significantly changed. Other significantly altered genes encode molecules whose functions in the brain are not well defined and mostly associated with structural functions and myelin organization (**Fig.4B** and *Supplementary Table 3B*).

While gene expression profiling data in this study need verification using additional methods, it is remarkable that many changes are associated with activation of brain plasticity factors and changes in sleep / circadian regulation that are known to be implicated in the pathogenesis of depression and antidepressant treatment (Mellman *et al.*, 2002; Wainwright and Galea, 2013). Moreover, a number of highlighted gene changes were also shown to affect the elements of insulin receptor-mediated signalling that could be expected with the use of compounds that like DS stimulate these processes.

Effects of Dicholine Succinate on behaviour and hippocampal pGSK-3 β in naïve mice

While the effects of either treatment on the latency to the first episode of floating were not significant, mice treated with higher doses of imipramine and DS had significantly shorter duration of this behaviour (**Fig.5A**). This is in line with recent findings treated with DS via food and tested in the tail suspension and FST and together suggests that this treatment diminishes the symptoms of learned helplessness (Costa-Nunes *et al.*, 2015). Coinciding with these results, another insulin sensitizer, rosiglitazone, was reported to reduce immobilization and floating behaviours in mouse tail suspension and forced swim tests respectively (Eissa Ahmed

et al., 2009). Such effects well documented for other antidepressants and are regarded as a manifestation of antidepressant-like activity (Porsolt, 1998, Willner, 2005).

Treatment with DS decreased anxiety scores as shown by increased time spent in the open arms of the elevated O-maze indicating its anxiolytic and anti-stress effect (**Fig.5B**). Such effects are well documented for compounds with anxiolytic activity (Willner, 2005). Elevated anxiety was found to parallel induction of a depressive-like syndrome (Willner *et al.*, 1987; Willner, 2005; Krishnan *et al.*, 2007; Strekalova *et al.*, 2011). No changes in vertical activity were found in the DS-treated group at no time period of the observation in the novel cage test, suggesting a lack of general effects on locomotion and a specificity of the above-described effects on measures of depression- and anxiety-like behaviours (**Fig.5C**).

Because on one hand, insulin, via the IRS/PI3K/Akt pathway, regulates GSK-3 β activity (Cross *et al.*, 1995) and on another hand, mice exposed to a two-day forced swimming revealed decreased hippocampal levels of phosphorylated at Ser9 (inactive) form of GSK-3 β (Markova *et al.*, 2014), we assessed potential effects of DS on the latter measure as well. We found that DS at the dose in which it exerted an antidepressant effect in this test, precluded stress-induced reduction of *p*GSK-3 β in this study that was comparable to the effect of imipramine. The present finding is in line with an inhibitory effect of insulin on GSK-3 β activity. Given accumulating evidence for similar effects of other antidepressant interventions and, in general, for the role of elevated function of GSK-3 β in promoting mood disorders and neurodegeneration (Doble *et al.*, 2003; Li and Jope, 2010), these results suggest that the above-described effects on *p*GSK-3 β can underlie an antidepressant and pro-cognitive action of DS. As GSK-3 β plays a key role in the induction of NMDA-receptor-dependent LTD (Peineau *et al.*, 2009; Bradley *et al.*, 2012) the effects of DS on

hippocampal gene expression of NR2A subunit of this receptor can be related to the changes in the *p*GSK-3 β levels in this study.

Conclusions

Although a link between DS treatment and its mechanism of action in the distinct mouse models applied here remains to be determined, the present study argues for the potential of DS to generate an antidepressant-like effect in various conditions, including those in which the mechanisms of action of other sensitizers of the insulin receptor are effective. A lack of signs of toxicity of choline succinate in mammals (Shivapurkar *et al.*, 1986; Maekawa *et al.*, 1990) at the dosage ranges of DS found effective in our study favours its potential practical use.

We conclude that the insulin receptor sensitizer DS ameliorates depressive-like features in mice whose induction was associated with chronic stress as well those which were not. In a model of stress-induced anhedonia, DS preserved normal contextual fear conditioning, hippocampal gene expression of NMDA receptor subunit NR2A, the NR2A/NR2B ratio and increased REM sleep rebound after acute predation. In a model of elderly depression, DS restored normal sucrose preference and altered gene expression of 27 genes of the hippocampus and the prefrontal cortex most of which are involved in brain plasticity and sleep/circadian regulation. Finally, young DS-treated C57BL6 mice had reduced signs of learned helplessness through lowered scores of floating, similarly to the imipramine-treated group. Also, like imipramine-treated mice, DS-treated mice demonstrated preserved hippocampal levels of the phosphorylated (inactive) form of GSK-3 β that was lowered by forced swimming in pharmacologically naïve animals. Thus, even though a variety of experimental techniques and determined physiological, behavioural and molecular read-outs of a depressive-like state dose not quite allow a connection these findings to each other, they all point towards an antidepressant-like role for DS

at different levels and in different contexts. Consequently, this further highlights the enhancement of insulin receptor signalling as a potential target of pharmacotherapy of depressive disorder, while exactly how this mechanism results due to the effects of DS reported here, remains to be discovered.

Conflict of Interest

The research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. The authors declare no conflict of interest.

Authors' Contributions

B.C. and J.C.N. carried out the chronic stress experiment, tissue collection, statistical analysis, prepared the figures and took part in drafting of the manuscript; R.C. and A.S. organised and carried out EEG study on chronically stressed mice; Y.B. performed gene expression Illumina analysis; N.M. and A.K. performed study in old mice and participated in the ELISA and RT PCR assays; H.S. participated in the design of the study and coordination; K.P.L. participated in the coordination of the study and contributed to the drafting of the manuscript; T.S. conceived of the study, participated in its design and coordination and drafted the manuscript. All authors read and approved the final manuscript.

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Figure legends

Figure 1. Study flows. (A) Study of the effects of Dicholine Succinate in a chronic stress model.

Abbreviations: DS: dicholine succinate-treated; ST: sucrose test; FC: fear conditioning; T: training; R: recall test; B: baseline; S: stress; D: day of experiment. (B) Schematic of the 10-day chronic stress procedure. **Abbreviations:** TS: tail suspension stress, R: restraint stress, D: day, N: night Rat: rat exposure stress (C) Study of the effects of Dicholine Succinate in a model of elderly depression; D: day of experiment. **Abbreviations:** ST: sucrose test (D) Study of the effects of Dicholine Succinate in naïve mice. **Abbreviations:** NC: novel cage test; OM: O-maze test; FST D1: forced swim test day 1; FST D2: forced swim test day 2; D: day of experiment.

Figure 2. Effects of Dicholine Succinate in a chronic stress model. (A) Sucrose preference was significantly lowered in stressed vehicle treated animals as compared to their controls as well as to DS-treated stressed mice ($p < 0.001$ vs control and DS respectively). (B) In contextual fear conditioning, the percentage of time spent with freezing during a baseline (pre-training) period was similarly low in all experimental groups ($p > 0.05$). The vehicle stressed group showed memory loss as indicated by significantly lesser freezing compared to both non-stressed controls ($p < 0.01$) and DS treated stressed mice ($p < 0.01$). (C) Slow wave sleep was not different between the not treated stressed or DS treated stressed (groups ($p > 0.05$)). Power spectra analysis revealed no differences between the groups ($p > 0.05$). (D) REM sleep was significantly increased in stressed DS treated mice as compared to stressed not treated animals ($p < 0.05$); number of animals per group as indicated above. Power spectra analysis revealed no differences between the groups ($p > 0.05$). (E) mRNA expression of NR2A, as well as the NR2A/NR2B ratio were elevated in the non-treated stress group, as compared to controls ($p < 0.05$, NR2A; and $p < 0.01$ NR2A/NR2B ratio), but not in stressed DS-treated mice ($p > 0.05$). No changes were observed for NR2B mRNA expression. **Abbreviations:** Con: control group; VEH: vehicle-treated; DS: dicholine succinate-treated; B: baseline conditions; S: stressed. * $p < 0.05$ vs controls, # $p < 0.05$ vs. DS-treated group.

Figure 3. Effects of Dicholine Succinate in a model of elderly depression. (A) In 18 month aged animals, only the DS treated group showed an increase in sucrose preference ($p < 0.05$) while non-treated animals had no increased sucrose preference ($p > 0.05$). (B) Total sucrose intake

was not changed in animals without treatment ($p>0.05$) while DS-treated animals had an increase in total sucrose intake ($p<0.05$). **(C)** No groups showed any difference for total water intake ($p>0.05$, not treated and $p>0.05$, DS). **(D)** Also there were no differences in the total liquid consumption for the not treated animals nor for the DS treatment group ($p>0.05$).

Figure 4. Gene expression profiling of brain of old mice treated with Dicholine Succinate. A significant change in over 1.5 folds from vehicle-treated aged control as found with 27 genes in the **(A)** hippocampus and 4 genes in the **(B)** prefrontal cortex. For the criteria of gene selection, see the text.

Figure 5. Study of the effects of Dicholine Succinate in naïve mice. **(A)** No differences were observed in the latency to float for any group ($p>0.05$) while there was a significant effect of total time spent floating ($p<0.005$). Tukey's revealed that groups receiving higher treatment doses had significantly less floating ($p<0.01$, imipramine and $p<0.05$, DS). **(B)** In the elevated O-maze test mice treated with a dose of 75 mg/kg/day of dicholine succinate, displayed significantly longer duration in the open arms, with no significant changes to a latency to exit, total number of exits, or risk assessment behaviour in comparison with control animals ($p<0.05$). **(C)** No changes were observed between DS treated animals and controls at any time point ($p>0.05$, see the text) or for total rearings ($p>0.05$). **(D)** Tukey's revealed a significant reduction in $pGSK-3\beta$ for untreated animals subjected to FST compared to naive control ($p<0.05$). There was no difference for imipramine-treated mice subjected to FST ($p>0.05$) or DS-treated mice subjected to FST ($p>0.05$), in comparison with naive control group. $pGSK-3\beta$ levels were also significantly lower in untreated animals exposed to FST as compared to imipramine treated subjected to FST ($p<0.05$) and DS treated subjected to FST ($p<0.05$) groups. **Abbreviations:** DS: dicholine succinate-treated; FST: subjected to a forced swim test. * $p<0.05$ vs controls, # $p<0.05$ vs. FST non-treated group.

Figure 1

Figure 2

Figure 3

Figure 4

A FORCED SWIM TEST

B O-MAZE

C NOVEL CAGE TEST

D GSK-3 β

Figure 5

Supplementary data

Rat exposure while in a small container

Mice were introduced into cylindrical containers, which were placed into a rat home cage for 15h (over-night, from 18h00 to 9h00). Containers were made from customized transparent plastic, size 15cm x Ø 8 cm, with holes in covers ($\varnothing < 0.5\text{cm}$), which ensured protection of the mouse from the rat, but allowed visual and odor contact.

Social defeat stress

Social defeat took place during the dark phase under red light to enable visual control over the resident-intruder confrontation. In a preliminary test, aggressive individuals of the CD1 mouse strain that were able to attack the counter-partners in less than 60 sec without injuring them were selected for this procedure; these animals were introduced in the home cages of mice from the stress group during social defeat sessions for 5 min. During social defeat stress, test mice typically showed flight response, submissive posture and vocalization. Pairs of animals were carefully observed in order to exclude any physical harm. In rare cases of its incidence, overly aggressive individuals were immediately removed from the cage of resident mice. After a 5-min period of social defeat C57BL/6 mice were introduced into small containers and placed inside the CD1 cage, where they stayed for a 3h-period. Thereafter, a 5-min social defeat procedure was repeated again. In order to randomize the procedure, the same pairs of C57Bl6 and CD1 mice were never put together.

Tail suspension stress

Mice were submitted to the tail suspension procedure by hanging them by their tails. The procedure was done during the dark phase of the animals' light cycle.

Restraint Stress

Animals were placed inside a plastic tube (internal diameter 26 mm) for 2 h during the dark phase of the light cycle and kept in a dark experimental room.

Real-time PCR assay

RNA extraction was performed as previously described (Costa-Nunes *et al.*, 2014).

Cycling conditions and sequences of primers used are indicated in *Table 1*.

Table 1. Cycling conditions and sequences of primers used in RT PCR assay

mRNA target	Primer sequence (5' → 3')		Annealing Temperature (C°)
NR2A	Forward	GCTACACACTCTGCACCAATT	64
	Reverse	CACCTGATAGCCTTCCTCAGTGA	
	TaqManProbe	6-FAM TGGTCAATGTGACTTGGGATGGCAABHQ-1	
NR2B	Forward	CTGCATGCGGAATACAGTC	56
	Reverse	TTGATGTAGCCTGGTTCCT	
	TaqManProbe	6-FAM CAGAAGCGCATCATCTCTGAGAATBHQ-1	
GAPDH	Forward	TGCCACCAACTGCTTAG	54
	Reverse	GGATGCAGGGATGATGTTC	
	TaqManProbe	HEX ATCACGCCACAGCTTCCAGA BHQ-1	

The qPCR was performed in a 25 µl reaction volume containing a 10xPCR Buffer (2.5µl), 25 mmol MgCl₂ (4 µl), 10 mmol dNTPs (2µl), specific forward and reverse primers at 20 pmol/µl concentration (1 µl), 5pmol/µl Taq Man probe (1.5 µl), cDNA (2µl), 5 u/µl Taq DNA polymerase (1 µl) (Beagle, st. Petersburg, Russia), and ddH₂O (10 µl). All samples were run in duplicate. Cycling was performed at 95 C° for 5 min followed by a 45-cycle amplification at 95°C for 10s, then at the annealing temperature (see Table 1) for 15 s and at the temperature 72°C for 20 s.

Results of the qPCR measurements were expressed as Ct values, where Ct is defined as the threshold cycle of PCR at which amplified product was 0.05 % of normalized maximal signal. We used the comparative Ct method and computed the difference between the expression of the gene of interest and GAPDH expression in each cDNA

sample ($2^{-\Delta\Delta Ct}$ method). Results are expressed as folds of expression compared to the mean values of expression in non-stressed control animals (Couch *et al.*, adapted from Livak and Schmittgen 2001).

ELISA of pGSK-3 β

Hippocampus was homogenized in buffer containing 10 mM Tris (pH7,4), 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM Na₄P₂O₇, 10% glycerol, 2mM Na₃VO₄. Protease inhibitor cocktail (Sigma, USA) was added immediately prior to homogenization. The GSK-3 β [pS9] ELISA kit (Invitrogen Corporation, USA) was used for detection and quantification of the level of GSK-3 β protein phosphorylated at serine residue 9. A monoclonal capture antibody specific for GSK-3 β has been coated onto the wells of the 96-well plate provided. During the first incubation, standards of known GSK-3 β [pS9] content and unknown samples are pipetted into the wells and the GSK-3 β antigen binds to the immobilized (capture) antibody. After washing, a rabbit antibody specific for GSK-3 β [pS9] is added to the wells. During the second incubation, this antibody serves as a detection antibody by binding to the immobilized GSK-3 β protein captured during the first incubation. After washing, a horseradish peroxidase-labelled anti-rabbit IgG is added. This binds to the detection antibody to complete the four-member sandwich. After a third incubation and washing to remove all the unbound enzyme, a substrate solution (TMB) is added, which is acted upon by the bound enzyme to produce color. The intensity of this colored product is directly proportional to the concentration of GSK-3 β [pS9] present in the original specimen. All procedures were done according the instruction manual and the optical density of experimental plates were measured at 450 nm using a plate reader (Wallac 1420 VICTOR, USA). The results were normalized to total protein level in tissues homogenates. Protein concentrations were determined by the biuret assay using bovine serum albumin as a standard (Gornall, A. G., Bardawill, C. J., and David, M. M. (1949) J Biol Chem 177, 751-766).

Table 2. EEG power spectra parameters in chronically stressed mice were not affected by DS.

Treatment	Delta	Theta	Alpha	Sigma	Beta 1	Beta 2
<u>SLOW WAVE SLEEP</u>						
Vehicle baseline	39.95±0.5	32.15±0.8	14.10±0.9	3.55±0.4	5.04±0.2	5.21±0.7
Vehicle stress	40.40±0.7	33.10±1.9	13.40±0.8	3.19±0.6	4.72±0.3	5.19±0.8
DS baseline	41.10±0.8	32.60±0.9	14.25±1.0	3.39±0.3	4.88±0.1	3.78±0.5
DS stress	40.01±1.6	34.00±2.1	13.97±0.9	3.28±0.4	4.73±0.3	4.00±0.7
<u>REM SLEEP</u>						
Vehicle baseline	24.32 ±1.4	41.79±0.9	17.05±0.7	3.56±0.3	5.98±0.6	7.30±0.7
Vehicle stress	26.67±2.3	40.52±0.45	15.60±1.2	3.48±0.4	5.86±0.5	7.87±1.1
DS baseline	23.65±1.7	39.86±1.2	17.87±0.9	3.64±0.2	6.53±0.4	8.45±1.1
DS stress	24.18±1.9	40.50±1.0	16.90±0.9	3.60±0.3	6.21±0.5	8.61±1.4

Table 3A. List of differentially expressed genes in the hippocampus of old mice treated with DS.

Gene name	Gene Symbol	Ratio*	p-Value
Activity-regulated cytoskeleton-associated protein	Arc	2,097	0,0000819
Neuronal pentraxin II	Nptx2	2,034	0,0000107
Serum/glucocorticoid regulated kinase 1	Sgk1	1,83	0,001816
TATA-binding protein-associated factor 2N	Taf15	1,67322	0,00019865
Dynactin 1	Dctn1	1,65636	0,00115384
VGF nerve growth factor inducible	Vgf	1,62473	0,00047794
Regulator of G-protein signaling 4	Rgs4	1,58555	0,00017529
Early growth response 1	Egr1	1,57936	3,45E-06
IQ motif containing GTPase activating protein 2	Iqgap2	1,57442	0,00069018
proline-rich coiled-coil 2A	Bat2	1,5569	3,56E-05
HtrA serine peptidase 1	Htra1	1,52536	1,02E-05
GATA zinc finger domain containing 2B	Gatad2b	1,52186	1,42E-05
Secretory leukocyte peptidase inhibitor	Slpi	1,51893	0,00199586
FBJ murine osteosarcoma viral oncogene homolog B	Fosb	1,51512	6,34E-06
Solute carrier family 23 (ascorbic acid transporter), member 3	Slc23a3	1,50125	4,12E-06
Histone cluster 1, H1c	Hist1h1c	-1,5352	0,00180782
Circadian Associated Repressor Of Transcription	Gm129	-1,5496	5,65E-05
Sperm associated antigen 7	Spag7	-1,5573	1,41E-05
Enhancer of rudimentary homolog (Drosophila)	Erh	-1,5667	4,47E-07
Solute carrier family 27 (fatty acid transporter), member 1	Slc27a1	-1,6106	1,63E-05
ST3 beta-galactoside alpha-2,3-sialyltransferase 5	St3gal5	-1,6226	5,94E-07
Cold inducible RNA binding protein	Cirbp	-1,6348	9,62E-06
D site of albumin promoter (albumin D-box) binding protein	Dbp	-1,6382	4,01E-10
Oligodendrocytic myelin paranodal and inner loop protein	Opalin	-1,8266	0,00016628

*Ratio=(mRNA abundance DISU/ mRNA abundance Control) Unadjusted p value <0.001 and |fold change| >1.5 (see the text).

Table 3B. List of differentially expressed genes in the prefrontal cortex of old mice treated with DS.

Gene name	Gene Symbol	Ratio*	p-Value
Activity-regulated cytoskeleton-associated protein	Inhba	1,66369	0,00075
Neuronal pentraxin II	Stmn4	-1,9122	4,73E-06
Serum/glucocorticoid regulated kinase 1	Opalin	-1,7873	0,000254
TAF15 RNA	Gabarapl1	-1,5367	1,21E-07

*Ratio=(mRNA abundance DISU/ mRNA abundance Control) Unadjusted p value <0.001 and |fold change| > 1.5 (see the text)

Chapter 4

Tlr4 upregulation in the brain accompanies depression- and anxiety-like behaviors induced by a high-cholesterol diet

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Abstract

An association between metabolic abnormalities, hypercholesterolemia and affective disorders is now well recognized. Less well understood are the molecular mechanisms, both in brain and in the periphery, that underpin this phenomenon. In addition to hepatic lipid accumulation and inflammation, C57BL/6J mice fed a high-cholesterol diet (0.2%) to induce Non-Alcoholic Fatty Liver Disease (NAFLD), exhibited behavioural despair, anxiogenic changes, and hyperlocomotion under bright light. These abnormalities were accompanied by increased expression of transcript and protein for Toll-like receptor 4, a pathogen-associated molecular pattern (PAMP) receptor, in the prefrontal cortex and the liver. The behavioural changes and *Tlr4* expression were reversed ten days after discontinuation of the high-cholesterol diet. Remarkably, the dietary fat content and body mass of experimental mice were unchanged, suggesting a specific role for cholesterol in the molecular and behavioural changes. Expression of *Sert* and *Cox1* were unaltered. Together, our study has demonstrated for the first time that high consumption of cholesterol results in depression- and anxiety-like changes in C57BL/6J mice and that these changes are unexpectedly associated with the increased expression of TLR4, which suggests that TLR4 may have a distinct role in the CNS unrelated to pathogen recognition.

Key Words: Western diet; cholesterol; affective disorders; depression; anxiety; impulsivity; hepatosteatosis; toll-like receptor four (*Tlr4*); CNS; mouse.

Introduction

In man the prevalence and coincidence of both depression and obesity are growing steadily (de Wit *et al.*, 2010). An emerging body of clinical and experimental evidence suggests that excessive nutrition, generally attributed to a so-called Western diet (a diet enriched with saturated fat and cholesterol), and the ensuing hypercholesterolemia, obesity and insulin resistance contribute to the pathogenesis of affective disorders and interfere with pharmacotherapy (Sonawalla *et al.*, 2002). Translational studies using this type of dietary challenge in rodents revealed deficient synaptic plasticity and increased production of IL-1 β in the hippocampus of Wistar rats after foot shock (Sobesky *et al.*, 2014), blunted responses to antidepressant treatment in a chronic stress depression model (Isingrini *et al.*, 2010), and signs of hepatosteatosis and liver inflammation, such as over-expression of genes of the macrophage activation marker Cd68, and the cytokine Tnf- α in C57BL/6J mice (Comhair *et al.*, 2011). Another study in toll-like receptor 4 (*Tlr4*) deficient mice revealed that these mice were more insulin sensitive, than WT mice after being fed a high-fat diet (Shi *et al.*, 2006). However, these studies did not investigate potential changes of parameters of emotionality and related molecular changes in the CNS, and the link between peripheral and central effects of a high-fat diet was not addressed.

A condition of non-alcoholic fatty liver disease (NAFLD) can be induced by a 3-week exposure of young C57BL/6J mice to a diet containing 0.2% cholesterol (Comhair *et al.*, 2011). In this model, cholesterol and female sex, but not the degree of saturation of the fats was found to be the key factors that determine the degree of hepatic steatosis and inflammation. Here, we used this model to investigate the effects of increased cholesterol intake on depression- and anxiety-like behaviours and on *Tlr4*, *Sert* (*Slc6a4*), and *Cox1* gene expression to discover whether the changes in *Tlr4* expression observed in the periphery might also occur in the CNS.

Methods

Animals

Studies were performed on 3-month-old female C57BL/6J from the Gulbenkian Institute of Science, Oeiras, Portugal. Mice were housed in groups of five under a reversed light–dark cycle, under standard laboratory conditions (see *Supplementary data*). All experiments were carried out in accordance with the European Communities Council Directive for the care and use of laboratory animals.

Study flow with a model of non-alcoholic fatty liver disease (NAFLD)

This study used a recently validated model of non-alcoholic fat dystrophy of the liver (Comhair et al. 2011). Mice were exposed for three weeks either to a regular laboratory diet with an energy content of 4.0 kcal/g (Mucedola Srl, Settimo Milanese, Italy) or a diet of the similar composition with 0.2% (w/w) of cholesterol (HC diet) and an energy content of 4.6 kcal/g (Research Diets Inc, New Brunswick, NJ, USA; see *Supplementary information*); their body-weight and 24-h food intake were evaluated throughout the experimental period (see *Supplementary information*, SI Figure 1). One set of mice received the HC diet or standard chow for three weeks before behavioural testing and sacrifice, another set was exposed to the same regime, but then a 10-day wash-out period with standard chow followed before further behavioural testing and sacrifice. After this period of refeeding, the NAFLD-like hepatosteatosis has been shown to resolve itself (Itagaki et al. 2013). For more detailed investigation of potential behavioural changes, several tests were used. In order to avoid any interference between the tests on the same animal, different behaviours were studied in different groups of mice. Respective control mice were tested simultaneously with experimental mice (see *supplementary information* for full details of the testing regime).

The assessment of histological integrity of liver and hepatic triglyceride content was performed as described elsewhere (Comhair *et al.*, 2011). RNA extraction from brain

and liver tissue was carried out as described previously on animal that had been perfused with sterile heparinised-saline (Blond *et al.*, 2002; Campbell *et al.*, 2005). Quantitative RT-PCR (qPCR) was performed to evaluate expression of *Tlr4*, *Sert*, *Tlr2*, *Il-1b*, *Tnf* and *Cox1* using glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) as the housekeeping gene. Results were calculated as fold increase compared to control animals and normalized to the means of this group (details of all experimental techniques are given in *Supplementary data* and Table 1). An ELISA for TLR4 was employed to explore protein expression levels and the LAL test for endotoxin was performed on plasma collected immediately prior to perfusion (please see the *supplementary information* for details).

Statistics

Data were analysed with GraphPad Prism version 6.0 for Macintosh (San Diego, CA, USA) using unpaired t-tests or 2-way ANOVA with Bonferoni post hoc-testing. The level of confidence was set at 95% ($p < 0.05$). For the analysis of the metabolic data (weight, food intake, and energy intake) multiple t-tests were used with a FDR set at 1%.

Results

Mice subjected to a high-cholesterol diet display impulsivity and increased depressive-, and anxiety-like behaviours

Mice exposed to the HC diet displayed signs of elevated anxiety when tested under subtle lighting (5lux); significantly shorter time was spent in the open arms of elevated O-maze ($p = 0.02$, $t = 2.661$) and lit box of dark/light box ($p = 0.02$, $t = 2.365$), as well as reduced mean distance to the wall in the open field ($p = 0.041$, $t = 2.262$) (Fig.1B-D). Mice re-exposed to a normal diet spent the same amount of time in the lit box and exhibited the same mean distance to the wall compared to the control group ($p = 0.27$, $t = 1.161$ and $p = 0.60$, $t = 0.533$, respectively; Fig.1C,D). In the forced

swim and tail suspension tests, animals fed the highcholesterol diet showed a significantly decreased latency to float or to immobility compared to control animals ($p=0.04$, $t=2.381$, and $p=0.03$, $t=2.526$, respectively; Fig.1E, F). The duration of floating immobility behaviour in the dietary challenged group were significantly higher than in control animals ($p=0.007$, $t=3.361$ and $p=0.04$, $t=2.345$, respectively; Fig.1 E, G). Together these results suggest that increased behavioural despair, a sign of a depressive-like state, is induced in mice that received increased dietary cholesterol.

When the more stressful 25-Lux illumination was used in the dark-light box, open field and novel cage, the high-cholesterol group spent significantly longer time in the lit box and had an increased number of exits therein ($p=0.04$, $t=2.226$ and $p=0.04$, $t=2.174$, respectively; Fig.1H). Similarly, increased vertical activity in the novel cage test and mean speed of horizontal movement in the open field was revealed in the animals subjected to a highcholesterol diet, compared to the control group ($p=0.009$, $t=3.082$ and $p=0.03$, $t=2.354$; Fig.1I, J). Such behavioural changes that are indicative of increased impulsivity and general behavioural invigoration are often associated with an elevated stress response (Strekalova *et al.*, 2005). Ten days after discontinuation of the high-cholesterol diet, both horizontal and vertical activities measured under bright light returned to normal ($p=0.53$, $t=0.6448$ and $p=0.55$, $t=0.617$; Fig.1I,J).

High-cholesterol diet induces NAFLD-like syndrome and Tlr4 upregulation without increasing body mass

Mice assigned to the control or the high-cholesterol diet had similar body weights before (mean: 20.3 ± 1.4 g and 19.9 ± 1.4 g, respectively; $p=0.577$, $t=0.568$) and at all time points during the experiment (*see Supplementary Fig 1*, $p>0.05$; body weight averaged across experiment was 21.1 ± 1.2 g and 21.2 ± 1.4 in control and high-cholesterol diet groups, respectively; $p=0.92$, $t=0.099$). The average daily intake of

the high-cholesterol diet was significantly lower than that of control diet (averaged food intake across entire period of dietary intervention: $2.89 \pm 0.26\text{g}$ and $2.30 \pm 0.35\text{g}$, respectively; $p=0.001$, $t=3.670$), but the daily intake of calories did not differ between the groups (SI Figure 1). This is likely to reflect an adjustment of the mice to the higher caloric content of the high-cholesterol diet.

Compared with the control mice, the high-cholesterol diet group revealed compromised liver tissue integrity, in agreement with previous observations (Comhair *et al.*, 2011). The appearance of intracellular vacuoles was due to fat deposition in the tissue (Fig.2A, middle image). The liver of the animals re-fed standard chow after the HC diet (bottom image) and control (top image) animals appeared normal. The colorimetric assay showed significantly increased liver concentration of triglycerides in mice on the high-cholesterol diet as compared to control animals ($p=0.0003$, $t=6.001$; Fig.2A, middle panel). The liver mRNA concentrations of *Tlr4* were significantly elevated in the high-cholesterol group as compared to controls ($p=0.03$, $t=2.533$; Fig.2A,). However, there was no difference in the circulating levels of endotoxin as revealed by the LAL test. In the brain, 2-way ANOVA revealed that diet had a significant impact on *Tlr4* gene expression (79% of variation; $p<0.0001$) that was independent of the brain region (1.62% of variation; $p=0.242$). In the prefrontal cortex (PFC) ($p<0.001$, $t=5.165$) and hippocampus ($p<0.001$, $t=6.89$), mRNA concentrations of *Tlr4* were significantly higher than in the control group (Fig 2B, left panel), while mRNA concentrations of *Sert* ($p>0.05$, $t=0.271$) and *Cox1* ($p>0.05$, $t=1.199$) were not altered (Fig 2B, middle and right panels). An ELISA revealed that TLR4 protein was significantly upregulated in the PFC ($p<0.05$, $t=2.130$), but not in the hippocampus following exposure to the HC diet compared to control. After a 10-day reversal to the control diet, mRNA concentrations of *Tlr4* were no longer different in the prefrontal cortex ($p>0.05$, $t=1.291$, Fig.2C, left panel) and in the hippocampus ($p>0.05$, $t=1.918$ Fig. 2C, left panel) between mice from the high-

cholesterol group and control mice. As before, expression levels of *Sert* and *Cox1* were unchanged (Fig.2C, middle and right panels). We also examined gene expression for *Tnf*, *Il1b* and *Tlr2*, but the transcripts were expressed at a very low level and were unaffected by diet (data not shown).

Discussion

The present study with a recently validated model of NAFLD (Comhair *et al.*, 2011) has demonstrated that a 3-week exposure of young naïve C57BL/6J mice to dietary cholesterol in amounts of 0.2% of daily food intake can evoke profound behavioural modifications and molecular changes in the brain and liver. This is the first report of the behavioural changes associated with this diet, and these changes are consistent with depression and anxiety-like behaviour. Based on previously validated protocols used in this part of the experiment, the data presented here suggest that mice exposed to high amounts of dietary cholesterol display behavioural changes that are similar to those induced in a model of stress-induced depression (Strekalova *et al.*, 2004, 2005; Couch *et al.*, 2013). The signs of impulsivity observed under bright light have also been shown to be associated with the response to chronic stress in mice (Sanchez, 1997; Strekalova *et al.*, 2005). The diet-induced changes observed here, though unexpected, further support the argument that a high-cholesterol diet can induce a depression-like syndrome in a similar manner to the changes induced by a chronic stress model.

Liver steatosis, dystrophy, and accumulation of triglycerides have been previously described for this model, but the over-expression of a molecular marker of inflammation *Tlr4* has not. However, it has been shown that fatty acids can interact with TLR4 on adipocytes and macrophages in the periphery, and that *Tlr4*^{-/-} animals are protected against insulin resistance induced by a high-fat diet. Meanwhile, no previous study has reported that *Tlr4* is induced in the CNS as a response to a high-

cholesterol diet. A significant increase of gene expression of *Tlr4* was found in both the hippocampus and the prefrontal cortex, two important structures of the limbic system, but significant changes in protein were only detectable in the prefrontal cortex. This results were surprising as, in general, we have often found that changes in transcript are usually reflected by downstream changes in protein though not always to the same magnitude. We have previously shown that the prefrontal cortex behaves differently, in terms of cytokine induction, compared to the hippocampus (Couch et al. 2013). As the behavioural changes identified are most likely to reflect molecular changes in the prefrontal cortex than in the hippocampus, the putative relationship between TLR4 expression and the behavioural changes we observed remains consistent with our findings. The level of *Tlr4* expression returned to normal ten days after reversion to the control diet, and was accompanied by a normalization of vertical activity under stressful testing conditions. Notably, mRNA expression of *Sert* and *Cox1* was not altered in any of the experimental groups. We have previously shown that changes in *Sert* and *Cox1* expression are associated with the occurrence of stress-induced anhedonia (Couch *et al.*, 2013). Thus despite the behavioural similarities in the diet-induced depressive-like state and the chronic stress paradigm there are clear molecular disparities. However, increased expression of *Tlr4* in the hypothalamus of female hamsters has been shown to be associated with increased anxiety-like behaviour and stress response but the rationale for exploring this gene was not given or discussed (Shannonhouse *et al.*, 2014). Interestingly, the social separation method employed in the Shannonhouse study induced *Tlr4* expression only in female mice, and it should be noted that we used female mice in the present study because they exhibit a more pronounced phenotype. Pharmacological blockade of TLR4 has been shown to reduce indices of anxiety in the elevated plus maze test (Okun *et al.*, 2012), but LPS-induced activation of TLR4 is known to increase parameters of anxiety (Rico *et al.*, 2010). Taken together our results suggest that

central upregulation of *Tlr4* might underlie the changes in the emotionality caused by the exposure to a high-cholesterol diet as reported here.

Hepatic *Srebf1* and *Fasn* are also increased during high intake of cholesterol (Comhair *et al.*, 2011), and are found to increase circulating endotoxins (Cani *et al.*, 2008). This, in turn, can lead to elevated plasma concentrations of TNF- α , IL-1 β , IL-6, IL-10 and cortisol, and the increased measures of anxiety and worsening of mood (Kullmann *et al.*, 2013). Thus the increased *Tlr4* expression in the CNS might be the consequence of increased circulating levels of cytokines. However, our failure to detect changes in *Tnf*, *Il-1*, *Tlr2*, or *Cox1* expression argue for a rather more indirect pathway. However, it is important to note that we only examined expression at the selected behavioural time points and it is possible that abnormal cytokine expression may have been induced either before or after these particular points in time. The overexpression of *Tlr4* can compromise the so-called neural plasticity, a term that was proposed for multiple aspects of aberrant brain plasticity underlying mechanisms of depression (Wainwright and Galea, 2013), since its deficiency enhances proliferation and neuronal differentiation (Rolls *et al.*, 2007) and improves spatial reference memory (Okun *et al.*, 2012). Interestingly, a high-fat diet that caused overexpression of IL-6 was recently reported to impair synaptic plasticity in rats (Sobesky *et al.*, 2014). Thus future studies might explore whether altered brain plasticity as a consequence of *Tlr4* overexpression may be a cause for the changes in emotionality found in the present study.

Remarkably, in the model employed here, the body weight of mice on the high-cholesterol diet remained unchanged. This, together with the fact that the fat content of the diet was normal, excludes the contribution of obesity or excessive amounts of exogenous or endogenous fat, or other pathogenic elements of a Western diet, apart from dietary cholesterol, to the central and peripheral abnormalities found here in the employed NAFLD model.

In summary, our study demonstrates for the first time that the exposure of naïve mice to high amounts of dietary cholesterol can result in depression- and anxiety-like changes, impulsivity and this is associated with systemic and central upregulation of TLR4 mRNA and protein.

Conflict of Interest

The authors declare no conflict of interest.

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Figure Legends

Figure 1. Study flow and effects of a high-cholesterol diet on parameters of emotionality. (A) Schematic of the study. (B-D) Anxiety-like behavior was increased in animals kept on a high-cholesterol diet for 3 weeks in as revealed by the (B) O-maze, (C) dark/light box and (D) open field tests; this anxiety-like behavior returned to normal after the wash-out period in the two latter tests. (E-G) Depressive-like behavior was elevated in mice exposed to high cholesterol diet as revealed by the (E) forced swim and (F,G) tail suspension tests; such depressive-like behaviour was no different from control following the wash-out period in the tail suspension test. (H-J) Mice exposed to high-cholesterol diet displayed signs hyperactivity and invigoration in brightly illuminated (H) dark/light box, (I) open field and (J) novel cage that was not found in mice re-exposed to normal diet, in the two latter tests (I, J). (* $p < 0.05$ vs. control, t-test). Data are shown as mean $\pm$ SEM, $n = 6+$.

Figure 2. The effects of a high-cholesterol diet on (A) liver and (B&C) the brain. (Ai) Photomicrographs of haematoxylin and eosin-stained sections showing the normal appearing liver in animals feed a control diet (top) versus the high-cholesterol diet for 3 weeks (middle) and those re-fed the standard chow for 10 days (bottom). Note the vacuolation in the middle image. Scale bar 20 μ m. (Aii) Concentration of triglycerides in the liver, (Aiii) mRNA expression of *Tlr4*, and (Aiv) the absence of any increase in circulating levels of endotoxin. (B) mRNA concentrations of *Tlr4*, *Sert* and *Cox1* in the hippocampus and prefrontal cortex immediately after a 3-week dietary intervention and ELISA data revealing that there is an increase in TLR4 protein expression in the PFC. (C) Ten days after reversal to the control diet gene expression returned to normal (* $p < 0.05$ vs. control, t-test and ANOVA, see the text). Data are shown as mean $\pm$ SEM, $n = 6+$.

Figure 1

i. ii. iii.

B CNS gene expression after exposure to a high cholesterol diet

C CNS gene expression after discontinuation of a high cholesterol diet

Figure 2

Chapter 5

Animal models of depression and drug delivery with food as an effective dosing method: evidences from studies with celecoxib and dicholine succinate

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Chapter
5.

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Abstract

Multiple models of human neuropsychiatric pathologies have been generated during the last decades which frequently use chronic dosing. Unfortunately, some drug administration methods may result in undesirable effects creating analysis confounds hampering model validity and preclinical assay outcomes. Here, automated analysis of floating behaviour, a sign of a depressive-like state, revealed that mice, subjected to a three-week intraperitoneal injection regimen, had increased floating. In order to probe an alternative dosing design that would preclude this effect, we studied the efficacy of a low dose of the antidepressant imipramine (7mg/kg/day) delivered via food pellets. Antidepressant action for this treatment was found while no other behavioural effects were observed. We further investigated the potential efficacy of chronic dosing via food pellets by testing the antidepressant activity of new drug candidates celecoxib (30 mg/kg/day) and dicholine succinate (50 mg/kg/day), against standard antidepressants imipramine (7mg/kg/day) and citalopram (15 mg/kg/day) utilizing the forced swim and tail suspension tests. Antidepressant effects of these compounds were found in both assays. Thus, chronic dosing via food pellets is efficacious in small rodents, even with a low drug dose design, and can prevail against potential confounds in translational research within depression models applicable to adverse chronic invasive pharmacotherapies.

Key words: pre-clinical models of depression, dosing route, imipramine, citalopram, celecoxib, dicholine succinate, animal welfare, mouse.

Introduction

The challenge to propose new powerful therapeutics for neuropsychiatric disorders, including antidepressants, has raised important questions regarding the efficiency of preclinical approaches currently being used (Munos, 2009; Piggot *et al.*, 2010; Araragi and Lesch, 2013). Numerous limitations of the models of human neuropsychiatric pathologies have been intensively discussed during the last years (Insel, 2007; Insel and Sahakian, 2012; Strekalova *et al.*, 2013). Apart from a general problem of translational research, basic practical issues with animal models of neuropsychiatric conditions, however seemingly trivial, can essentially affect the validity of preclinical models, yet these can be addressed and resolved.

As with translational models in small rodents, these issues concern laboratory and procedural settings in animal studies. A number of experimental conditions have been shown to result in potential confounds for the practical application of animal models. The principals of these factors are commonly considered to include: the circadian phase of manipulations (Sato *et al.*, 2005; Beeler *et al.*, 2006), cage enrichment (Frick *et al.*, 2003; Wolfer *et al.*, 2004; Munne *et al.*, 2011), lighting conditions (Strekalova *et al.*, 2005; Fukushiro *et al.*, 2010), handling (Wahlsten *et al.*, 2003; Longordo *et al.*, 2011; Cloutier *et al.*, 2014), vibration (Ringgold *et al.*, 2013), the adverse taste of food or water (Strekalova *et al.*, 2008; Strekalova and Steinbusch, 2010), and a presence of and manipulations by an experimenter (Bohlen *et al.*, 2014; Sorge *et al.*, 2014). They are sometimes believed to result in the remarkable variability in results that are extensively reported by the literature (Crabbe *et al.*, 1999; Mandillo *et al.*, 2008; Strekalova *et al.*, 2008, 2011). The method and duration of dosing of experimental animals is one of the important sources of such confounds (Wahlsten *et al.*, 2003; Sousa *et al.*, 2006; Azar *et al.*, 2011).

Various types of invasive treatments in rodents were shown to induce pain, inflammation and distress, despite the proper use of standardized methods of application, especially when prolonged dosing is employed (Gärtner *et al.*, 1980; Cloutier and Newberry, 2008; Thiele and Navarro, 2014). Obviously, this raises issues that concern not only the quality of studies, in which such dosing methods are used, but also animal welfare and ethical aspects. Nonetheless, in many cases long and invasive drug administration to small rodents is problematic to avoid. This often applies for instance when non-water soluble compounds have to be chronically administered, for example, during experimental conditions for which the induction of a desirable syndrome in an animal and / or the occurrence of the therapeutic drug's effect require a long time. The latter experimental situations are particularly typical for testing drugs in rodent models of depression where, for example, the induction of some key elements of depressive syndrome may take 2-12 weeks (Willner, 2005; Strekalova *et al.*, 2011; Harro, 2013) and the occurrence of an antidepressant's effect, with most of the classical antidepressants, develop after 3-4 weeks of treatment (Willner, 2005; Strekalova *et al.*, 2006; Overstreet, 2012).

In order to avoid the negative effects of chronic invasive dosing on overall animal welfare and experimental outcomes from standard models of depression, we evaluated the efficacy of drug delivery via food pellets in mice. First, we studied the effects of a three-week daily intraperitoneal vehicle injection in the mouse forced swim test, a common scheme of testing for the antidepressant-like effects of various treatments (Cryan *et al.*, 2005a; Overstreet, 2012; Harro, 2013). As this manipulation resulted in an increase of floating scores, a measure of "behavioural despair", indicating a "pro-depressant" effect of daily intraperitoneal injections for the experimental animals, we probed an alternative way of dosing using food pellets.

Though drug delivery with voluntarily consumed food is one of the common methods of dosing, its use in laboratory research is quite limited. While in many cases this

mode of pharmacological treatment is seen as advantageous because it enables the maintenance of a steady blood concentration for the drug, in contrast to bolus drug administration. However, it is sometimes viewed as insufficiently reliable due to its reliance on food intake and the variable bioavailability of some compounds depending on their delivery route (Pottenger *et al.*, 2000; Volvert *et al.*, 2008).

However, given that, in a reasonable proportion of the experimental situations, the consummatory behaviour of laboratory animals is not altered and the standard pharmaca, whose bioavailability and metabolism are well known not to be sensitive to the treatment method, are used; dosing with the voluntary intake of food pellets can probably be exploited much more frequently. Apart from the obvious benefits of animal wellbeing, the delivery of investigational drugs with food pellets can increase the validity of translational models as it simulates a human equivalent therapeutic dosing route.

In this study we first used, via food pellets, a low dose of a classical antidepressant reference drug, imipramine (7mg/kg/day), for which chronic administration via drinking water for 3 weeks was recently reported to evoke an antidepressant effect in a model of stress-induced anhedonia (Cline *et al.*, 2014). A low dose of antidepressant was selected because we sought to evaluate the usefulness of this dosing method at the lowest possible dosage limit which is used in other means of drug administration, and because imipramine may exert side-effects when applied in higher concentrations (Strekalova *et al.*, 2013). The effects of imipramine delivered with self-made food pellets were tested in the forced swim test, as well as, in order to exclude potential non-specific effects of treatment, in a battery of behavioural tests including: dark/light box, O-maze, novel cage, open field and two-bottle sucrose test. Finally, to verify the applicability of this defined dosing method with food pellets, we tested the effects of new potential antidepressants, celecoxib, a non-water-soluble compound, at the dose 30 mg/kg/day which was selected based on previously

published data (Myint *et al.*, 2007; Maciel *et al.*, 2013), and dicholine succinate whose dose was applied at 50 mg/kg/day based on previous results (Cline *et al.*, 2012) (Strekalova and Anthony, *unpublished results*), in the forced swim and tail suspension tests. Imipramine, applied at 7 mg/kg/day (Pottenger *et al.*, 2000; Cline *et al.*, 2012; Malatynska *et al.*, 2012; Strekalova *et al.*, 2013), and citalopram, 15 mg/kg/day (Strekalova *et al.*, 2006, 2013; Cline BH *et al.*, 2014), were used as pharmacological references.

Materials and Methods

Animals and housing

Three month old C57BL/6N male mice were supplied by Instituto Gulbenkian de Ciência, Oeiras, Portugal, and housed individually in standard laboratory conditions under a reverse 12:12h cycle (lights on at 21:00). Behavioural tests took place from the onset of the dark phase of the light cycle (9:00h). The testing was carried out in a dark, quiet room in morning hours. All procedures were in accordance to the European Union's Directive 2010/63/EU, Portuguese law Law-Decrees DL129/92 (July 6th), DL197/96 (October 16th) and Ordinance Port.131/97 (November 7th). This project was approved by the Ethical Committee of the New University of Lisbon.

Study flow with chronic intraperitoneal injections

This study used a broadly applied treatment, in small rodents, of chronic intraperitoneal injections (Gärtner *et al.*, 1980). We have chosen to expose mice to a three-week daily intraperitoneal injections of NaCl at volume 0.01 ml/g body weight (for scheme of study flow, see **Fig.1A**). Control mice were not treated but handled daily. Starting from the next day after this period, mice were tested in the two-day forced swim test as previously described (Malatynska *et al.*, 2012; Cline *et al.*, 2014). Behavioural data were scored using Noldus EthoVision XT 8.5 (Noldus Information

Technology, Wageningen, the Netherlands). Number of mice per group is indicated in *Figure legends*.

Study flow with chronic imipramine delivery via food pellets

As a next step, we exposed mice to self-made food pellets (*see below*) that contained imipramine, for four weeks. Prior to starting treatment, animals were balanced upon body weight. The calculation of the used concentration of imipramine in food pellets was based on a daily food intake of experimental mice that constituted 2.9 ± 0.26 g, and a desirable dosage of 7 mg/kg/day. The selection of this dose was based on previously obtained data that showed the efficacy of the dose (Cline *et al.*, 2014) and a lack of such with chronic imipramine delivery via drinking water at a dose of 2.5 mg/kg (Strekalova and Markova, *unpublished results*), in mice. Control mice received a regular diet. Before the start and after four weeks of dosing, all mice were tested in the sucrose preference test, O-maze test and in the Dark/light box, as described elsewhere (Markova *et al.*, 2013; Costa-Nunes *et al.*, 2014) (*see below*). After two and four weeks of dosing, locomotor activity of all mice was studied in the novel cage and open field tests, as described elsewhere (Cline *et al.*, 2012; Malatynska *et al.*, 2012; Costa-Nunes *et al.*, 2014) (*see below*). At the end of behavioural testing, a two-day forced swim test with 6 min sessions was performed as previously described (Malatynska *et al.*, 2012; Cline *et al.*, 2014) (*see below*; for scheme of study flow, *see Fig.1B*). Number of mice per group is indicated in *Figure legends*.

Study flow with chronic delivery via food pellets of new candidates to antidepressants

Next, we subjected mice to food pellets that contained imipramine, citalopram, celecoxib or dicholine succinate (*see below*), for four weeks. Prior to starting treatment, animals were balanced upon body weight. The latter two drugs are regarded as compounds with potential antidepressant activity (Myint *et al.*, 2007; Cline *et al.*, 2012; Maciel *et al.*, 2013). The calculation of drug concentrations was based on daily food intake of experimental mice, and desirable doses: 7 mg/kg/day,

15 mg/kg/day, 30 mg/kg and 50 mg/kg respectively. Control mice received regular diet. A two-day tail suspension test and a two-day forced swim test were carried out during four consecutive days after the termination of the dosing period, as described elsewhere (Malatynska *et al.*, 2012; Markova *et al.*, 2013) (for scheme of study flow, see **Fig.1C**). Number of mice per group is indicated in *Figure legends*.

Preparation of pellets

Imipramine Hydrochloride (Sigma-Aldrich, Munich, Germany), citalopram (Lundbeck, Copenhagen, Denmark) or celecoxib (Pfizer, Berlin, Germany) were added to a commercial chow (Mucedola SRL, Milan, Italy) that was turned to powder by a blender. Small amounts of distilled water were added, food pellets of a similar size to commercial pellets were formed and dried overnight (16h) at 60°. New pellets were prepared twice a week in order to refresh the food supply of experimental groups. The content of drugs was adjusted to the dose indicated above and was based on the consumption of normal diet that was averaged over 3 days. Food pellets containing dicholine succinate (Buddha Biopharma Oy Ltd, Helsinki, Finland) were prepared in a similar way, using a 7% solution of the compound; the content of drug was adjusted to the above-mentioned daily dose of this drug.

Behavioural tests

Forced swim test. The Porsolt forced swim test has been used as described elsewhere (Malatynska *et al.*, 2012; Cline *et al.*, 2014). Mice were subjected to two 6 min swimming sessions spaced 24 h apart in a transparent cylinder (Ø 17 cm) filled with water (+23 °C, water height 13 cm, height of cylinder 20 cm, illumination intensity 25 Lux). Floating behaviour was defined by the absence of any directed movements of the animals' head and body and was scored with Noldus EthoVision XT 8.5 (Noldus Information Technology, Wageningen, the Netherlands). Using this method, the latency of the first episode of floating and the duration of floating behaviour were recorded during the 6 min swimming session, on day 1 and day 2 of the test. Latency

to begin floating was scored as time between introduction of the animal into the pool and the first moment of complete immobility of the entire body for a duration of >3 seconds. The total time spent floating, number of floating episodes, mean velocity and distance moved were scored for the entire duration of the test using post-test video footage.

Dark/light box. The dark/light box (Technosmart, Rome, Italy) consisted of two plexiglass compartments, one black/dark (15 cm x 20 cm x 25 cm) and one lit (30 cm x 20 cm x 25 cm), connected by a tunnel. Anxiety-like behaviour was assessed by earlier validated measures (Malatynska *et al.*, 2012; Markova *et al.*, 2013). Mice were placed into the dark compartment, from where they could visit the lit box, illuminated by light of 25 Lux intensity. The latency of the first exit to the light compartment, the total duration of time spent in the lit box and the number of visits to this anxiety-related compartment were scored by visual observation over 5 min.

Elevated O-maze. The apparatus (Technosmart, Rome, Italy), which consisted of a circular path (runway width 5.5 cm, diameter 46 cm), was placed 50 cm above the floor. Two opposing arms were protected by walls (height 10 cm), and the illumination strength was 25 Lux. The apparatus was placed on a dark surface in order to reduce reflection and maintain control over lighting conditions during testing. Anxiety-like behaviour was assessed using previously validated parameters (Cline *et al.*, 2014; Costa-Nunes *et al.*, 2014). Mice were placed in one of the closed-arm compartments of the apparatus. The latency of the first exit to the anxiety-related open compartments of the maze, the total duration of time spent therein and the number of exits to the open arms were scored during a 5-min observation period.

Novel cage test. The novel cage test was performed to assess vertical activity, as described elsewhere (Cline *et al.*, 2014). Mice were introduced into a standard plastic cage the size of their home cage filled with small amounts of fresh sawdust. The

number of exploratory rearings was counted under red light during a 5-min period.

Open field. The open-field apparatus consisted in four square arenas (50cm x 50cm x 50cm), made of wood covered by white resopal. Mice were put in the center and their behaviour was recorded on camera for 10 min. The open field was illuminated with white light (25 Lux). Distanced moved and mean instant speed were analysed off-line using the Any-maze software (Stoelting Co, Wood Dale, IL, USA), as described elsewhere (Pawluski *et al.*, 2012).

Sucrose test. Animals were given 8 hours of free choice between two bottles of either 1% sucrose or normal drinking water, as described elsewhere (Cline *et al.*, 2014). At the beginning and end of the period the bottles were weighed and consumption calculated. The beginning of the test started with the onset of the dark (active) phase of animals' cycle. To prevent the possible effects of side-preference in drinking behaviour, the position of the bottles in the cage was switched at 4 hours, halfway through testing. No previous food or water deprivation was applied before the test. The 1%-sucrose solution is used in tests performed across the experiment. Percentage preference for sucrose is calculated using the following formula:

$$\text{Sucrose Preference} = \left(\frac{V_{\text{Sucrose solution}}}{(V_{\text{Sucrose solution}} + V_{\text{Water}})} \right) \times 100\%$$

Tail suspension test. The protocol used in this study was adapted from a previously proposed procedure (Malatynska *et al.*, 2012; Markova *et al.*, 2013). Mice were subjected to the tail suspension by being hung by their tails with adhesive tape to a rod 50 cm above the floor for 6 min. Animals were tested in a dark room where only the area of the modified tail suspension construction was illuminated by a spotlight from the ceiling; the lighting intensity on the height of the mouse position was 25 Lux. The trials were recorded by a video camera positioned directly in front of the mice while the experimenter observed the session from a distance in a dark area of

the experimental room. This procedure was carried out twice with a 24-h interval between tests. The latency of the first episode of immobility, the total duration of this behaviour and mean velocity were scored using Noldus EthoVision XT 8.5 (Noldus Information Technology, Wageningen, the Netherlands) according to the protocol that was previously validated (Malatynska *et al.*, 2012). In accordance with the commonly accepted criteria of immobility, the immobility behaviour was defined as the absence of any movements of the animals' head and body. The latency of immobility was determined as the time between the onset of the test and the first bout of immobility.

Statistical analysis

Data were analysed with GraphPad Prism version 5.00 for Windows (San Diego, CA, USA). Two-tailed unpaired *t*-tests were applied for two-group, two-tailed comparisons of independent data sets, as the distribution was normal. One-way ANOVA was used followed by a *post-hoc* Dunnett for a comparison of more than two groups with a control; repeated measures ANOVA was used for analysis of repeated measures. The level of confidence was set at 95% ($p < 0.05$) and data are shown as mean $\pm$ SEM.

Results and Discussion

Effects of 3-week intraperitoneal vehicle injections on floating behaviour

Behaviour analysis revealed that animals subjected to injections displayed a non-significant decrease of latency to float as compared to control animals (Day 1: $p = 0.11$, $t = 1.73$; Day 2: $p = 0.91$, $t = 0.12$, **Fig.2A**, unpaired two-tailed *t*-test). The number of floating episodes and the duration of floating in the chronically injected group were significantly higher than in control animals (Day 1: $p = 0.0037$, $t = 3.89$ and $p = 0.016$, $t = 3.06$; Day 2: $p = 0.0016$, $t = 4.30$ and $p = 0.11$, $t = 1.77$, respectively; **Fig.2B,2C** unpaired two-tailed *t*-test), mean velocity of swimming and distance moved were non-

significantly decreased (Day 1: $p=0.25$, $t=1.25$ and $p=0.31$, $t=1.09$; Day 2: $p=0.18$, $t=1.43$ and $p=0.15$, $t=1.56$, respectively, **Fig.2D,2E**, unpaired two-tailed t -test). This suggests increased “behavioural despair”, a sign of depressive like state, in mice that received chronic manipulations with intraperitoneal injections.

Similar results were obtained in our previous experiments which demonstrated that three and four-week daily injections in chronically stressed mice increased the number of individuals exhibiting signs of anhedonia, a reduced sensitivity to reward, in a sucrose preference test (Strekalova *et al.*, 2008, 2011). Other studies showed that chronic intraperitoneal injections in rats evoke ultrasonic vocalizations at 22-kHz range, indicative of a negative emotional state that was reduced by pre-exposure of experimental animals to handling (Cloutier *et al.*, 2014). These “pro-depressive” like changes found in this study, could be potentially induced by well-recognized pathogenetic elements of depression, such as stress of manipulation (Silverman and Sternberg, 2012) and pain experience (Miller and Cano, 2009; Goffer *et al.*, 2013), repeated situations of unescapable stress and helplessness (Pryce *et al.*, 2011), as well as inflammation (Dantzer *et al.*, 2011).

Effects of chronic imipramine delivery via food pellets on floating behaviours and other variables

In order to assess the efficacy of an alternative chronic dosing design that could preclude the adverse changes in behaviour described above, we evaluated the effects of a four-week dosing of imipramine via food pellets in the forced swim test and supplementary behavioural paradigms. Animals subjected to imipramine treatment, showed a significant increase in the latency to float and decreased immobility time, when compared to control animals (Day 1: $p=0.0002$, $t=5.19$ and $p=0.0008$, $t=4.42$; Day 2: $p=0.18$, $t=1.43$ and $p=0.0011$, $t=4.28$, respectively, **Fig.3A**, unpaired two-tailed t -test). Thus, an applied low dose of antidepressant treatment delivered with food pellets induced an antidepressant-like effect in the present study.

This result is in line with our previous findings that showed a 3-week low dose administration of imipramine to C57BL6J mice via drinking water reduced such depressive symptoms as stress-induced decrease in sucrose intake and preference, hyperlocomotion and elevated aggressive behavior (Cline *et al.*, 2014). Similar behavioural results were obtained in the chronic stress depression model with CD1 mice (Cline *et al.*, 2012) and in a model of elderly depression in 18-month-old C57BL6N mice (Malatynska *et al.*, 2012). The low dose imipramine antidepressant effects were accompanied by preservation of normal activity of brain peroxidation enzymes which were suppressed by chronic stress (Cline *et al.*, 2014). These effects are typical for antidepressant effect manifestations induced by tricyclics in rodents (Von Frijtag *et al.*, 2002; Cryan *et al.*, 2005a).

Further, in order to rule out potential effects of imipramine administration on anxiety, locomotion and liquid intake that were previously reported in mice treated with this drug at a dose of 15/mg/kg in C57BL6N mice, we performed supplementary tests in all mice. In both anxiety paradigms, dark-light box and O-maze, animals treated with imipramine showed no significant differences in their behaviour from the control group: in latency of the exit to the anxiety-related areas, lit box and open arms ($p=0.94$, $t=0.08$ and $p=0.59$, $t=0.55$, respectively; unpaired two-tailed t -test), time spent in the lit box and open arms ($p=0.80$, $t=0.26$ and $p=0.28$, $t=1.14$, respectively; unpaired two-tailed t -test) and numbers of exits to these zones ($p=0.87$, $t=0.17$ and $p=0.13$, $t=1.63$, respectively; unpaired two-tailed t -test **Fig.3B,3C**). In locomotory tests, in comparison with control mice, animals treated with imipramine exhibited normal vertical activity, as shown by the number of rearings in novel cage, (Week 2: $p=0.33$, $t=1.01$; Week 4: $p=0.54$, $t=0.63$, unpaired two-tailed t -test), as well as unchanged horizontal locomotion in the open field.

In the latter test, no difference between groups was found in distance travelled (Week 2: $p=0.97$, $t=0.038$; Week 4: $p=0.31$, $t=1.05$) or mean instant velocity (Week 2:

$p=0.98$, $t=0.026$; Week 4: $p=0.84$, $t=0.21$, **Fig.3D**, unpaired two-tailed t -test). In a two-bottle sucrose preference test, there were no significant differences in water intake, sucrose solution intake and sucrose preference between the groups ($p=0.47$, $t=0.75$; $p=0.32$, $t=1.04$; $p=0.20$, $t=1.35$, respectively; unpaired two-tailed t -test, **Fig.3E**). Finally, body weight was not different between control and imipramine-treated groups ($p=0.20$, $t=1.37$, *data not shown*, unpaired two-tailed t -test). There are no statistical significance using a repeated measures ANOVA (*data not shown*).

Thus, the employed dosing with imipramine did not affect basic physiological variables, such as locomotion, liquid consumption and body weight. Also, it did not affect the parameters of anxiety and sucrose ingestion, as reported in some studies that employ higher amounts of tricyclics (File and Tucker, 1986; Mogensen *et al.*, 1994; Willner, 2005; Strekalova *et al.*, 2013). The discrimination between antidepressant- versus antianxiety-like effects, and lack of evidences for general effects on physiological parameters, suggests that low dose imipramine treatment via voluntary food pellet intake can serve as an optimal pharmacological reference in animal models of depression that require prolonged antidepressant treatment of small rodents.

Effects of chronic delivery via food pellets of new candidates to antidepressants in the forced swim and tail suspension tests

Next, we sought to investigate whether the defined method of antidepressant dosing with food pellets can be applicable with the testing of new drug candidates, one of which, celecoxib, is not soluble in water and; therefore, is problematic to deliver to the animals chronically. As such, we exposed a cohort of animals to food pellets containing new drug candidates, dicholine succinate or celecoxib. In addition, we used imipramine or citalopram as the antidepressant references.

In the forced swim test, one-way ANOVA revealed significant differences between the groups in the latency to float, total time spent floating and velocity (Day 1: $p=0.0054$, $F=4.24$; $p=0.049$, $F=2.60$ and $p=0.22$; $F=3.18$, respectively, **Fig.4A**). *Post-hoc* Dunnett test showed that on Day 1, in comparison with the control group, the latency to swim was increased in animals treated with imipramine or dicholine succinate ($p<0.05$, $q=3.17$ and $p<0.01$, $q=3.20$), the duration of immobility was decreased in the imipramine-treated animals ($p<0.05$, $q=2.67$) and velocity was elevated in the dicholine succinate-treated group ($p<0.05$, $q=2.62$). On Day 2 of the forced swim test, one-way ANOVA showed a trend to a statistically significant difference in the latency of floating and no differences in the duration of floating or velocity ($p=0.059$, $F=2.46$; $p=0.48$, $F=0.89$ and $p=0.40$, $F=1.04$, respectively, **Fig.4B**). Dunnett *post-hoc* test revealed a significant increase in latency to float in the imipramine-treated group ($p<0.05$; $q=2.96$). As a reduction of the parameters of floating behaviour in the forced swim test is a well-established measure of antidepressant activity of various compounds (Cryan *et al.*, 2005a; Harro, 2013), these data suggest that the applied treatment with imipramine or dicholine succinate induces an antidepressant effect and that the employed dosing was effective.

With the tail suspension test, one-way ANOVA showed that on Day 1, there were statistically significant differences between the groups in the latency and duration of immobility ($p=0.007$, $F=4.09$; $p<0.0001$, $F=3.43$ and $p<0.0001$, $F=3.43$, respectively, **Fig.4C**); a strong tendency to differences in velocity were found ($p=0.0505$, $F=2.57$). *Post-hoc* Dunnett test revealed a significant difference in the latency of immobility from the control group in imipramine-treated animals ($p<0.01$, $q=3.380$), but not in other treatment groups.

All groups that received pharmacological treatment had significantly reduced duration of immobility in comparison to control mice (imipramine-treated: $p<0.001$, $q=4.50$; citalopram-treated: $p<0.001$, $q=5.02$; dicholine succinate-treated: $p<0.05$,

$q=3.07$; celecoxib-treated: $p<0.05$, $q=2.65$; Dunnett *post-hoc* test). In comparison to control group, velocity was significantly increased in imipramine- and dicholine succinate-treated groups ($p<0.05$, $q=2.55$; $p<0.05$, $q=2.56$, respectively; Dunnett *post-hoc* test).

On Day 2 of the tail suspension test, statistically significant differences between the groups were found in the latency of immobility, the duration of immobility and velocity ($p=0.0159$, $F=3.43$; $p<0.0002$, $F=6.90$ and $p=0.012$, $F=3.65$, respectively, one-way ANOVA; **Fig.4D**). Dunnett *post-hoc* test showed a significant increase of the latency of immobility in imipramine- and dicholine succinate-treated animals, as compared with controls ($p<0.01$, $q=3.48$ and $p<0.05$, $q=2.75$, respectively). All treated groups had significantly reduced duration of immobility, as compared with control mice (imipramine-treated: $p<0.001$, $q=4.46$; citalopram-treated: $p<0.001$, $q=4.31$; dicholine succinate-treated: $p<0.01$, $q=3.39$; celecoxib-treated: $p<0.001$, $q=4.24$ Dunnett *post-hoc* test). Velocity was significantly increased in comparison with control mice in the imipramine- and dicholine succinate-treated groups ($p<0.05$, $q=2.97$; $p<0.05$, $q=2.59$, respectively; Dunnett *post-hoc* test). Since a decrease of immobility behaviour in the tail suspension test is generally considered as a manifestation of the antidepressant activity of various treatments (Cryan *et al.*, 2005b; Harro, 2013), these results evidence an antidepressant-like effect of the applied drugs, and again, the efficacy of the tested method of drug administration.

Conclusions

Thus, as a desirable alternative to invasive dosing, such as chronic intraperitoneal injections, the administration of various drugs via food pellets can be very efficient. The results from our study are in line with other successful attempts to avoid adverse drug delivery methodologies in translational research that showed, for example, the efficacy of treatment with analgesic therapy delivered via food in rats which were

subjected to surgery (Jessen *et al.*, 2007). The use of such methods could be particularly needed when repeated drug administration to stressed, operated or immunodeficient laboratory animals is necessary and therefore could greatly improve not only animal welfare but also the validity of animal models.

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Figure legends

Figure 1. Experimental design. A - Schematic timeline of studies with vehicle injections; Chronic delivery with food pellets of imipramine (B) and new drug candidates to antidepressants (C). i.p. - intraperitoneal injection; FST=forced swim test; TST=tail suspension test; DLB=dark-light box; OM= O-maze; NCT= novel cage test; OF=open field; ST= sucrose test; d=days.

Figure 2. Chronic intraperitoneal injections increase depressive-like behaviour in the forced swim test. In comparison with control group, mice that received daily intraperitoneal saline injections over the course of three weeks had a non-significantly reduction of the latency of floating (A), significant increase of the total number of floating episodes (B) and total time spent floating (C). There were no significant differences in mean velocity (D) or distance swum (E) between control and injected groups. * $p < 0.05$; ** $p < 0.01$ vs. control (unpaired *t*-test). Con: control group, ($n=7$); i.p. injection: a group of mice subjected to intraperitoneal injections with a vehicle ($n=5$). All data are means $\pm$ SEM.

Figure 3. Effects of dosing with imipramine via food pellets on floating scores and other behaviours. (A) In comparison with control group, mice that received imipramine in food pellets over four weeks showed an increase of latency to float and total duration of floating in the forced swim test. No significant differences between the groups were found in parameters of anxiety (B) in the dark-light box and (C) O-maze tests. (D) Control and imipramine-treated mice showed similar numbers of rearings in the novel cage, distance travelled and mean velocity in the open field test. (E) In the two-bottle sucrose preference test, water intake, sucrose solution intake and sucrose preference were not different between imipramine-treated and control groups. ** $p < 0.01$, *** $p < 0.001$ vs. control (unpaired *t*-test). Con: control group, ($n=7$); Imi-food: imipramine-treated group ($n=8$). All data are means $\pm$ SEM.

Figure 4. Effects of chronic delivery via food pellets of new candidates to antidepressants in the forced swim and tail suspension tests. (A) On Day 1 of the forced swim test, as compared with control, imipramine-treated animals elicited an increase in latency to float and reduced

time spent floating, and dicholine succinate-treated groups displayed an increase swim velocity. **(B)** On Day 2 of the forced swim test, imipramine-treated group had higher latency to float in comparison to control mice; no other differences between treated and control groups were found. **(C)** On Day 1 of the tail suspension test, there was a significant increase of the latency of immobility and velocity in imipramine- and dicholine succinate-treated groups, as compared to controls. All treated groups showed a significant reduction of total time spent immobile, as compared to control animals. **(D)** On Day 2 of the tail suspension test, in comparison to control group, an increase of the latency of immobility was found in imipramine-treated group and an increase of velocity was observed in both imipramine- and dicholine succinate-treated mice. All animals that received a treatment, demonstrated a significant reduction of total time spent immobile, in comparison to control group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control (one-way ANOVA with Dunnett *post-hoc* tests). All groups were $n=10$. **Con:** control group; **Imi-food:** imipramine-treated group; **Cit-food:** citalopram-treated group; **DS-food:** dicholine-succinate-treated group; **Cel-food:** celecoxib-treated group. All data are means $\pm$ SEM.

A **B** **C** *Figure 1*

Figure 2

Figure 3

Figure 4

Chapter 6

Insulin receptor sensitizer, dicholine succinate, prevents both Toll-like receptor 4 (TLR4) upregulation and affective changes induced by a high-cholesterol diet in mice.

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Abstract

Background: High cholesterol intake in mice induces hepatic lipid dystrophy and inflammation, signs of non-alcoholic fatty liver disease (NAFLD), depressive- and anxiety-like behaviours, and the up-regulation of brain and liver Toll-like receptor 4 (*Tlr4*). Here, we investigated whether dicholine succinate, an insulin receptor sensitizer and mitochondrial complex II substrate would interact with these effects. **Methods:** C57BL/6J mice were given a 0.2%-cholesterol diet for 3 weeks, alone or along with oral DS administration, or a control feed. Outcomes included behavioural measures of anxiety/depression, and *Tlr4* and peroxisome-proliferator-activated-receptor-gamma coactivator 1b (*PPARGC1b*) expression. **Results:** 50mg/kg DS treatment for 3 weeks partially ameliorated the cholesterol-induced anxiety- and depressive-like changes. Mice were next treated at the higher dose (180mg/kg), either for the 3-week period of dietary intervention, or during its last two weeks. Three-week DS administration normalized behaviours in the forced swim and O-maze tests and abolished the *Tlr4* up-regulation in the brain and liver. The delayed, 2-week DS treatment had similar effects on the *Tlr4* expression and largely rescued above-mentioned behaviours. Suppression of *PPARGC1b*, a master regulator of mitochondrial biogenesis, by the high cholesterol diet, was prevented with a 3-week administration, and markedly diminished during a 2-week administration of DS. None of treatments prevented hepatic dystrophy and triglyceride accumulation. **Limitations:** Other conditions have to be tested to define possible limitations of reported effects of DS. **Conclusions:** DS treatment did not alter the pathomorphological substrates of NAFLD syndrome in mice, but ameliorated its molecular and behavioural consequences, likely by activating mitochondrial functions and anti-inflammatory mechanisms.

Key words: Western diet; depression; anxiety; insulin receptor sensitizers; toll-like receptor four (*Tlr4*); mice

Introduction

An emerging body of human and animal experiments has shown that a “Western diet” (high in saturated fat and/or cholesterol) – with ensuing obesity, hypercholesterolemia and insulin resistance – is associated with higher rates of affective disorders, and decreases therapeutic efficacy of antidepressants (Pomytkin *et al.*, 2015; Shelton and Miller, 2010; Sonawalla, 2002; de Wit *et al.*, 2010). Whilst the peripheral mechanisms of the association between metabolic factors and affective disorders has received some attention in the literature (Castanon, *et al.*, 2015; Kishi and Elmquist, 2005), very few studies have addressed the central molecular pathways involved, which could be explored as potential targets for pharmacotherapy (Andre *et al.*, 2014; Lucas and Maes, 2013; Scheen, 2009).

Insulin resistance, including deficient insulin receptor signalling, is regarded as a crucial pathogenic mechanism of affective changes associated with over-nutrition and metabolic syndrome (Kan *et al.*, 2013; Pomytkin *et al.*, 2015; Pyykkönen *et al.*, 2011). Recent studies have shown that insulin itself and “neuronal insulin receptor sensitizers” have anti-depressant-like properties (Eissa Ahmed and Al-Rasheed, 2009; Gupta *et al.*, 2014; Igarashi *et al.*, 2008; Kemp *et al.*, 2011; Mittal *et al.*, 2009; Rasgon *et al.*, 2010; Saubermann *et al.*, 2002). These compounds increase neuronal mitochondrial biogenesis, are anti-inflammatory, and ameliorate neuronal damage (Ali *et al.*, 2006; Asghar *et al.*, 2007; Nuccio *et al.*, 2015; Salehi-Sadaghiani *et al.*, 2012; Strum *et al.*, 2007; Zhao *et al.*, 2006). Given the crucial role of mitochondria in synaptic function (Maeder *et al.*, 2014) and evidence of lowered mitochondrial ATP and enzyme production, and inhibited respiratory chain and glucose metabolism during a depressive-like state (Gardner *et al.*, 2003; Madrigal *et al.*, 2001; Videbech *et al.*, 2000), it is remarkable that insulin receptor sensitizers substantially ameliorate mitochondrial functions in the brain (De Nuccio *et al.*, 2015; Dello Russo *et al.*, 2002; Crenshaw *et al.*, 2015). Notably, mitochondrial effects of insulin receptor sensitizers

result in increased resting-state functional connectivity of hippocampal CA1 area to the hypothalamus and ventral thalamus (Crenshaw *et al.*, 2015); currently, compromised brain connectivity is regarded as a biomarker of severe depression (Kaiser and Pizzagalli, 2015).

Antidepressant-like effects have also been reported for the non-esterified salt, dicholine succinate (DS), a mitochondrial complex II substrate, which enhances insulin-induced neuronal insulin receptor autophosphorylation in a dose-dependent manner (Persiyantseva *et al.*, 2013; Storozheva *et al.*, 2008; Storozhevykh *et al.*, 2007), a mechanism for receptor activation (Wei *et al.*, 1995). In a model of stress-induced anhedonia, DS, rescued normal contextual fear conditioning, increased hippocampal N-methyl-D-aspartate (NMDA) receptor subunit NR2A expression (and increased the NR2A/NR2B ratio), and increased rapid-eye-movement (REM) sleep rebound after acute predation (Cline *et al.*, 2015, 2012). In a model of elderly depression, DS restored normal hedonic behaviours and increased the expression of neural plasticity factors in the hippocampus (Cline *et al.*, 2015). Also, DS normalized hippocampal levels of the phosphorylated (inactive) form of glycogen synthase kinase three-beta (GSK3- β) during swim test in mice (Cline *et al.*, 2015) and improved tail suspension behaviour (Costa-Nunes *et al.*, 2015).

Given the link between insulin receptor-mediated signalling and nutritional/metabolic factors (Kan *et al.*, 2013; Pyykkönen *et al.*, 2011), we hypothesized that DS can improve the conditions resulting from non-alcoholic fatty liver disease (NAFLD). To test this, we employed recently established NAFLD model, in which young female C57BL/6J mice are exposed to a feed containing 0.2% cholesterol for 3 weeks (Comhair *et al.*, 2011; Strekalova *et al.*, 2015). These changes are accompanied by behavioural despair, anxiety-like and impulsive behaviours, and elevated brain and liver levels of Toll-like receptor 4 (*Tlr4*). The present study

investigated whether DS could ameliorate the behavioural and molecular changes associated with NAFLD.

Methods

Animals

Three-month-old female C57BL/6J mice obtained from the Gulbenkian Institute of Science, Oeiras, Portugal were housed in groups of five under standard conditions and reversed lighting (*see Supplementary material*). All experiments were carried out in accordance with the European Communities Council Directive for the care and use of laboratory animals.

Study flow, dietary challenge and behavioural testing

Twenty-three mice were exposed for three weeks either to a regular diet (control group), with an energy content of 4.0 kcal/g (Mucedola Srl, Settimo Milanese, Italy), or a diet of similar composition with 0.2% (w/w) cholesterol and an energy content of 4.6 kcal/g (Research Diets Inc, New Brunswick, NJ, USA; *see Supplementary material*), using an established protocol (Comhair *et al.*, 2011; Strekalova *et al.*, 2015). Body weight and 24-h food intake was evaluated on Days 0, 14 and 21 of dietary challenge. Eight mice were treated with DS via drinking water at 50 mg/kg/day (*see p. 2.3*) on days 1-21 of the dietary intervention. DS-treated mice were tested using the O-maze on day 21, along with eight dietary challenged but pharmacologically naïve mice, and seven dietary control mice; lighting intensity was 5Lux (**Fig.1A**; details of all behavioural paradigms can be found in *Supplementary material*). Latency of exit to the open arms, time spent therein, and number of exits, were scored in all groups (Strekalova *et al.*, 2015). 3h after the O-maze, all mice were subjected to the 6-min forced swim test and 24h later, on Day 22, they were scored for latency to floating, and total duration of floating (Malatynska *et al.*, 2012; Strekalova *et al.*, 2015).

Next, seven mice fed a high fat diet were given a dose of 180 mg/kg of DS (*see* 2.3), and studied alongside pharmacologically-naïve (n=7) and dietary control (n=10) mice, as described above (**Fig.1B**). In addition, a group of mice were given high-cholesterol diet *alone* for 1 week, and then were given DS alongside for the remaining 2 weeks (n=8; **Fig.1B**). On days 21-22, all thirty-two mice were studied behaviourally as described above. On day 23, mice were sacrificed for brain and liver dissection and subsequent histological, molecular and biochemical analyses; the details of ex-vivo assays (*see* 2.4) are presented in *Supplementary material*.

Drug administration

DS was dissolved in tap water at 50 and 180 mg/kg/day, and provided for mice to drink *ad libitum* in water bottles as described elsewhere (Cline *et al.*, 2015; Costa-Nunes *et al.*, 2015).

Tissue collection and ex-vivo analyses

Mice were terminally anaesthetised and transcardially perfused with NaCl, liver and brains were dissected, and prefrontal cortex, hippocampus, hypothalamus and dorsal raphe were isolated as described elsewhere for a subsequent *in vitro* assays, where five animals from each group were analyzed (Couch *et al.*, 2013; Cline *et al.*, 2015). The assessment of histological integrity and triglycerides in livers were performed as described elsewhere (Comhair *et al.*, 2011; Strekalova *et al.*, 2015). RNA extraction was performed from snap-frozen tissue to evaluate expression of *Tlr4* and *PPARGC1b* using SYBR green-based technology (Primer Design Ltd.), as described previously (Couch *et al.*, 2013; Strekalova *et al.*, 2015). Results were corrected to the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) and expressed as fold increase compared to control animals and normalized to the means of this group.

Statistics

Data were analysed with GraphPad Prism version 5.0 (San Diego, CA, USA) using one- or two-way ANOVA with Tukey post-hoc testing. The level of significance was set at $p < 0.05$.

Results

Dosing with 50 mg/kg/day choline salt of succinic acid partially rescued affective changes induced by cholesterol diet

Body weight did not differ significantly between groups, suggesting that the dietary and pharmacological interventions did not grossly alter body composition ($p > 0.05$, ANOVA; see *Supplementary material, Fig.1A*). In line with previous findings, intake of the high-cholesterol diet was significantly lower than that of the control diet, and this was not altered by DS administration ($p < 0.05$, ANOVA; see *Supplementary material, Fig.1B*), likely due to the adjustment of mice to the higher caloric value of the high-cholesterol diet. In the O-maze, there was a significant difference between the groups with regards to latency to exit into the open arms, time spent therein and number of exits to this zone ($F = 3.98$, $p = 0.035$; $F = 4.58$, $p = 0.023$ and $F = 4.46$, $p = 0.025$, respectively, one-way ANOVA; Fig.2A-C), with increased latency, decreased time spent, and decreased number of exits in mice fed with cholesterol diet compared with control mice ($p < 0.05$, Tukey test, see *Supplementary Table 3*). Whilst there was a trend to normalizing of these behavioural features with DS treatment, there were no statistically significant differences between DS-treated mice with pharmacologically-naïve mice in terms of latency to exit into open arms, time spent therein and number of exits into open arms ($p > 0.05$, Tukey test).

In the forced swim test, there was a significant difference between the groups in terms of latency and duration of floating ($F = 5.86$, $p = 0.010$ and $F = 6.37$, $p = 0.007$ respectively; ANOVA, Fig.2D,E). Post-hoc comparisons revealed shortened latency to

float in pharmacologically-naïve mice fed with the high cholesterol diet compared with dietary control mice ($p<0.05$, Tukey test) but this was significantly ameliorated in the DS group ($p<0.05$, Tukey test, *see Supplementary Table 3*). Conversely, duration of floating was increased in pharmacologically naïve high cholesterol mice ($p<0.05$, Tukey test); there was a tendency to normalization with DS treatment, but this did not meet statistical significance. Due to the borderline effect noted with 50 mg/kg/day treatment with DS, we next investigated whether these behavioural effects become more prominent at a higher dose.

Both simultaneous and delayed dosing with 180 mg/kg/day choline salt of succinic acid ameliorated the affective changes induced by cholesterol diet

As in the previous study, there was no difference between the groups in body weight across the study ($p>0.05$, ANOVA; *see Supplementary material, Fig.3A*); similarly, the intake of the high-cholesterol diet was significantly lower than that of control diet and was not altered in DS-treated mice ($p<0.05$, one-way ANOVA).

The groups differed significantly in terms of time spent in the open arms and number of exits into the open arms of the O-maze ($F=3.50$, $p=0.029$ and $F=3.19$, $p=0.040$, respectively, one-way ANOVA; **Fig.2F-H**). Pharmacologically-naïve mice fed the high cholesterol diet spent significantly less time in the open arms compared with dietary control mice ($p<0.05$, Tukey test, *see Supplementary Table 4*) and this normalized significantly with 3 week DS treatment at 180 mg/kg/day ($p<0.05$, Tukey test). Similarly compared with dietary control mice, those given a high cholesterol diet trended to fewer exits to the open arms, but this did not reach statistical significance; however, DS treatment significantly increased the number of exits onto the open arms in mice given DS for 3 weeks compared with pharmacologically-naïve mice ($p<0.05$, Tukey test). Other differences between the groups did not reach statistical significance ($p>0.05$, ANOVA, Tukey test). Mice on the high cholesterol diet treated with the delayed DS regime during two last week of dietary intervention did not show

any changes in any behavioural measure on the O-maze as compared with control group ($p>0.05$, ANOVA, Tukey test; **Fig.2F-H**).

In the forced swim test, the groups differed significantly in terms of latency and duration of floating ($F=5.38$, $p=0.005$ and $F=5.92$, $p=0.003$, respectively; **Fig.2I,J**). Post hoc comparison showed that pharmacologically naïve mice fed a high cholesterol diet had a reduced latency to floating compared with dietary controls ($p<0.05$, Tukey test, see **Supplementary Table 4**), but DS treatment, either for the three or two-week treatment regime, increased latency significantly compared with naïve mice ($p<0.05$, Tukey test). The duration of floating was significantly increased in pharmacologically naïve high cholesterol mice compared with dietary controls ($p<0.05$, Tukey test), and this was significantly ameliorated with three-week, ($p<0.05$, Tukey test) but not two-week treatment with DS ($p>0.05$, Tukey test). No other comparisons between groups reached statistical significance ($p>0.05$, ANOVA, Tukey test). Thus, at the dose of 180 mg/kg/day, DS administration for the entire 3-week dietary period abolished behavioural changes induced by the high cholesterol diet. By comparison, a delayed, two-week administration of DS only partially ameliorated the behavioural effects of cholesterol supplementation.

Dicholine succinate treatment normalizes Tlr4 and PPARGC1b expression, but does not alter cholesterol-induced NAFLD syndrome

High cholesterol diet and treatment with DS had a significant impact on *Tlr4* gene expression ($F=108.0$, $p<0.0001$) that was independent of the CNS region ($p=0.195$, two-way ANOVA; **Fig.3A**). Expression of *Tlr4* was significantly higher in mice fed the high cholesterol diet without DS treatment compared with dietary controls, in the prefrontal cortex ($F=11.99$, $p<0.0001$), hippocampus ($F=8.99$, $p<0.0001$), hypothalamus ($F=5.66$, $p=0.009$), and dorsal raphe ($F=8.29$, $p<0.0001$; **Fig.3A**). This effect was obliterated with DS treatment, with significantly decreased expression in all anatomical regions, for high cholesterol-fed mice treated with DS vs

pharmacologically naïve mice, including prefrontal cortex, hippocampus, hypothalamus and dorsal raphe ($p<0.0001$, see *Supplementary Table 5*), but no significant differences between mice fed a normal diet compared with mice fed high cholesterol diet but receiving DS treatment in all these brain areas ($p>0.05$, Tukey test).

Similarly, *Tlr4* gene expression in the liver was significantly altered ($F=7.56$, $p=0.002$; one-way ANOVA, **Fig.3A**). *Post hoc* comparisons showed significant increased mRNA levels of *Tlr4* in the liver of mice exposed to high cholesterol diet ($p<0.05$, Tukey test, see *Supplementary Table 5*) but not in dietary challenged mice dosed with DS for 3 or 2 weeks ($p<0.05$, Tukey test), as compared to control. Animals exposed to cholesterol and treated with DS either for 3 or 2 weeks, showed significantly diminished liver *Tlr4* expression, in comparison with non-treated group fed with cholesterol-containing diet ($p<0.05$, Tukey test).

There were also significant differences between groups in terms of *PPARGC1b* gene expression ($F=123.0$, $p<0.0001$; one-way ANOVA), which were independent of CNS region ($F=0.54$, $p=0.660$; two-way ANOVA; **Fig.3B**). In all CNS regions, including prefrontal cortex, hippocampus, hypothalamus and dorsal raphe, *PPARGC1b* mRNA levels were significantly lower for mice fed with high cholesterol diet and no treatment and mice treated with DS for 2 weeks compared with the dietary control group ($p<0.0001$, Tukey test, see *Supplementary Table 6*).

Mice fed with high cholesterol diet and treated with DS had elevated *PPARGC1b* expression in all above-listed CNS structures compared with both pharmacologically naïve mice fed with cholesterol diet and mice given the delayed 2-week treatment ($p<0.001$, Tukey test). In most CNS regions, including hippocampus, hypothalamus and dorsal raphe, *PPARGC1b* expression was not significantly different between high cholesterol DS treated mice compared with normal diet control ($p>0.05$, Tukey test)

but was decreased in the prefrontal cortex ($p < 0.05$, Tukey test), showing that gene expression was returned to broadly normal levels with 3 week DS treatment. There were no significant differences in *PPARGC1b* expression between mice fed high cholesterol diet with no treatment, and those given the delayed 2-week regime, in any CNS region studied ($p > 0.05$, Tukey test). No other statistically significant changes in the brain levels of *PPARGC1b* mRNA were found.

Hepatic *PPARGC1b* gene expression was significantly different between treatment groups ($F = 8.50$, $p = 0.001$; ANOVA, **Fig.3B**). Hepatic *PPARGC1b* expression was significantly decreased in pharmacologically naïve mice exposed to high cholesterol diet ($p < 0.05$, Tukey test, see **Supplementary Table 6**) and mice dosed with DS for 2 weeks ($p < 0.05$, Tukey test). However, mice fed the high cholesterol diet with 3 week DS treatment had increased *PPARGC1b* levels compared with pharmacologically naïve mice ($p < 0.05$, Tukey test), but were not significantly different from mice fed a normal diet ($p > 0.05$, Tukey test).

Compared with mice fed a normal diet, the high-cholesterol diet groups revealed compromised liver tissue integrity, irrespectively to the scheme of dosing with DS (**Fig.3C**). The appearance of intracellular vacuoles was due to fat deposition in the tissue as previously reported (Comhair *et al.*, 2011; Strekalova *et al.*, 2015). There was limited difference between the four high fat groups, although there appears to be optical amelioration in the two treated groups compared with no treatment (**Fig.3C**). Dosing with DS for 3 weeks seems to have preserved integrity compared with a 2-week treatment with DS, although variation in tissue made this difficult to establish. Hepatic levels of triglycerides were significantly different between the groups ($F = 12.56$, $p = 0.0002$, ANOVA; **Fig.3D**). The colorimetric assay showed significantly elevated levels of triglycerides in mice on the high-cholesterol diet as compared to control animals ($p < 0.05$, Tukey test). This increase was found in mice treated with DS for 3 or 2 weeks as well ($p < 0.05$, Tukey test, see **Supplementary Table**

7). There was no difference in this measure between treatment groups for mice fed with high cholesterol diet ($p>0.05$, Tukey test).

Discussion

In this study we showed that treatment with insulin receptor sensitizer dicholine succinate ameliorated anxiety- and depressive-like behaviours induced by a high cholesterol diet, and normalized expression of pro-inflammatory factor *Tlr4* and mitochondrial activity marker *PPARGC1b*, which were expressed abnormally high and low after high cholesterol diet treatment respectively. These effects were most pronounced when the dose of DS of 180 mg/kg/day was used for the entire period of a 3-week dietary challenge, and appeared to be limited when the onset of this treatment was delayed by one week. Liver steatosis and dystrophy were not significantly changed by the DS administration. As such, the improvement of affective changes in mice exposed to elevated dietary cholesterol and treated with the insulin receptor sensitizer DS was likely realized via the normalization of the secondary mechanisms of NAFLD syndrome, such as inflammation and mitochondrial functions, rather than of its primary features of liver steatosis and dystrophy.

We have previously reported sharply elevated brain and liver mRNA and protein levels of *Tlr4* associated with increased anxiety, hyperlocomotion and “behavioral despair” using this NAFLD model (Strekalova *et al.*, 2015). Here, both 3- and 2-week administration of DS at the dose 180 mg/kg/day rescued normal gene expression of *Tlr4* in a liver and all four investigated brain structures, including the hippocampus and the prefrontal cortex. This was accompanied by a normalization of anxiety- and depressive-like behaviours. Our data and other recent reports in the literature show a link between TLR4 expression, anxiety-like behaviour and stress in rodents (Okun *et al.*, 2012; Rico *et al.*, 2010; Shannonhouse *et al.*, 2014), which suggests that the

ameliorative behavioural effects of DS shown in this experiment may be due to a suppressive action of DS on *Tlr4*.

In comparison to the behavioural effects of a 3-week treatment with DS, the dosing with DS for 2 weeks with a delayed onset of 1 week had limited efficacy. Both treatments similarly prevented the brain and liver *Tlr4* overexpression, but no significant restorative action of a 2-week DS administration on the marker of mitochondrial activity *PPARGC1b* was found, which might explain the above-mentioned discrepancy in behavioural results. While this discrepancy can also be due to differing protein levels of TLR4 protein, our data may suggest a partial role of TLR4 in the regulation of affective changes in mice.

PPARGC1b is a member of family of peroxisome proliferator activated receptor gamma coactivators, which induces nuclear-encoded genes regulating mitochondrial oxidative metabolism and beta-oxidation of fatty acids in the brain, liver and other tissues, particularly during over-nutrition (Lin *et al.*, 2003; Scarpulla, 2008; Sonoda *et al.*, 2007). *PPARC1b* knock-out mice have increased susceptibility to hepatic steatosis induced by high fat diet, insulin resistance, reduced mitochondrial biogenesis and behavioural inhibition (Lelliott *et al.*, 2006; Sonoda *et al.*, 2007; Vianna *et al.*, 2006). In contrast, mutant mice overexpressing *PPARGC1b* revealed enhanced biosynthesis of mitochondria and basal oxygen consumption, increased energy expenditure and reduced lipid accumulation under these conditions (Kamei *et al.*, 2003; Lin *et al.*, 2002; Meirhaeghe *et al.*, 2003). Available data suggest that the treatment of mice housed on a high cholesterol diet with DS could enhance lipid metabolism and mitochondrial functions via a restoration of basal *PPARGC1b* expression, and this might preserve normal brain functions and behaviour.

In summary, our study demonstrates for the first time that the dosing with insulin receptor sensitizer DS can ameliorate depressive- and anxiety-like behaviours, as well

as changes in the molecular markers of inflammation and mitochondrial function, which are evoked by dietary cholesterol. Given data showing a functional relationship between insulin resistance, TLR4 and mitochondrial functions, the precise mechanisms of which are not yet fully elucidated (Shi *et al.*, 2006), we hypothesize that the molecular effects of DS shown in this report are mediated by the stimulation of insulin receptor signalling. They are likely to involve secondary effects associated with increased insulin receptor signalling, such as amelioration of glucose tolerance and neuronal mitochondria functions resulting in normalized brain connectivity, an important factor in depression (Maeder *et al.*, 2014; Pomytkin *et al.*, 2015). While DS induced no significant effects on hepatic histology or triglyceride content, our findings nonetheless suggest a potential therapeutic role of insulin receptor sensitizers, an emerging class of antidepressant remedies, for the treatment of affective symptoms during NAFLD syndrome.

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The founding source had no influence on any aspect of work presented in this publication.

Conflict of Interest

The authors declare no conflict of interest.

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Figure legends

Figure 1. Schematics of the study with administration of DS (A) at the dose 50 mg/kg and at the dose 180 mg/kg **(B)**, for 3-weeks or for 2 weeks, with late onset.

Figure 2. Effects of DS on behavioral correlates. Partial efficacy of treatment with DS at the dose of 50 mg/kg on the parameters of anxiety-like behavior: the **(A)** latency of the first exit **(B)** time spent in open arms **(C)** number of exits to open arms, in the elevated O-maze, and on depressive-like behavior: the latency **(D)** and **(E)** duration of floating in the forced swim test. Preventive effects of administration of DS at the dose of 180 mg/kg on the parameters of anxiety-like behavior: the **(F)** latency of the first exit **(G)** time spent in open arms **(H)** number of exits to open arms, in the elevated O-maze, and on depressive-like behavior: the latency **(I)** and **(J)** duration of floating in the forced swim test. * $p < 0.05$ vs. control, # $p < 0.05$ vs. HC diet, Tukey test). Data are shown as mean $\pm$ SEM.

Figure 3. Effects of DS on gene expression, triglyceride levels and hepatosteatosi. Administration of DS at the dose of 180 mg/kg precludes dietary-induced **(A)** *Tlr4* up-regulation in the brain and liver and **(B)** a suppression of a mitochondrial activity markers PPARGC1b, but does not alter **(C)** hepatosteatosi (Haematoxylin and eosin-stained sections: scale bar 20um) nor **(D)** triglyceride levels in the liver. * $p < 0.05$ vs. control, # $p < 0.05$ vs. HC diet, Tukey test). Data are shown as mean $\pm$ SEM.

A Experimental design. Dosing with 50 mg/kg

B Experimental design. Dosing with 180 mg/kg

Figure 1

DOSING WITH 50 MG/KG

DOSING WITH 180 MG/KG

Figure 2

Figure 3

Supplementary material

Animals

Studies were performed using 3-month-old female C57BL/6J from the Gulbenkian Institute of Science, Oeiras, Portugal. C57BL/6J mice were housed in groups of five under a reversed 12-hour light–dark cycle (lights on: 21:00 h) starting from the day of animals' transportation in the laboratory with food and water provided *ad libitum*, under controllable laboratory conditions ($22 \pm 1^\circ\text{C}$, 55% humidity). Animals were handled weekly during the changes of cages by care takers, as well as when experimental procedures were performed. All mice had a tissue paper in their cages and also placed on the bedding material.

All experiments were carried out in accordance with the European Communities Council Directive for the care and use of laboratory animals 2010/63/EU and Portuguese Law-Decrees DL129/92 upon approval by the Ethical and Welfare Committee of the New Lisbon University on animal care and welfare.

Elevated O-maze

The apparatus (Technosmart, Rome, Italy), which consisted of a circular path (runway width 5.5 cm, diameter 46 cm), was placed 20 cm above the floor. Two opposing arms were protected by walls (height 10 cm), and the illumination strength was 5 Lux. The apparatus was placed on a dark surface in order to reduce reflection and maintain control over lighting conditions during testing. Anxiety-like behaviour was assessed using previously validated parameters (Cline *et al.*, 2015; Costa-Nunes *et al.*, 2014, 2015, Strekalova *et al.*, 2015). Mice were placed in one of the closed-arm compartments of the apparatus. The latency of the first exit to the anxiety-related open compartments of the maze, the total duration of time spent therein, and the number of exits to the open arms were scored during a 5-min observation period; all behaviours were scored manually.

Forced swim test

The Porsolt forced swim protocol has been adapted from previously established method (Malatynska *et al.*, 2012; Strekalova *et al.*, 2015). Mice were subjected to two 6-min swimming sessions, on Days 1 and 2, spaced 24 h apart, in a transparent cylinder (Ø 17 cm) filled with water (23 °C, water height 13 cm, height of cylinder 20 cm, illumination intensity 25 Lux). Behavior was scored on Day 2. Floating was defined by the absence of any directed movements of the animals' head and body and was scored manually; criteria of scoring were previously validated using Noldus EthoVision XT 8.5 (Noldus Information Technology, Wageningen, The Netherlands) and CleverSys (CleverSys, Reston, VA, USA). Using this method, the latency of the first episode of floating and the duration of floating behaviour were scored. Latency to begin floating was scored as time between introduction of the animal into the pool and the first moment of complete immobility of the entire body for a duration of >3 seconds. The total time spent floating was scored for the entire duration of the test using post-test video footage.

Tissue collection and in vitro assays

Mice were terminally anaesthetised with sodium pentobarbitone and transcardially perfused with NaCl, brains were dissected and prefrontal cortex, hippocampi, hypothalamus, raphe dorsalis and liver were isolated and stored at -80°C until use as described elsewhere (Couch *et al.*, 2013; Cline *et al.*, 2015; Costa-Nunes *et al.*, 2014). Pieces of liver were separately stored for a subsequent histological and triglyceride analysis as previously described (Strekalova *et al.*, 2015).

Quantitative RT-PCR (qPCR)

RNA Extraction and Quantitative RT-PCR: RNA extraction was performed as previously described from specifically microdissected snap-frozen brain regions (Blond *et al.*, 2002, Campbell *et al.*, 2005). Specific primers were designed by the Primer Design Centre (Roche) and are given in the table below (*see Supplementary*

Table 1). Standard curves were generated using synthetically designed amplicons of known copy number (Primer Design Ltd., UK). Quantitative RT-PCR was run using Roche Mastermix and FAM PCR probes. Results are expressed as number of copies of target per 1 ng input RNA corrected to the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (Gapdh). Data were calculated as fold increase compared to control animals and normalized to housekeeping gene.

Triglyceride assay

The triglyceride colorimetric assay from Cayman chemicals (Cayman Europe, Tallinn, Estonia) was used for this analysis. In brief, small segments of liver (range: 25.0mg – 34.0mg; median: 30.05mg) were placed in 100µL of the cell lysis buffer, and homogenized using an electronic tissue disruptor. Samples were then frozen at -80°C to use freeze-thaw for mechanical disruption of the tissue. Once thawed, 10µL of each samples were placed in a 96 well plate, with 150µL of enzyme mixture (lipoprotein lipase, glycerol kinase, glycerol phosphate oxidase and peroxidase). A triglyceride standard curve was used with the following concentrations:

Triglyceride standard	Concentration (mg/dL)
1	200
2	100
3	50
4	25
5	12.5
6	6.25
7	3.125
8	0

This was read using a plate reader with absorbance at 550 nm. The 0 mg/dL standard was used as a null reference, and the absorbance value of this standard was subtracted from all other samples and standards to give a corrected absorbance value (CA). The standard curve was used to construct the linear equation $y = mx + c$, and the concentration of triglycerides in the samples was calculated using a formula:

$$\text{Triglycerides}_{(\text{mg/dL})} = [(CA) - c]/x]$$

This gives the concentration of triglycerides in the samples, which is then divided by 10,000 to give the total amount of triglyceride in the original lysis buffer. This value was then divided by the number of mg of tissue homogenized into the lysis buffer, multiplied to convert to μg triglyceride to end up with a value of μg triglyceride / mg of liver tissue.

Liver morphology assay

Small segments of liver tissue were fresh frozen, and stored at -80°C until use. Tissue was attached using embedding medium to the cryostat apparatus (Leica Microsystems, Nusslock, Germany) and $20\mu\text{m}$ sections were cut and mounted on ionized microscope slides. The slides were dried over night at room temperature, and then dehydrated using increasing concentrations of ethanol (70% - 95% - 100%) and placed in xylene. The tissue was subsequently rehydrated using the reverse procedure, and then stained using a basic protocol:

- Haematoxylin solution – 5 mins
- Acidified 70% ethanol (1% HCl) – 5 mins
- Scott's tap water – 5 mins
- Eosin Y solution – 5 mins
- Running tap water – 5 mins
- Scott's tap water – 5 mins

The slides were then taken through increasing concentrations of ethanol to dehydrate, and then placed in xylene as a clearing agent. Slides were mounted with coverslips using DPX hydrophobic mounting medium, and allowed to air dry at room temperature. Representative images were acquired using Leica firecam software at 20 x magnification (Leica Microsystems, Nusslock, Germany).

Supplementary Tables

Table 1. Sequences of primers used

mRNA target	Primer sequence (5' → 3')	
<i>Tlr4</i>	Forward	GAGGCAGGAGGTCTGAGGT
	Reverse	GACAGCCAGCAAGACCAAG
<i>PPARGC1b</i>	Forward	CTCCAGTTCCGGCTCCTC
	Reverse	CCCTGTGCTCTCACGTCTG
<i>Gapdh</i>	Forward	ACCCCTTCATTGACCTCAACTACATG
	Reverse	CCTTCTCCATGGTGGTGAAGAC

Table 2. Composition of Control and High Cholesterol diets

	Control diet	High Cholesterol diet
Saturated / unsaturated fat	5.2%	7.6%
Saturated (SFA) ,% of fat	12.9%	14.2 %
Monounsaturate(MUFA), % of fat	76.3%	72.1 %
Polyunsaturated (PUFA), % of fat	10.8%	13.7 %
Carbohydrates (en%)	70.0%	81 %
Protein (%)	24.6%	11%
Choline (%)	0.2%	0.2%

The composition of the diets with respect to the content of carbohydrates, saturated / unsaturated fat, protein were similar in control and high cholesterol diets, apart from a presence of cholesterol.

Table 3. Summary of group comparisons in O-maze and forced swim behaviors: study with DS administration at the dose 50 mg/kg/day (see **Figure 2A-F**)

Groups	O-maze test			Forced swim test	
	Latency of exit	Time spent in open arms	Number of exits	Latency of floating	Duration of floating
Control diet vs. HCD	q=4.28, p=0.0176	q=3.84, p=0.0343	q=3.73, p=0.0401	q=4.52, p=0.0121	q=4.91, p=0.0065
Control diet vs. HCD + DS 50 mg/kg/day	q=2.37, p=0.2390,ns	q=1.11, p=0.7146,ns	q=0.31, p=0.9733,ns	q=0.95, p=0.7842,ns	q=1.63, p=0.4936,ns
HCD vs. HCD + DS 50 mg/kg/day	q=1.98, p=0.3611,ns	q=2.82, p=0.1402,ns	q=3.54, p=0.0531,ns	q=3.70, p=0.0419	q=3.39, p=0.0649,ns

HCD – high cholesterol diet; ns – not significant ($p>0.05$).

Table 4. Summary of group comparisons in O-maze and forced swim behaviors: study with DS administration at the dose 180 mg/kg/day (see **Figure 2E-J**)

Groups	O-maze test			Forced swim test	
	Latency of exit	Time spent in open arms	Number of exits	Latency of floating	Duration of floating
Control diet vs. HCD	q=2.88, p=0.1987,ns	q=4.06, p=0.0374	q=2.34, p=0.3676,ns	q=4.27, p=0.0258	q=4.08, p=0.0358
Control diet vs.HCD + DS 180 mg/kg/day 3-wk	q=0.243, p=0.9981,ns	q=1.13, p=0.9936,ns	q=2.02, p=0.4926,ns	q=1.39, p=0.7604,ns	q=2.38, p=0.3507,ns
Control diet vs. HCD + DS 180 mg/kg/day 2-wk	q=1.24, p=0.8155,ns	q=0.37, p=0.8532,ns	q=1.77, p=0.5992,ns	q=0.36 p=0.9939,ns	q=0.72, p=0.9555, ns
HCD vs. HCD + DS 180 mg/kg/day 3-week treatment	q=2.88, p=0.1988,ns	q=3.94, p=0.0452	q=3.95, p=0.0444	q=5.22, p=0.0050	q=5.83, p=0.0017
HCD vs. HCD + DS 180 mg/kg/day 2-wk	q=1.60, p=0.6719,ns	q=2.82, p=0.2145,ns	q=0.60, p=0.9738,ns	q=4.40, p=0.0208	q=3.22, p=0.1281,ns
HCD + DS 180 mg/kg/day 3-wk vs. HCD + DS 180 mg/kg/day 2-wk	q=1.37, p=0.7675,ns	q=1.35, p=0.7760,ns	q=3.49, p=0.0882,ns	q=0.99, p=0.8961,ns	q=2.92, p=0.1914,ns

HCD – high cholesterol diet; ns – not significant ($p>0.05$)

Table 5. Summary of group comparisons in *Tlr4* gene expression: study with DS administration at the dose 180 mg/kg/day (see Fig.3A).

Groups	Brain structures				Liver
	Prefrontal cortex	Hippocampus	Hypothalamus	Dorsal raphe	
Control diet vs. HCD	q=11.99, p< 0.0001	q=8.99, p< 0.0001	q=5.66, p=0.0009	q=8.29, p< 0.0001	q=5.05, p=0.0122
Control diet vs. HCD + DS 180 mg/kg/day 3-wk treatment	q=1.42, p=0.7491, ns	q=1.95, p=0.5181, ns	q=1.63, p=0.6596, ns	q=1.61, p=0.6676, ns	q=0.51, p=0.9836, ns
Control diet vs. HCD + DS 180 mg/kg/day 2-wk late treatment	q=2.93, p=0.1729, ns	q=3.02, p=0.1527, ns	q=2.58, p=0.2732, ns	q=1.99, p=0.4977, ns	q=0.73, p=0.9538, ns
HCD vs. HCD + DS 180 mg/kg/day 3-wk treatment	q=13.41, p< 0.0001	q=10.94, p< 0.0001	q=7.29, p< 0.0001	q=9.89, p< 0.0001	q=5.56, p=0.0059
HCD vs. HCD + DS 180 mg/kg/day 2-wk late treatment	q=14.93, p< 0.0001	q=12.01, p< 0.0001	q=8.24, p< 0.0001	q=10.28, p< 0.0001	q=5.78, p=0.0043
HCD + DS 180 mg/kg/day 3-wk treatment vs. HCD + DS 180 mg/kg/day 2-wk late treatment	q=1.52, p=0.7078, ns	q=1.07, p=0.8723, ns	q=0.95, p=0.9081, ns	q=0.39, p=0.9929, ns	q=0.22, p=0.9985, ns

HCD – high cholesterol diet; ns – not significant (p>0.05)

Table 6. Summary of group comparisons in PPARGC1b gene expression: study with DS administration at the dose 180 mg/kg/day (see Fig.3B).

Groups	Brain structures				Liver
	Prefrontal cortex	Hippocampus	Hypothalamus	Dorsal raphe	
Control diet vs. HCD	q=11.18, p< 0.0001	q=11.09, p< 0.0001	q=11.02, p< 0.0001	q=10.81, p< 0.0001	q=6.35, p=0.0019
Control diet vs.HCD + DS 180 mg/kg/day 3-wk treatment	q=3.90, p=0.0371	q=1.81, p=0.5790,ns	q=1.09, p=0.8665,ns	q=1.58, p=0.6824,ns	q=2.15, p=0.4479,ns
Control diet vs. HCD + DS 180 mg/kg/day 2-wk late treatment	q=10.03, p< 0.0001	q=10.39, p< 0.0001	q=9.84, p< 0.0001	q=9.70, p< 0.0001	q=5.32, p=0.0083
HCD vs. HCD + DS 180 mg/kg/day 3-wk treatment	q=7.28, p< 0.0001	q=9.28, p< 0.0001	q=9.93, p< 0.0001	q=9.24, p< 0.0001	q=4.20, p=0.0408
HCD vs. HCD + DS 180 mg/kg/day 2-week late treatment	q=1.15 p=0.847,ns	q=0.70 p=0.9593,ns	q=1.19, p=0.8355,ns	q=1.11, p=0.8599, ns	q=1.03, p=0.8859,ns
HCD + DS 180 mg/kg/day 3-wk treatment vs. HCD + DS 180 mg/kg/day 2-wk late treatment	q=6.13, p=0.0003	q=8.58, p< 0.0001	q=8.75, p< 0.0001	q=8.12, p< 0.0001	q=3.17, p=0.1542,ns

HCD – high cholesterol diet; ns – not significant (p>0.05)

Table 7. Summary of group comparisons in hepatic triglyceride levels: study with DS administration at the dose 180 mg/kg/day (see Fig.3D).

Groups	
Control diet vs. HCD	q=5.94, p=0.0034
Control diet vs. HCD + DS 180 mg/kg/day 3-wk treatment	q=7.96, p=0.0002
Control diet vs. HCD + DS 180 mg/kg/day 2-wk late treatment	q=6.77, p=0.0010
HCD vs. HCD + DS 180 mg/kg/day 3-wk treatment	q=2.02, p=0.4995, ns
HCD vs. HCD + DS 180 mg/kg/day 2-wk late treatment	q=0.93, p=0.8328, ns
HCD + DS 180 mg/kg/day 3-wk treatment vs. HCD + DS 180 mg/kg/day 2-wk late treatment	q=1.19, p=0.8294, ns

HCD – high cholesterol diet; ns – not significant ($p>0.05$)

Supplementary Figures

Figure 1. Body weight and food intake during challenge with cholesterol diet and dosing with DS at the dose of 50 mg/kg/day (A). Body weight was similar between the groups receiving control diet, cholesterol diet, or cholesterol diet and dosing with DS at the dose 50 mg/kg/day. (B). Food intake was similar in mice that were fed with cholesterol diet regardless the dosing with DS, and was lower in both of these groups than in control animals. Data are expressed as means $\pm$ SEMs.

A **B**

Figure 2. Body weight and food intake during challenge with cholesterol diet and dosing with DS at the dose of 180 mg/kg/day during two or three weeks. **(A).** Body weight was similar between the groups receiving control diet, cholesterol diet, or cholesterol diet and dosing with DS at the dose 180 mg/kg/day, either for two or three weeks. **(B).** There was no difference in the food intake between the groups that were fed with cholesterol diet, regardless their dosing with DS. All three groups had lower food intake than control mice. Data are expressed as means $\pm$ SEMs.

Chapter 7

Discussion and impact

Affective pathology is a complex construct which encompasses a pathological disturbance in primary emotions, rapidly shifting from neutral to intense perception, associated to dysfunctional coping. In rodents, assessing affective pathology, translated mostly by anxiety- and depressive-like states, is challenging and requires a combined analysis of behavioural, physiological and molecular data in elaborate paradigm constructs. In this regard, we aimed to establish new, reliable and robust models, sensitive to the action of pharmacological compounds such as insulin receptor sensitizers or antidepressants.

Knowing the role of NMDA receptor-mediated neurotransmission in the aetiology of stress-related cognitive deficits and behavioural abnormalities, as well as the hypothesis of different roles of NR2A/B receptors regulating the mechanisms of learning and emotion processing, we have employed ethological stressors and

assessed their effect on building an affective pathology phenotype in mice (**Chapter 2**).

We found stress-induced increases in hippocampal expression of NR2A and NR2A/NR2B ratio, previously linked to elevated anxiety, impulsivity and aggression, home cage hyperactivity and increases in circulating corticosterone, also present in our behavioural and biochemical data. Moreover, studies have reported a link between these molecular changes and a disruption of long-term memory, but not short-term learning. In summary, our newly validated mouse model of affective pathology accurately resembles the consequences of experiencing chronic stress in humans, and proves useful for translational studies on the effect of putative pharmaca which may compensate or revert this condition.

As diverse etiological factors may contribute to a state of affective pathology, we aimed to characterize the effects of a neuronal insulin receptor sensitizer on a plurality of already established rodent models, which included chronic stress, elderly depression and naïve mice, evaluating several behavioural, molecular and sleep variables, characterized as biological correlates of a depressive state and adaptive response in mice (**Chapter 3**).

Firstly, in the model of chronic stress, pre-treatment with our compound prevented stress-induced memory impairment, possibly through augmented choline acetyltransferase activity, which increases neurogenesis and is considered to be neuroprotective; extended the duration of REM sleep, but not SWS, a mechanism described as an adaptive response to stress, that loses efficacy with ageing and in neuropsychiatric disorders; and increases hippocampal gene expression of NMDA receptor subunit NR2A, as well as NR2A/B ratio.

Secondly, the delivery of this insulin sensitizer in a model of elderly depression has resulted in the restoration of the hedonic deficit similarly to a classical tricyclic

antidepressant and gene expression profiling demonstrates an upregulation of genes encoding for factors of synaptic plasticity, regulation of sleep and circadian rhythm, known to be implicated in the pathology of depression.

Thirdly, the administration of dicholine succinate to naïve mice has reduced parameters of learned helplessness and decreased anxiety scores in behavioural tests in a similar way to established antidepressants and other thiazolidinediones, without inespecific locomotory side-effects. Furthermore, as insulin regulates GSK-3 β activity, treatment precluded the reduction of its inactive form similarly to antidepressants, possibly establishing a link to the effects of insulin receptor sensitizers and hippocampal NMDAr expression.

All of the abovementioned effects support the hypothesis of an antidepressant-like role for insulin receptor sensitizers, at different levels and in different contexts for each model. Together with the lack of signs of toxicity in mammals at effective dosage ranges, the enhancement of insulin receptor signalling may be regarded as a potential pharmacotherapeutic target.

A growing body of clinical and experimental data suggests that excessive nutrition conveyed by the so-called Western diet (*e.g.* high-cholesterol, high-fat, high-salt, high-sugar), and ensuing hypercholesterolemia, obesity and insulin resistance contribute to the pathogenesis of affective disorders and interfere with pharmacotherapy. We considered the use of this type of dietary challenge to investigate the effects of increased cholesterol intake on behaviour and molecular markers, comparing the changes observed in the periphery to those occurring in the central nervous system, and attempting to establish a link to the development affective pathology (**Chapter 4**).

For the first time, profound behavioural modifications and molecular changes were observed in the brain and liver in association with this diet, along with depression

and anxiety-like behaviour, and similar to stress-induced depression. A significant increase in *Tlr4* gene expression was found in both the hippocampus and the prefrontal cortex, albeit increased protein levels were only detectable in the prefrontal cortex. Expression levels returned to baseline values after reversion to control diet, accompanied by a normalization of anxiety- and depression-related behaviours. Unexpectedly, body weight of mice on the high-cholesterol diet remained unchanged, excluding the contribution of obesity or excessive amounts of fat. This methodology was shown to be effective in inducing dietary-associated affective changes, with prospected value in translational pharmacological and pre-clinical studies.

As a translational approximation to the human clinic and potential use of various compounds as a food supplement, we aimed to evaluate whether it would be effective to deliver compounds by an oral route in mice (**Chapter 5**). We demonstrated that the delivery of an insulin receptor sensitizer by oral route is effective to elicit antidepressant-like effects similarly to those of the antidepressant imipramine that was used as a reference drug.

Together, we have supported pharmacological delivery of dicholine succinate with food as an effective method of dosing that opens possibilities of the application of this compound with food. Apart from that, our study showed that drug delivery with pellets can be a desirable alternative to invasive dosing, which may greatly improve animal welfare, particularly with the need for repeated or chronic drug administration to physically vulnerable laboratory animals.

Lastly, we investigated the effect of oral delivery of an insulin receptor sensitizer in a non-alcoholic fat liver disease mode induced by a high-cholesterol diet, characterized by hepatic lipid dystrophy, inflammation, depressive- and anxiety-like behaviours and increased expression of TLR-4 (**Chapter 6**). Treatment with the selected compound

ameliorated diet-induced anxiety- and depressive-like behaviours, likely linked to the normalized expression of pro-inflammatory factor TLR4 and mitochondrial activity marker PPARGC1b, which were expressed abnormally high and low, respectively.

Our results suggest that the seen improvement is associated with the underlying normalization of the secondary mechanisms of NAFLD syndrome, such as inflammation and mitochondrial functions, rather than of its primary features of liver steatosis and dystrophy, since the use of this insulin sensitizer did not elicit changes on the latter. These evidences support a potential use of insulin receptor sensitizers for the treatment of affective symptoms during NAFLD syndrome.

Concluding remarks

This work pioneered to robustly use ethological stressors and high amounts of dietary cholesterol as accurate methodologies to reliably mimic cognitive and emotional abnormalities associated with human phenotypes. Combined with these models, age-induced and naïve learned helplessness were successful tools to study the effects of insulin receptor sensitization, gathering evidence which supports the potential to generate effective antidepressant-like effects in various conditions, restoring baseline levels of brain plasticity and function, memory, gene expression and normal behaviours, albeit the underlying mechanisms are not yet fully understood.

Altogether, insulin receptor sensitization has shown a sound potential in rodent models of depression of various etiologies. In line with existing literature, our data supports the vision that enhancement of insulin receptor mediated signalling may be a new promising strategy of pharmacotherapy of affective disturbances in human clinic. Thus, the use of compounds of a similar nature to the insulin receptor sensitizer dicholine succinate can be beneficial for future clinical use in a plurality of metabolic and neuropsychiatric disorders.

Valorization

Relevance for society

Diabetes and neuropsychiatric disorders are major health and economic burdens in the next decades. It is estimated that 9% of the world's adult population suffers from diabetes, and alarmingly, over 13% are obese and risking developing the disease, which accounts for 1.5 million direct deaths per year. In other hand, recent reports reveal that over 400 million adults suffer of depression and 47.5 million of elderly dementia, 60-70% directly linked to Alzheimer's disease, the most prevalent neuropsychiatric disorder, recently considered to be "brain diabetes" or a Type III diabetes.

Insulin resistance is the hallmark of diabetes and metabolic syndrome and has been associated with depression. Whereas depression increases the risk for diabetes, people with diabetes are twice as likely to experience depression when compared with the general population. A growing body of evidence in the later years and of the present work has enlightened the role of compromised brain insulin receptor signalling in the aetiology of neuropsychiatric disorders including depression.

Recently, several types of insulin receptor sensitizers have been offered as a potential remedies to tackle affective disturbances. Namely, several classes of pharmacologically active compounds have been developed lately. They include meglitinides and sulfonylureas, which increase a release of insulin by the beta islets in the pancreas; biguanides, which decreases liver gluconeogenesis and increases number of receptors recruited to the membrane and thiazolidinediones, which increase mitochondrial biogenesis and improve lipid metabolism.

Unfortunately, and albeit successful results from the application of these compounds as antidiabetic and even antidepressant agents, associated side effects may vary from

osteoporosis, cardiovascular complications, increase in body weight and food cravings, kidney failure, cancer, severe hypoglycaemia and other undesirable consequences.

In the present studies, dicholine succinate, an endogenous molecule, has been shown to elicit an antidepressant-like effect in the pre-clinical models of depressive-like state of various origins. This opens the possibilities to consider this substance as a candidate to additive pharmacotherapy of depression, in particular when this disorder is associated with metabolic syndrome. Also, it can be used during obesity as a food supplement to counteract negative effects of high amounts of dietary cholesterol and fat. It is expected that the use of dicholine succinate will be not accompanied by significant side effects that are known for the treatment with other insulin receptor sensitizers. The results in this thesis also support the view that dicholine succinate can be used during senile depression. It can be particularly useful for patients suffering from the combination of above listed syndromes that are often co-morbid. Thus, the application of dicholine succinate as a food supplement and as a drug can be of medical and economic importance.

Apart from a discovery of a potential of dicholine succinate being a candidate substance during several forms of affective pathology, our studies have contributed in the optimization and development of animal models of investigated syndromes. A model of affective pathology associated with dietary cholesterol challenge, was newly developed. Models of stress-induced and aging-related affective syndromes were optimized in terms of the use of labour and other resources. Finally, we validated oral ways of chronic dosing with antidepressants, which allows reducing costs for translational studies based on chronic dosing of animals, and significantly ameliorating animal welfare. We have probed and confirm the effectiveness of a delivery of drugs with food or drinking water, which could be particularly useful in future experiments with long protocols and on immunodeficient animals.

Target groups

In our studies, we have found beneficial effects of the applications for the insulin receptor sensitizer dicholine succinate as an antidepressant during stress, aging and metabolic conditions associated with consumption of Western-diet type of food. Therefore, we consider our target groups to be 1) patients with depression associated with obesity / metabolic syndrome, 2) patients suffering from elderly depression, with or without metabolic syndrome, 3) individuals with a risk of a development of obesity / metabolic syndrome. For the first two target groups, one can anticipate the usefulness of dicholine succinate as an adjustment therapy of depression that can increase the effect of main antidepressant treatment and allow to decrease its dose, thus, leading to a less pronounced side effects of a conventional drug.

As potential outlook of presented work, we foresee a potential interest of pharmaceutical companies to complete necessary tests and develop dicholine succinate as a formulation for pharmacotherapy of depression and associated symptoms, and / or a food supplement, like other endogenous molecules or elements (e.g., fluoride). It is likely that the use of dicholine succinate as a food supplement as for instance, would generally help to improve a public health. It is expected that the production of the compound will be relatively inexpensive and is also widely available for all parts of the population. Nowadays this could be especially important. Given the fact that a lifestyle in developed countries involves lots of stressful life events and the consumption of high-fat, salty and sweet foods is increasing, the use of dicholine succinate can be beneficial in the prevention of metabolic syndrome, obesity and associated with these disturbances depression. Also, a general tendency of a global population to age makes potential use of dicholine succinate a promising tool of prevention with pathological developments related to elderly.

From an economical point of view, filing of a patent for the use of dicholine succinate could be an option to go for. The use should be defined for conditions where dicholine succinate have shown its efficacy in pre-clinical models, such as depressive pathology associated with metabolic syndrome, senile depression and a risk of the development of these conditions.

Activity / Products

The major finding in my studies that is described in this dissertation is that the enhancement of insulin receptors by dicholine succinate protects mice from the deleterious effects of a development of affective symptoms during stress, during stress, aging and challenge with high amounts of dietary cholesterol. Also, dicholine succinate is increasing expression of genes related to neuronal plasticity and survival. As the models used have a high translational validity to what has been described in the human clinic, we hypothesize that patients could benefit from the similar properties of dicholine succinate as they are hereby described in pre-clinical experiments.

Innovation

The work hereby presented has been innovative in various regards. First, we have validated the induction of affective pathological disturbances, characterized by molecular and behavioural phenotypes, in mice models of stress-induced depression, using an optimized protocol that reduces labour and time costs (Chapter 2,3). Second, we have proposed a new model of affective changes that are resulting from a diet with high cholesterol content (Chapter 4 and 6). Third, we have validated the efficacy of chronic dosing with food and water of antidepressant drugs, that, we believe, greatly helps to optimise economic costs for experimental procedure that require such dosing, and contribute to the animal welfare. Finally, we have shown that insulin receptor signalling is a mechanism that is implicated in the affective changes

during exposure to a Western diet-like diet and that it can be targeted by insulin receptor sensitizers, such as dicholine succinate.

Implementation

In line with the abovementioned relevance for the scientific and medical communities, society and industry, the implementation of the knowledge generated in the current dissertation is also multidimensional, as already discussed. From an academic perspective, results have been or will be published in peer-reviewed international journals and presented at national and international conferences.

Biography

João Pedro da Costa Alvares Viegas Nunes was born on April 26th 1987 in Lisbon, Portugal. In 2004 he has obtained his high school degree in Natural Sciences. Driven by a fascination for biodiversity, he enrolled in a Biology course at the Science Faculty of the University of Lisbon, where an interest in behaviour and neuroscience developed, obtaining his bachelor degree in 2007. For one year, João was intern at cell cycle regulation groups at the Gulbenkian Institute for Science, Oeiras, Portugal to increase his competence in molecular biology techniques. In the fall of 2008, he started his Master courses in Human Biology and Environment, with senior internship at the lab of Dr. Tatyana Strekalova, in a joint collaboration between the University of Lisbon and the School for Mental Health and Neuroscience (MHeNS, from Maastricht University, obtaining his degree from Lisbon in May 2011 with a thesis in the field of neuropsychopharmacology entitled “Behavioural effects of chronic administration of imipramine in food and water regarding anxiety and depression paradigms on naïve C57BL/6N male mice”, co-supervised by Dr. Jodi Pawluski, from Maastricht University. Ever since, João has collaborated in multiple research projects, with affiliation to MHeNS as a member of Dr. Tatyana Strekalova. From main collaborations with the University of Oxford, UK, and Prof. Dr. Daniel C. Anthony, University of Wuerzburg, Germany, and Prof. Dr. Klaus-Peter Lesch, also a member of Maastricht University and University of Strasbourg, France, and Dr. Brandon H. Cline, João has produced quality research, published in peer-reviewed journals and scientific meeting, hereby presented, under the main supervision of Prof. Dr. Harry Steinbusch, in order to obtain his doctoral degree.

Publications

Strekalova T, **Costa-Nunes J**, Veniaminova E, Kubatiev A, Lesch KP, Chekhonin V, Evans M, Steinbusch HWM. Insulin receptor sensitizer choline salt of succinic acid prevents both TLR4 upregulation and affective changes induced by a high-cholesterol diet in mice. *J Affect Disord*. (submitted 15.09.2015, Revised 25.10.2015).

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* Authors contributed equally.

Thesis defenses from MHeNS - School for Mental Health and Neuroscience

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Rohit Shetty: **Understanding the Clinical, Immunological and Genetic Molecular Mechanisms of Keratoconus.** Supervisors: Prof.dr. R.M.M.A. Nuijts / Prof.dr. C.A.B. Webers.

Christine van der Leeuw: **Blood, bones and brains; peripheral biological endophenotypes and their structural cerebral correlates in psychotic disorder.** Supervisor: Prof.dr. J. van Os; Co-supervisor: Dr. M. Marcelis.

Sanne Peeters: **The Idle Mind Never Rests; functional brain connectivity across the psychosis continuum.** Supervisor: Prof.dr. J. van Os; Co-supervisor: dr. M. Marcelis.

Nick van Goethem: **$\alpha 7$ nicotinic acetylcholine receptors and memory processes: mechanistic and behavioral studies.** Supervisor: Prof.dr. H.W.M. Steinbusch; Co-supervisor: Dr. J. Prickaerts.

Nicole Leibold: **A Breath of fear; a translational approach into the mechanisms of panic.** Supervisor: Prof.dr. H.W.M. Steinbusch; Co-supervisors: Dr. K.R.J. Schruers / Dr. D.L.A. van den Hove.

Renske Hamel: **The course of mild cognitive impairment and the role of comorbidity.** Supervisor: Prof.dr. F.R.J. Verhey; Co-supervisors: Dr. I.H.G.B. Ramakers / Dr. P.J. Visser.

Lucia Speth: **Effects of botulinum toxin A injections and bimanual task-oriented therapy on hand functions and bimanual activities in unilateral Cerebral Palsy.** Supervisors: Prof.dr. J. Vles; Prof.dr. R. Smeets; Co-supervisor: Dr. Y. Janssen-Potten, Adelante Hoensbroek.

Yuan Tian: **The effects of Lutein on the inflammatory pathways in age-related macular degeneration (AMD).** Supervisors: Prof.dr. C. Webers; Prof.dr. A. Kijlstra, WUR; Co-supervisor: Dr. M. Spreeuwenberg; Dr. H. Tange.

Peggy Spauwen: **Cognition and Type 2 diabetes; the interplay of risk factors.** Supervisors: Prof.dr. F. Verhey; Prof.dr. C. Stehouwer; Co-supervisor: Dr. M. van Boxtel.

Marc Hilhorst: **Crescentic glomerulonephritis in ANCA associated vasculitis.** Supervisors: Prof.dr. J. Cohen-Tervaert; Co-supervisor: Dr. P. van Paassen.

Martin Gevonden: **The odd one out: exploring the nature of the association between minority status and psychosis.** Supervisors: Prof.dr. J-P. Selten; Prof.dr. J. Booij, Uva; Prof.dr. I. Myin-Germeys.

Bart Biallosterski: **Structural and functional aspects of sensory-motor Interaction in the urinary bladder.** Supervisors: Prof.dr. Ph. Van Kerrebroeck; Prof.dr. S. De Wachter, UvAntwerpen; Co-supervisors: Dr. G. van Koeveering; Dr. M. Rahnama'i.

Alexandra König: **The use of information and communication technologies (ICT) for the assessment of patients with Alzheimer's Disease and related disorders.** Supervisors: prof.dr. F. Verhey; prof.dr. Ph. Robert, Nice, Fr; Co-supervisors: dr. P. Aalten; dr. R. David, Nice, Fr.

Micheline Chenault: **Assessing Readiness for Hearing Rehabilitation.** Supervisors: prof.dr. M.P.F. Berger; prof.dr. B. Kremer; Co-supervisor: dr. L.J.C. Anteunis.

Anand Vinekar: **Retinopathy of Prematurity. Recent advances in tele-medicine screening, risk factors and spectral domain optical coherence tomography imaging.** Supervisor: prof.dr. C.A.B. Webers; Co-supervisor: dr. N.J. Bauer.

Fleur van Dooren: **Diabetes and Depression: exploring the Interface between Pathophysiological and Psychological factors.** Supervisors: prof.dr. F.R.J. Verhey; prof.dr. J.K.L. Denollet, UvT; prof.dr. F. Pouwer, UvT; Co-supervisor: dr. M.T. Schram.

Gabriëlla Pons van Dijk: **Taekwondo and physical fitness components in middle-aged healthy volunteers; the Sekwondo study.** Supervisors: prof.dr. J. Lodder; prof.dr. H. Kingma; Co-supervisor: dr. A.F. Lenssen.

Yara Pujol López: **Development and psychoneuroimmunological mechanisms in depression.** Supervisor: prof.dr. H.W.M. Steinbusch; Co-supervisors: Dr. G. Kenis; Dr. D. van den Hove; Dr. Aye Mu Myint, München.

Romina Gentier: **UBB⁺¹; an important switch in the onset of Alzheimer's disease.** Supervisors: Prof. H. Steinbusch; Prof. D. Hopkins; Co-supervisor: Dr. F. van Leeuwen.

Sanne Smeets: **Insights into insight: studies on awareness of deficits after acquired brain injury.** Supervisor: Prof. C. van Heugten; Prof. R. Ponds; Co-supervisor: Dr. I. Winkens

Kim Beerhorst: **Bone disease in chronic epilepsy: fit for a fracture.** Supervisor: Prof. A. Aldenkamp; Prof. R. van Oostenbrugge; Co-supervisor: Dr. P. Verschuure.

Alex Zwanenburg: **Cerebral and cardiac signal monitoring in fetal sheep with hypoxic-ischemic encephalopathy.** Supervisor: Prof. T. Delhaas; Prof. B. Kramer; Co-supervisors: Dr. T. Wolfs; Dr. P. Andriessen, MMC.