Article Type: Review

Effects of ketone bodies in Alzheimer's disease in relation to neural hypometabolism, β-amyloid toxicity and astrocyte function

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Abstract

Diet supplementation with ketone bodies (acetoacetate and β-hydroxybuturate) or medium-length fatty acids generating ketone bodies has consistently been found to cause modest improvement of mental function in Alzheimer's patients. It was suggested that the therapeutic effect might be more pronounced if treatment was begun at a preclinical stage of the disease

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as an 'Accepted Article', doi: 10.1111/jnc.13107

instead of well after its manifestation. The preclinical stage is characterized by decade-long glucose hypometabolism in brain, but ketone body metabolism is intact even initially after disease manifestation. One reason for the impaired glucose metabolism may be early destruction of the noradrenergic brain stem nucleus, locus coeruleus, which stimulates glucose metabolism, at least in astrocytes. These glial cells are essential in Alzheimer pathogenesis. The β-amyloid peptide Aβ interferes with their cholinergic innervation, which impairs synaptic function due to diminished astrocytic glutamate release. Aβ also reduces glucose metabolism and causes hyperexcitability. Ketone bodies are similarly used against seizures, but the effectively used concentrations are so high that they must interfere with glucose metabolism and de-novo synthesis of neurotransmitter glutamate, reducing neuronal glutamatergic signaling. The lower ketone body concentrations used in Alzheimer's disease may owe their effect to support of energy metabolism, but might also inhibit release of gliotransmitter glutamate.

Key words: Aβ; Alzheimer's disease; astrocytes; gliotransmitter; hypometabolism; subcortical nuclei

1. Introduction: Overview

A moderate improvement in Alzheimer's disease symptomatology has been seen after supplementation of the diet with relatively small amounts of ketone bodies. These amounts are much smaller than those that can be effective against seizures, and the mechanism(s) of action are probably very different. The effect of ketone body supplementation is interesting because Alzheimer's disease is preceded by decades of glucose hypometabolism, and earlier dietary intervention might have greater therapeutic effect. Moreover, the potent toxicity of the soluble oligomeric Aβ peptide is being realized and this compound is used experimentally. A previously suggested hypothesis that initial damage of subcortical nuclei is important for development of the disease is getting renewed support. While it is well known that microglia play a major role in Alzheimer's disease, it is becoming obvious that impairment of astrocytic functions is also important. These cells are essential for the pathogenesis of Alzheimer's disease, due to their large contribution to brain energy metabolism and inflammatory and anti-inflammatory events. Recent research has also shown the importance of their release of glutamate and other compounds as gliotransmitters.

2. Brain hypometabolism precedes clinical signs of Alzheimer's disease

Frackowiak et al. (1981) found a decline in cerebral blood flow and mean cerebral oxygen utilization, i.e., hypometabolism, which correlated with the severity of dementia. Global oxygen extraction ratio was not increased, providing evidence against chronic ischemia. Shortly afterwards De Leon et al. (1983) found that aged patients with senile dementia showed consistent diminutions in regional glucose use compared to elderly normal persons, also with significant correlation between hypometabolism and decreased cognitive functioning. Thirteen additional studies (till 2009) all showed glucose hypometabolism (Cunnane et al. 2011), and since then glucose hypometabolism has repeatedly been confirmed in Alzheimer's patients. In contrast, metabolism of ketone bodies is unaltered, at least in early stages of the disease (Castellano et al., 2015).

Alzheimer's patients expressing the lipoprotein ApoE4, a risk factor for the disease, show a more severe medial temporal hypometabolism than matched ApoE4-negative patients in spite of lower global amyloid burden (Lehmann et al., 2014). Brain glucose metabolism is also reduced in late-middle-aged healthy ApoE4 carriers (Reiman et al., 2005). A confounding factor is that glucose metabolism decreases in healthy aging, but the decrease is more pronounced in ApoE4 carriers than in age-matched non-carriers (Reiman et al., 2005). When this decline is taken into account, the most severe hypometabolism seems to occur in younger Alzheimer's patients. Thus, glucose hypometabolism in brain may indicate a risk for future development or worsening of dementia (Dukart et al., 2013). Consistent with this, reduced glucose metabolism can be demonstrated not only in ApoE4 carriers but also in other persons at risk for developing Alzheimer's disease decades before the onset of the disease (Mosconi et al., 2008). Since mitochondrial DNA is maternally inherited in humans, it is interesting that brain glucose metabolism is lower in elderly with a maternal family history of Alzheimer's disease than in those with a paternal family history and in controls with a negative family history of Alzheimer's disease (Mosconi et al., 2007). Furthermore, in cognitively normal persons between 32 and 72 years of age with a family history of Alzheimer's disease, brain glucose metabolism is lowest in those where both parents had suffered from the disease, intermediate in those with a maternal history of the disorder and highest in those with a paternal family history (Mosconi et al., 2014). The importance of brain energy metabolism is also indicated by the observation that reduction of blood pressure in hypertonic patients is associated with memory decline (Glodzik et al., 2014).

More detailed information about brain metabolism can be obtained by studies of the metabolic fate of ^{13}C-labeled glucose or the astrocyte-specific substrate acetate by nuclear magnetic resonance (^{13}C-NMR). Nilsen et al. (2014) performed a ^{13}C-NMR study in the transgenic McGill-R-Thy1-APP rat model of Alzheimer's disease. In frontal cortex the tricarboxylic acid (TCA) cycle turnover was reduced in both neurons (glutamatergic and GABAergic) and astrocytes. Pyruvate carboxylation, an astrocytic metabolic process, which is essential for *de novo* synthesis of transmitter glutamate and GABA via the glutamine-glutamate-GABA cycle (see legend of Figure 1), was decreased, and the level of glutamate, glutamine, GABA and aspartate was reduced in some regions. This observation is in agreement with a pronounced decrease of pyruvate carboxylase activity in brains of patients having suffered from Alzheimer's disease (Hertz et al., 2000). Nielsen et al. (2014) found the contents of glucose and of [1-^{13}C]glucose in brain to be unchanged (indicating that uptake across the blood-brain barrier was not reduced). In another rat model of Alzheimer's disease the metabolic response to forepaw stimulation as well as evoked neural activity were reduced by ~50% (Sangahalli et al., 2013). This may reflect synaptic degeneration and/or glutamate deficiency. A small ^{13}C-MRS study in Alzheimer's patients using ^{13}C labeled glucose and with no attempt to study astrocytic metabolism showed also a clear-cut reduction of neuronal TCA cycle turnover rate, which was combined with reduced formation of glutamate from glutamine (Lin et al., 2003). Another study from the same group using ^{13}C-labeled acetate in Alzheimer's patients and measuring bicarbonate (CO_2) formation correlated an increase in astrocytic TCA cycle metabolism with an increase in the presumed astrocytic marker myo-inositol and glial activation as an indication of inflammation (Sailasuta et al., 2011). This is consistent with elevated activity of beta-glucuronidase, a lysosomal enzyme, which occurs in reactive astrocytes (McGeer et al., 1989), a hallmark of Alzheimer's disease. Increased physiological activity by 'activated' astrocytes (as indicated by up-regulation of glial fibrillary acidic protein (GFAP)) has also been shown by Seidel et al. (2015). However, the 'activated' astrocytes may well be metabolically and functionally different from astrocytes in normal brain.

Glucose hypometabolism is dangerous not only because it acutely limits the metabolic capabilities of the cells, but also because it leads to oxidative stress. Oxidative stress due to glucose deprivation is well known in human tumor cells (Lee et al., 1998; Blackburn et al., 1999) and in primary cultures of rat thymocytes (Aulwurm et al., 2000). The first step of most glucose degradation is conversion to pyruvate by cytosolic glycolysis, initiated by hexokinase-catalyzed glucose phosphorylation. The hexokinase isoforms I an II are also bound to the mitochondrial membrane (Polakis et al., 1985; Henderson et al., 2006; Sun et al., 2008). The binding occurs to the Voltage-Dependent Anion Channel (VDAC), also known as mitochondrial porin, located at the outer membrane (Pastorino and Hoek, 2008). This channel is associated with the ADP/ATP carrier (adenine nucleotide translocator), which mediates the exchange of ADP and ATP through the inner mitochondrial membrane (Vyssokikh et al., 2003; Azoulay-Zohar et al., 2004). Appropriate function of VDAC is essential to prevent oxidative damage and cell death since the channel also transports reactive oxygen species (ROS) (Shoshan-Barmatz et al., 2015). Hexokinase binding to the mitochondrial membrane reduces channel conductance and ROS transport (Shoshan-Barmatz et al., 2015). Thus, besides the acute effect on energy supply, continued glucose hypometabolism and hexokinase hypoactivity may be an important inducer of oxidative stress, known to play a major role in Alzheimer's disease (Subbarao et al., 1990; Mosconi et al., 2008). However, formation of mitochondrial reactive oxygen species (ROS) is also increased by β-amyloid, which accumulates in mitochondria before its deposit as extracellular amyloid plaques (Readnower et al., 2011). Mitochondrial Ca^{2+} mobilization may play a role in this process (Camello-Almarez et al., 2006). An association between β-amyloid toxicity, mitochondrial dysfunction, oxidative stress and neuronal damage and death in Alzheimer's disease has been emphasized by Albrekkan and Kelly-Worden (2013). Recently an ATP synthase has also been recognized on the surface of neural cells which is inhibited by the β-amyloid peptide, Aβ or Abeta (Schmidt et al., 2008; Xing et al., 2012)

2. Toxicity of Aβ1-42 and related peptides

Aβ1-42 is a soluble cleavage product of Amyloid Precursor Protein (APP). APP is a family of transmembrane proteins that can be cleaved at several sites. Neurons are the principal source of APP in the normal cerebral cortex (Cole et al., 1991), but both astrocytes and microglia can rapidly synthesize APP, when stimulated by neuronal injury (Siman et al., 1989; Töpper et al., 1995). APP is most commonly cleaved by α-secretase at a site on the extracellular, N-terminal domain, close to the cell membrane, yielding a soluble, diffusible APP fragment, sAPPα, which is neuroprotective and may play a role during development (Ho et al., 1996; Barger et al., 2000). APP can also be cleaved a little further from the cell membrane by β-secretases, leading to the release of sAPPβ. Although this protein is only slightly different from sAPPα, it does not share the neuroprotective effect of sAPPα. Combined action of β-secretases with γ-secretases, which act intracellularly, generates β-amyloid peptide (Aβ) 40 or 42 together with peptides of different length (Zhang and Xu, 2007). Although it has been suggested that Aβ in the brain only becomes toxic when it precipitates from solution and forms aggregates, the soluble oligomeric polypeptide itself induces a multitude of functional abnormalities in brain tissue (Zilberter et al., 2013). Consistent with this, the research focus is shifting from amyloid plaques toward soluble oligomeric Aβ as the toxic species, which strongly correlates with the severity of dementia (McLean et al., 1999).

Aβ is toxic to primary cultures of rodent hippocampal neurons (Barger et al., 1995; Bales et al., 1998) and inhibits long-term potentiation in co-cultures (Lambert et al., 1998). Aβ1-42 is neurotoxic, although it has no effect on $[Ca^{2+}]_i$ in neurons but rapidly increases $[Ca^{2+}]_i$ in nearby astrocytes in hippocampal co-cultures (Abramov et al., 2003). This effect is dependent on extracellular Ca^{2+}, and it may be the reason for an increased rate of generation of ROS causing mainly neuronal death and depletion of glutathione, a ROS scavenger (Morris et al., 2014), in both neurons and astrocytes. Malfunctioning Ca^{2+} homeostasis in astrocytes (Lim et al., 2014) may also affect Ca^{2+}-dependent gliotransmitter release and in line with this Aβ induces glutamate release in cultured astrocytes (Talantova et al., 2013). In the additional presence of neurons the released glutamate causes extrasynaptic NMDA receptor activation and synaptic loss. Similar observations have been made in normal astrocytes of hippocampal slices, where acute exposure to Aβ1-42 acts rapidly at the α7 nicotinic cholinergic receptor, where it increases $[Ca^{2+}]_i$ and releases gliotransmitter glutamate that subsequently activates neuronal NMDA receptors (Pirttimaki et al., 2013). The same authors found that spontaneous astrocytic $[Ca^{2+}]_i$ elevations took place at increased frequency in a 3-4 month-old mouse Alzheimer model, in which synaptic or cognitive deficits had not yet developed. This indicated enhanced gliotransmission, probably due to increase in Aβ. White matter myelin and oligodendrocytes are also damaged by Aβ (Jantaratnotai et al., 2003).

A study in neuronal-astrocytic co-cultures by Abeti et al. (2011) showed a direct and rapid inhibition by Aβ of astrocytic and neuronal glucose uptake. It also demonstrated a rapid decrease in rate of oxygen consumption and mitochondrial depolarization in astrocytes. This was caused by activation of astrocytic poly(ADP-ribose) polymerase 1 (PARP-1), a nuclear DNA repair enzyme, and it was triggered by oxidative stress. These events, as well as neuronal death after 24 hrs, could all be prevented by addition of pyruvate (Abeti et al., 2011; Abeti and Duchen, 2012) and might thus be secondary to inhibited metabolism of glucose. The data obtained by Abeti and Duchen (2012) suggested that PARP-1 activation initially depletes mainly cytosolic NAD^+ which is required for glycolysis but not for oxidation of substrates that enter the TCA-cycle directly, such as β-hydroxybutyrate and pyruvate. Abnormal NAD(P)H signaling together with neuronal hyperactivity and interictal-like spontaneous synchronized discharges that began after 40 min of exposure to Aβ1-42 has also been shown in hippocampal slices (Zilberter et al., 2013). Whether or not this is enough to explain the increased incidence of seizures in Alzheimer's patients (Spencer, 2014) is unknown. In addition, Zilberter et al. (2013) showed that synaptic function, long-term potentiation and GABA-ergic activity were impaired. All effects of Aβ were prevented by medium supplementation with pyruvate (5 mM) and β-hydroxybutyrate (4 mM), concentrations that are high compared to those in blood after administration of octanoic acid in usual doses. Zilberter et al. (2013) also found that brain slices from a transgenic Alzheimer mouse model fed a standard diet showed signs of glucose hypometabolism (abnormal NAD(P)H signaling, reduced tolerance to hypoglycemia and two-fold reduction in brain glycogen level) together with some neuronal dysfunction. Dietary supplementation with *small* amounts of pyruvate or β-hydroxybutyrate (26 mg/day) for 5 weeks decreased neuronal hyper-excitability and prevented glycogen depletion. Pyruvate also prevented the development of age-dependent cognitive deficits in a mouse model of Alzheimer's disease without reducing amyloid and tau pathology (Isopi et al., 2014).

3. Subcortical nuclei regulate glucose metabolism, inflammation and release of gliotransmitter and are vulnerable in Alzheimer's disease

A contributing factor to the development of brain hypometabolism early in the prodromal stage of Alzheimer's disease may be impairment of adrenergic stimuli of brain metabolism. The brain receives its entire adrenergic stimulation from a small brain stem nucleus, locus coeruleus (Moore and Bloom, 1979). There is profound neurodegeneration and expression of neurofibrillary tangles in locus coeruleus in Alzheimer's disease (German et al., 1987; Zweig et al., 1988; Busch et al., 1997; Chan-Palay, 1991; Haglund et al., 2006; Grudzien et al., 2007; Takahashi et al., 2014). Clinical manifestations associated with this subcortical pathology unfortunately remain undetected in most pathological studies, because current NIH consensus criteria (Hyman et al., 2012) for diagnosis of Alzheimer's disease focus on cortical pathology. This is in spite of occasional suggestions during the last 25 years that Alzheimer's disease might be an anterograde degeneration originating in the brainstem and secondarily affecting the brain cortex (Hertz, 1989; Marien et al., 2004; Weinshenker, 2008; Simic et al. 2009; Chalermpalanupap et al., 2013). The most direct support for this hypothesis is a recent paper by Braak and del Tredici (2013) concluding that '*the most likely candidates for generating Aβ, in our view, are neurons of brainstem nuclei with diffuse projections to the cerebral cortex. In fact, the existence of Aβ plaques in the cerebellum (Braak et al., 1989) can only be explained by such a phenomenon, i.e., the release of A□eta via terminal axons of nerve cells with tau pathology, insofar as the neuronal types within the cerebellum itself do not develop tau-*

associated lesions'. The authors go on to explain that cerebellar neurons are well supplied with dense networks of fibers originating from brainstem nuclei, above all locus coeruleus, where abnormal tau occurs in remarkably young individuals (Braak et al., 2011; Braak and del Tredici, 2011; Elobeid et al., 2012).

Reduced noradrenergic input to the brain has important consequences. A well established reason for a detrimental effect of deficient noradrenergic innervation of cortical brain cells is interruption of normal anti-inflammatory effects in microglia and astrocytes. This topic has recently been authoritatively reviewed by Braun et al. (2014), a review which should be consulted for additional information. It concludes that an increase in intracellular levels of cAMP by noradrenergic stimulation of β-adrenergic receptors suppresses the activity of inflammatory transcription factors, causes alterations in nuclear localization of proteins, and induces gene expression via CREB activation. These effects reduce inflammatory events and contribute to neuroprotective actions by increasing expression of neurotrophic substances including BDNF, GDNF, and NGF. Their absence contributes to the progression of Alzheimer's disease.

A less known effect of noradrenergic innervation is that on energy metabolism. Besides stimulating glycogenolysis (Magistretti et al., 1981; Ververken et al., 1982; Subbarao and Hertz, 1990; Hertz et al., 2015) noradrenaline has additional effects on energy metabolism. After unilateral chemical lesion of locus coeruleus in rats the resulting noradrenaline depletion was not associated with changes in resting metabolism in cerebral cortex of anaesthetized rats (LaManna et al., 1981). However, the noradrenaline depletion slowed the transient metabolic response of both cytochrome oxidase and NAD^+ to sudden increases in energy demand produced by direct cortical electrical stimulation.

Cell culture experiments have shown that glycolysis in isolated astrocytes is stimulated by noradrenaline and the α_1-adrenergic agonist phenylephrine, but not by the α_2-adrenergic agonist clonidine. Both subtypes of α-adrenergic agonists stimulated TCA cycle activity, measured as production of labeled CO_2 from [1-^{14}C]glutamate or [1-^{14}C]pyruvate (Subbarao and Hertz, 1991; Chen and Hertz, 1999; Chen et al., 2000). The effect of α_2 agonists in these isolated cells is exerted on post-junctional receptors (Hertz et al., 2010). The effect on oxidative metabolism was less potent than the very potent effect of noradrenaline on glycolysis (Subbarao and Hertz, 1991), which therefore may be its primary target under normal conditions. These results are consistent with an increased release of lactate from the prefrontal cortex after local perfusion with noradrenaline (Takita et al., 1992) and inhibition of glucose utilization in most cortical regions by both non-selective α-adrenergic inhibitors and the α_2-adrenergic antagonist yohimbine (Savaki et al., 1982). The non-selective α-adrenergic effect is probably mediated via an increase in mitochondrial Ca^{2+}, causing a stimulation of metabolic rate of several TCA cycle enzymes including the pyruvate dehydrogenase complex (Denton, 2009). This enzyme shows a decreased activity in Alzheimer's disease (Sheu et al., 1985; Bubber et al., 2005). The effect of α_2-adrenergic inhibition in whole brain is most likely due to a reduction of adrenergic brain activity by stimulation of pre-junctional inhibitory autoreceptors, reducing stimulation of neurons and astrocytes, which both are all major targets for noradrenaline (Bekar et al., 2008; O'Donnell et al., 2012). However, with the exception of noradrenaline's effect on Na^+,K^+-ATPase activity (Hajek et al., 1996) metabolic effects of noradrenaline have to our knowledge not been demonstrated in isolated neurons, but the stimulation of Na^+,K^+-ATPase activity is likely to increase glucose utilization. Neuronal depolarization also causes increased respiration due to increased activity of Ca^{2+}-dependent intramitochondrial enzymes (Duchen, 1992), but it was not studied if noradrenaline may have a similar effect. Noradrenaline effects have also not been studied on pyruvate carboxylation in brain, but it is enhanced in liver by α-adrenergic stimulation (Garrison and Borland, 1979).

Other transmitters released from subcortical structures also include astrocytes in their targets. Recently, Chen et al. (2012) showed potentiation of neuronal responses in visual cortex to stimuli from nucleus basalis of Meynert (NBM), which was mediated by a cholinergic muscarinic increase of $[Ca^{2+}]_i$ in astrocytes. Muscarinic innervation of astrocytes and $[Ca^{2+}]_i$ response requires functional inositoltrisphosphate (IP_3) receptors (Takata et al., 2011; Chen et al., 2012). Cholinergic activation is normally associated with attention and vigilance, and Takata et al. (2011) demonstrated astrocytic involvement in cortical plasticity. They showed that combined stimulation of mouse whiskers and NBM enhanced whisker-evoked local field potential by an effect on muscarinic and N-methyl-D-aspartic acid (NMDA) receptors. The elevation of astrocytic $[Ca^{2+}]_i$ was blocked by muscarinic antagonists, and whisker plasticity could not be induced in IP_3 receptor type 2 knock-out mice. In wild-type mice NBM stimulation led to an increase in the extracellular concentration of the NMDA receptor co-agonist d-serine, and plasticity in the knock-out mice could be rescued by external supply of d-serine. Thus, stimulation of gliotransmitter release represents another important mechanism by which astrocytic stimulation of subcortical nuclei can modify function in the normal brain. This effect should be added to inhibition of inflammation and stimulation of glucose metabolism.

It has long been known that NBM is involved in Alzheimer pathology at an early stage (Saper et al., 1985; Rasool et al., 1986; Mesulam et al., 2004). Injection of Aβ into the rat NBM triggers extracellular elevation of excitatory amino acids and increase in Ca^{2+} uptake in the injected area as well as loss of cholinergic projections to neocortex (Harkany et al., 2000). Thus, Aβ causes impairment of normal cholinergic innervation by dual mechanisms, since it also leads to a direct cholinomimetic activation of cortical tissue as described by Talantova et al. (2013) and Pirttimaki et al. (2013). This makes it no wonder that augmentation of cholinergic activity by cholinesterase inhibitors has only marginal therapeutic effects in Alzheimer's disease.

4. Effects of ketogenic diet

Over the last ~10 years, a ketogenic diet has been used with some effect in patients suffering from Alzheimer's disease (see below) or Parkinson's disease (Vanitallie et al., 2005; Hashim and VanItallie, 2014). Veech et al. (2001) were the first to recommend a ketone-rich diet for treatment of these conditions; the suggested daily amount of β-hydroxybutyrate was relatively (but not extremely) high (100-150 g). Lower doses were used by the Henderson group, where Reger et al. (2004) demonstrated an acute beneficial effect of a single 40 ml oral dose of octanoate on memory in probable Alzheimer's patients with mild to moderate memory impairment (Figure 2). The positive response was restricted to patients who tested negative for APOE4, whereas APOE4-positive patients showed no effect or even a minor deterioration (in spite of the more pronounced hypometabolism in APOE4 carriers described in section 2). Less than 10% of total calories were supplied from the medium-chain fatty acid octanoate, which is metabolized via acetoacetate/β-hydroxybutyrate. The serum β-hydroxybutyrate concentration only increased to ~0.5 mM, but there was a significant correlation between the increase and the improvement of memory (Fig. 2). The effects are moderate, but earlier treatment of individuals at risk for developing the disease might have greater therapeutic impact. A second clinical trial of the same compound showed identical effects (Constantini et al., 2008). Additional studies found memory-enhancing effects after 45 and 90 days of treatment (Henderson et al., 2009) in human patients. The beneficial effects persisted 14 days after termination of the administration in a study comprising 152 patients (Henderson and Poirier, 2011), although they were reduced after the discontinuation of the drug. This study confirmed that the beneficial effect excluded APOE4 carriers, and it also showed that some single nucleotide polymorphisms in the genes of interleukin 1B (IL1B) and of the insulin degrading enzyme (IDE) played a role in determining the efficacy of the treatment. IDE degrades several short polypeptides including both insulin and Aβ (Miners et al., 2008). A commercial preparation with octanoate as the active ingredient (Axona) is now available on prescription.

Another commercial preparation (from a different company), 'Fuel for Thought', consisting of coconut oil fortified with octanoate presently undergoes a clinical trial in Alzheimer's disease. The same company has submitted a patent application for a β-hydroxybutyrate ester, which has been found capable of producing plasma ketone body levels comparable to those achieved by rigorous ketogenic diet after oral administration (Hashim and Vanitallie, 2014). Following administration of a single dose of the ketone monoester plasma levels of β-hydroxybutyrate and acetoacetate were elevated, while the intact ester was not detected (Clarke et al., 2012). A case report showing improvement in a single Alzheimer's patient treated with this preparation (~29g three times daily) has recently been published (Newport et al., 2015). The treatment was well tolerated and the patient improved markedly in daily activity performance, as evaluated by mood, affect, self-care, and cognitive ability. Noticeable improvements in conversation and interaction was found at higher compared to lower plasma levels of β-hydroxybutyrate, which reached levels of ~5 mM 1 hr after the administration but subsequently decreased rapidly (half-life 1-1.5 hr).'Virgin' (cold-pressed) coconut oil is also a good source of medium-chain fatty acids but in addition contains some higher fatty acids (mainly C-12 and C-14), (Shilling et al., 2013) but no glucose. All the fatty acids are metabolized via the ketone body acetoacetate. Unfortunately none of the fatty acids is odd-numbered. This might have improved the effect since each odd-numbered fatty acid gives rise to one molecule of succinyl CoA, which enters the TCA cycle directly, providing a new molecule of a TCA cycle intermediate. This might compensate for the reduced pyruvate carboxylase expression in Alzheimer brain. This concept is supported by the finding that supplementation of a ketogenic diet with triheptanoin, a triglyceride composed of three C-7 fatty acids, reduces the memory impairment in an Alzheimer mouse model and decreases astrocytic inflammatory responses (Aso et al., 2013).

None of these preparations 'cures' the neurodegenerative disease, but also Vanitallie (2013) pointed out that best results can be expected by early treatment before any neurodegeneration has occurred. Didic et al. (2011) similarly emphasized the importance of early diagnosis of Alzheimer's disease, because development of effective therapeutic agents requires reliable identification of patients when neuropathological changes in the brain are minimal. Although cognitive deficit, initially as isolated loss of ability to remember recent events (episodic memory) is generally the initial event leading to assessment of patients for Alzheimer's disease this can be a difficult task. This is because normal aging and mild cognitive impairment (MCI) also affect memory, but it may be possible to reveal differences from Alzheimer's disease in these conditions by psychological testing (Koen and Yonelinas, 2014). Moreover, biomarkers for Alzheimer's disease (Aβ1-42, tau, phosphorylated tau in cerebrospinal fluid; expression of specific genes) are becoming of increased value and this field is constantly developing with the aim of enabling non-invasive and early detection of the disease (Humpel and Hochstrasser, 2011).

Since cognitive impairment is the catastrophic result of Alzheimer's disease, learning experiments in animals are of importance for evaluating the influence of ketone bodies on memory establishment and its mechanisms. In a mouse model of Alzheimer's disease, Kashiwaya et al. (2013) found that a diet where 21.5% of the calories had been switched from carbohydrate to a β-hydroxybutyrate ester improved performance in some conventional memory tests. Other learning experiments have been carried out after exposure to oligomeric Aβ1-42. It inhibits learning when injected intracerebrally in mice (Mishra et al., 2013) or into the avian equivalent of the cerebral cortex in day-old chickens, 45 min before training (Gibbs et al., 2009, 2010).

During learning in the day-old chicken neuronal glucose uptake and metabolism is essential immediately after training, whereas astrocytic glucose uptake is needed 30 min later (Gibbs et al., 2008). In addition astrocytic pyruvate carboxylation also occurs immediately after training and is required for formation of glutamate, which is indispensable for learning (Gibbs et al., 2005). Based on this knowledge, Gibbs et al. (2009) investigated whether inhibition of learning caused by administration of Aβ1-42 to the avian equivalent of the mammalian cortex 45 min before training could be reversed by addition of different metabolic substrates (glucose, lactate, octanoate, β-hydroxybutyrate and the astrocyte-specific substrate acetate). None of them had any effect when injected before the neuronal glucose demand immediately after training.

However, memory loss after injection of Aβ1-42 was prevented when almost any of the metabolic substrates tested was injected 20 min post-training. This is illustrated in Fig. 3A for acetate, a further indication that the provision of metabolic substrate rescued learning by an effect on astrocytes. This effect must be exerted on energy metabolism, not pyruvate carboxylation (see Fig. 1 and its legend), since β-hydroxybutyrate was effective and since lactate had no effect immediately after training. The only substrate that did not rescue memory after Aβ1-42 injection in the Gibbs experiments was glucose (Fig. 3B), which was interpreted as an inhibition of either glucose uptake or glycolysis by Aβ.

It has often been concluded that insulin signaling is impaired in Alzheimer brain (e.g., Hoyer, 1991, 1998). However, insulin did not counteract the inhibition of learning in day-old chicken by Aβ1-42 (Fig. 3B), although it can rescue learning impaired by other means (Gibbs et al., 2009, 2010). This might be related to the absence of the insulin-sensitive glucose transporter GLUT-4 in chicken, although another insulin-stimulated transporter, GLUT-8 is present in chicken brain (Seki et al., 2003). An ability of increased glucose supply to cause some improvement of memory in Alzheimer's patients is contingent upon appropriate insulin function, but it is not known if this is due to a neuronal or astrocytic effect (Watson and Craft, 2004). That the memory-enhancing effect is exerted on the brain is shown by the observation that intranasal administration of insulin in patients with mild cognitive impairment or Alzheimer's disease causes improvements in memory tasks and glucose metabolism (Hölscher, 2014), although adult brain expresses little GLUT4 (Vannucci et al., 2000).

5. Potential mechanism(s) of action of ketogenic diets: supplementation of energy production or inhibition of glucose-dependent gliotransmitter function

The ketone bodies acetoacetate and β-hydroxybutyrate are of major nutritional importance in brain neonatally when their plasma concentrations are high and the blood-brain barrier expression of monocarboxylate transporters (MCTs) is substantial (Cremer et al., 1976). In adults their blood concentrations are normally ≤0.5 mM (Laffel, 1999), i.e., 10 times lower than that of glucose, and the expression of their transporter into brain is greatly reduced. Nevertheless, ketone bodies at saturating concentrations can cover up to 60% of brain metabolism in the slightly anesthetized rat or normal human (but total metabolism in the deeply anesthetized animal), whereas the remaining 40% must be supplied by glucose (Owen et al., 1967; Chowdhury et al., 2014). Moreover, although cultured glutamatergic neurons metabolize both glucose and lactate avidly, only utilization of glucose, not that of lactate, is increased during NMDA-induced synaptic activity (Bak et al., 2006). Therefore a very high intake of ketone bodies combined with reduced glucose intake may inhibit some aspects of brain function. On the other hand, less drastic supplementation of the diet with ketone bodies can support metabolism, including brain metabolism. The simplicity of ketone body metabolism, compared to that of glucose may be important for this function.

Fatty acids are metabolized in the liver to the ketone body acetoacetate, which is carried in blood, mainly after reduction to β-hydroxybutyrate, to the cells where it is used. Acetoacetate is metabolized intramitochondrially to two molecules acetyl coenzyme A, which enter the TCA cycle directly. β-Hydroxybutyrate is oxidized to acetoacetate, generating one molecule of NADH. This is similar to lactate oxidation to pyruvate with the exception that lactate oxidation occurs in the cytosol and competes with an oxidation occurring during glycolysis. In contrast to the simple metabolism of ketone bodies, glucose must first be converted in the cytosol to pyruvate by glycolysis (Fig. 1). This is a complicated process. One oxidation in the cytosol necessitates cytosolic-mitochondrial transport of reducing equivalents in the malate-aspartate shuttle. Oxidation of acetoacetate in the TCA cycle via acetyl CoA requires the presence of OAA, which can be deficient when glucose metabolism is severely inhibited, e.g., in diabetes. If that is the case ketone body accumulation leads to acidosis. Glucose metabolism is not compromised in Alzheimer's disease to a degree inhibiting ketone body metabolism as seen by the rapid decline in plasma β-hydroxybutyrate in the case study by Newport et al. (2014).

A strict 4:1 diet (grams of fat versus grams of protein plus carbohydrate) has shown therapeutic effect in some epileptic children (Vining et al., 1998). A less restrictive more palatable diet based on medium-chain fatty acid triglycerides as well as a modified Atkins diet (MAD) may also have some anti-epileptic effect (Liu and Wang, 2013; Sharma and Jain, 2014). However, additional seizure control can be obtained by switching from MAD to the classical 4:1 ketogenic diet (Kossoff et al., 2010). β-Hydroxybutyrate levels in blood correlate with seizure control, and children with levels above 4 mM are significantly more likely to show decreased seizure frequency than those with lower levels (Gilbert et al., 2000).

Considering that fat oxidation provides twice the amount of calories per g as oxidation of glucose or amino acids, the strict ketogenic diet is extremely low in glucose, which might be a reason for its therapeutic effect. Such a profound reduction of carbohydrates may deliver insufficient glucose to maintain brain functions dependent upon a certain utilization of glucose. This includes provision of the main excitatory transmitter glutamate, which is essential for conscious brain function, including learning (Gibbs and Hertz, 2005; Gibbs et al., 2007). Synthesis of glutamate occurs via the glutamine-glutamate cycle. The flux within this extremely active cycle amounts to 75% of total cortical glucose utilization (Sibson et al., 1998; Rothman et al., 2011). It is initiated by production of glutamate from glucose via the TCA cycle constituent α-ketoglutarate. This occurs in astrocytes but not in neurons, because neurons lack an enzyme, pyruvate carboxylase, required for net synthesis of TCA cycle constituents (Fig. 1). At least some glucose is required for the synthesis of glutamate. This is partly because the glutamate generated in glutamatergic neurons from glutamine is released into the mitochondrial matrix and depends upon transport in a 'pseudo-malate-aspartate-cycle' to reach the cytosol (Palaiologos et al., 1989; Bak et al., 2008). The function of this cycle requires a concomitant oxidative process in the cytosol, generally pyruvate formation (reviewed by Hertz, 2013; Verkhratsky et al., 2015). The energy derived from this glucose oxidation is not utilized in the glutamine-glutamate cycle but might be used for energizing synaptic activity, which requires glucose metabolism (Bak et al., 2006). This could be because the neuronal vesicular glutamate transporter, VGLUT requires glucose for optimum function (Ikemoto et al., 2003). The 4:1 ketogenic diet may therefore prevent or reduce seizures by compromising formation of the excitatory neurotransmitter glutamate. It is consistent with this hypothesis that replacement of glucose in the medium with β-hydroxybutyrate reduces availability of transmitter glutamate in cultured neurons (Lund et al., 2009). The inhibitory transmitter GABA is synthesized via astrocyte-generated glutamate which is also carried to neurons in the glutamine-glutamate (GABA) cycle. However, GABAergic activity may be better maintained if cycle activity is impaired, because a fraction of precursor glutamate is formed directly from glutamine without entrance into mitochondria and decarboxylated to GABA (the direct pathway). Another fraction undergoes transamination to α-ketoglutarate in the same way as in glutamatergic neurons (the indirect pathway), although with some added complexity (Waagepetersen et al, 2001; Walls et al 2010; 2011; Leke et al., 2011). Both pathways contribute to vesicular GABA and are involved in determining the GABAergic tone. Moreover released GABA is to a considerable extent re-accumulated directly into GABAergic neurons (Schousboe et al., 2013; see, however also Patel et al. 2015), whereas almost all released glutamate is taken up by astrocytes (Danbolt, 2001; Zhou and Danbolt, 2013) and returned to neurons in the glutamine-glutamate/GABA cycle, again requiring concomitant glycolysis. Thus, inhibition of glutamate production during anti-epileptic treatment with ketone bodies would not be accompanied by a sim ilar decrease in formation of the inhibitory transmitter GABA.

Some glutamate is also utilized in astrocytes, e.g., as a gliotransmitter released in response to receptor activation or for synthesis of glutathione. This glutamate is formed in a similar manner as astrocytic glutamate used for production of neurotransmitter glutamate and GABA, but obviously no glutamine-glutamate/GABA cycling is involved. The release of glutamate from astrocytes is orders of magnitude smaller than that from glutamatergic neurons (Vardjan et al., 2014), but it is important because the activated glutamate receptors are not located postsynaptically. This matters because stimulation of extrasynaptic glutamate receptors may jeopardize neuronal viability (Molokanova et al., 2014).

Whether astrocyte dysfunction and hypometabolism (Abeti et al., 2011) contribute to the neuronal malfunction in Aβ-treated hippocampal slices observed by Zilberter et al. (2013) is unknown. A potentially decreased metabolism may seem in disagreement with the maintained or increased astrocytic energy metabolism found by Sailasuta et al. (2011) in Alzheimer's patients. However, it should be recalled that this study dealt with metabolism of acetate and therefore provides no information about glucose metabolism. Glucose metabolism would be especially sensitive to damage induced by increased PARP-1- (Abeti et al., 2011; Abeti and Duchen 2012). Unfortunately, no study seems to specifically show glucose metabolism in astrocytes of Alzheimer's patients or in animal models of the disease.

The astrocyte-specific rescue of Aβ1-42-learning shown in Fig. 3A by β-hydroxybutyrate, pyruvate or acetate could be consistent with the astrocyte-selective PARP-1 effect. However, preliminary experiments by D. Song, L. Hertz and L. Peng also confirmed effects on ATP synthase by Aβ (Schmidt et al., 2008; Xing et al., 2012) in cultured astrocytes which differed when the substrate was glucose and when it was pyruvate or β-hydroxybutyrate. Nevertheless, there is also the possibility that the rescue might be due to prevention of gliotransmitter release, shown to be deleterious in the presence of Aβ by Talantova et al. (2013) and Pirttimaki et al. (2013). The reason for this is that in the presence of glucose in the medium addition of lactate or glutamate (substrates that like β-hydroxybutyrate enter the TCA cycle directly) causes a rapid decrease in intracellular glucose concentration in astrocytes (Prebil et al., 2011). As already mentioned, glucose is essential for the vesicular glutamate transporter, which is also operating in astrocytes, and a drastic decrease in glucose concentration might reduce or abolish release of gliotransmitter glutamate and ensuing excitatory events. Aβ-induced transmitter release from microglia (Wu et al., 2004) might be affected in a similar manner and reduce inflammation, since transmitter release from microglia also can be vesicular (Imura et 2013).

6. Concluding remarks

Alzheimer's disease as well as some forms of epilepsy can be treated more or less effectively with ketogenic diet. In epilepsy, it appears advantageous to administer the highest possible fraction of the patients' calorie need in the form of ketone bodies, which may reduce glucose metabolism sufficiently to impair glutamate production in neurons. Much lower doses of ketone bodies can have therapeutic effect in Alzheimer's disease by different mechanisms. Enabling ketone bodies to supply a fraction of needed ATP may partly compensate for the deficiency in glucose metabolism in Alzheimer's patients. An alternative mechanism of action could be to prevent or reduce gliotransmitter release of glutamate. Stimulation from subcortical nuclei can induce gliotransmitter release besides decreasing inflammation and enhancing metabolism. The early destruction of these nuclei has up till now provided little or no impetus for drug development, but the recent conclusions regarding their importance by Heiko Braak, a giant in Alzheimer research, may hopefully lead to a change.

Acknowledgements: The support of the Danish Council for Independent Research: Medical Sciences (FSS 11-108039) to Helle S. Waagepetersen is gratefully acknowledged. The authors have no conflicts of interest to declare.

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Figure legends

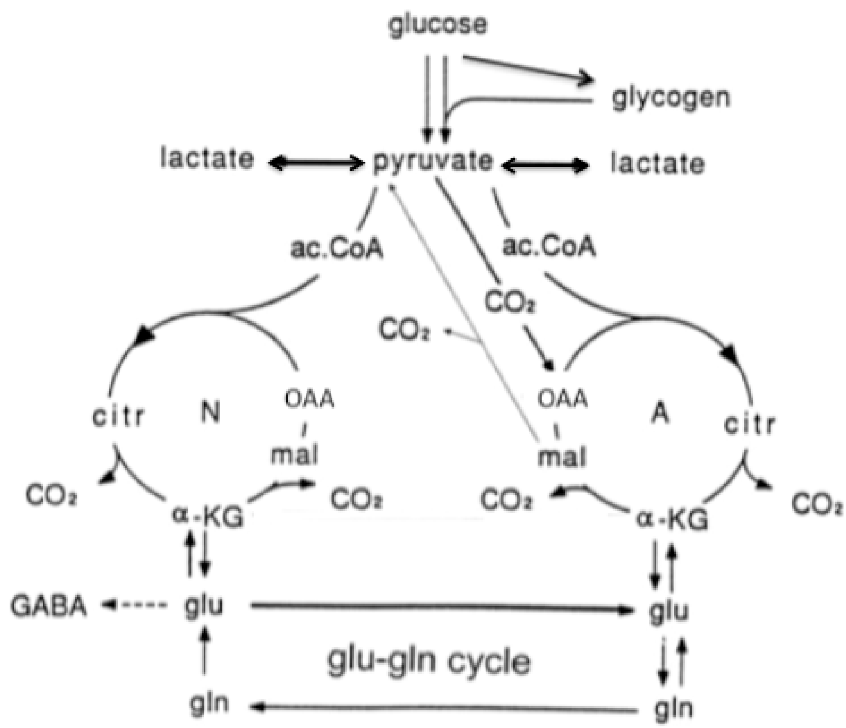
FIGURE 1. Cartoon of glucose metabolism via pyruvate in neurons (left - N) and astrocytes (right - A) and of glutamine–glutamate (GABA) cycling. One molecule glucose is metabolized by glycolysis in the cytosol to two molecules of pyruvate in a complex and strictly regulated pathway, where one oxidative process requires transfer of reducing equivalents to the mitochondria (see, e.g., Hertz and Dienel, 2002). In both neurons and astrocytes pyruvate metabolism via acetyl Coenzyme A (ac.CoA) leads to formation of citrate by condensation with pre-existing oxaloacetate (OAA) in the tricarboxylic acid (TCA), an end-result of the previous turn of the cycle. Citrate oxidation in the TCA cycle includes two decarboxylations, leading to re-formation of oxaloacetate, ready for another turn of the cycle, and to reduction of NADH, leading to large amounts of energy (ATP) via re-oxidation in the electron transport chain. Pyruvate carboxylation, which is active in astrocytes, but absent in neurons (reviewed in Hertz 2013), creates a new molecule of oxaloacetate, which after condensation with acetyl Coenzyme A, derived from a second molecule of pyruvate, forms a new molecule of citrate. α-Ketoglutarate (α-KG), one of the intermediates of the TCA cycle can leave the cycle to form glutamate (glu), catalyzed by either aspartate aminotransferase or glutamate dehydrogenase. Further metabolism by the cytosolic and astrocyte-specific enzyme glutamine synthetase leads to the formation of

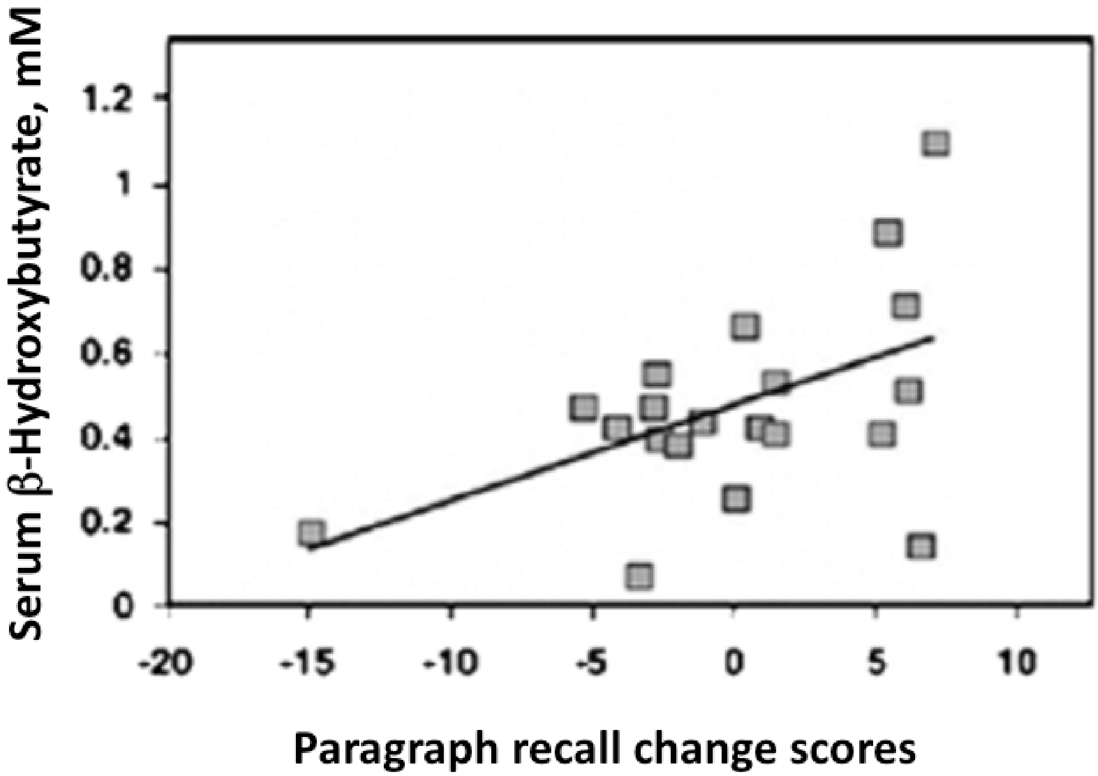
glutamine (gln). In glutamatergic neurons all glutamate formed by deamidation of glutamine enters the mitochondria (not shown) and is returned to the cytosol in a complex process, which requires simultaneous glucose metabolism. In GABAergic neurons this is only the case for some of the glutamate, whereas the remainder enters the cytosol directly. This difference may make GABA production less sensitive to replacement of glucose as the substrate with β-hydroxybutyrate in patients receiving very high amounts of ketone bodies to prevent seizures. After release from astrocytes glutamine is accumulated in glutamatergic and GABAergic neurons [lower line of the glutamine–glutamate(GABA) cycle (glu–gln cycle], converted to glutamate (and in GABAergic cells onward to GABA) and released as transmitter. Released glutamate is almost quantitatively re-accumulated in astrocytes, together with at least part of the released GABA [upper line of the glutamine–glutamate (GABA) cycle (glu–gln cycle)] and re-accumulated in the astrocytic cytosol. Here, about 75% is converted to glutamine and re-enters the glutamine–glutamate (GABA) cycle. The remaining ~25% is oxidatively degraded, via one of two partly different pathways. In both α-ketoglutarate is re-converted to malate. In one malate exits to the cytosol, is decarboxylated by cytosolic malic enzyme to pyruvate, which is oxidized in the TCA cycle via acetyl Coenzyme A. In the other malate does not exit the TCA cycle but may be further metabolized to α-ketoglutarate after condensation with acetyl Coenzyme A, allowing re-synthesis of another molecule of glutamate from only one molecule pyruvate. In either case the degraded glutamate must *in the long term* be replaced by a quantitatively similar production of glutamate from glucose, in the first case by complete de novo synthesis from one molecule glucose, in the second from one half of a glucose molecule. However, temporary fluctuations in the content of glutamate occur. The initial part of GABA metabolism is different, since all GABA is metabolized via succinate and α-ketoglutarate to glutamate. From Hertz, 2013.

FIGURE 2. Hydroxybutyrate levels at the time of cognitive testing and change in paragraph recall following acute treatment of Alzheimer's patients with octanoic acid. Each subjects received 40 ml of an octanoate triglyceride preparation or placebo 90 min before the cognitive test and the determination of plasma β-hydroxybutyrate. $r = 0.50$. $P=0.02$. From Reger et al., 2004.

Fig. 3. Ability of acetate, but not of glucose or insulin to restore learning after its inhibition by Aβ 1-42. A. Effect of different doses of acetate injected at 20 min after training in day-old chicks treated with 10 pmol/hemisphere of freshly prepared Aβ1–42 forty-five min before aversive training, carried out by briefly exposing the chicks to beads of two colors, of which one but not the other was tainted with a drug of aversive taste. The ability to remember association between color and aversive or non-aversive taste of an offered bead was determined 120 min post-training as the discrimination ratio between pecks on a bead of the previously aversive and the previously non-aversive color, of which neither was tainted. Perfect learning would result in a discrimination ratio of 1, and total inhibition would result in a ratio of 0.5. Normal learning generally results in a ratio of ~0.9, similar to that seen after administration of acetate. Results are means ± SEM for group sizes of 15–16, $*p < 0.005$ for difference from control (saline injection). Lactate, β-hydroxybutyrate and octanoic acid had similar effect as the high does of acetate (not shown). B. Inability of glucose or insulin to restore learning in the Aβ-treated chickens, although both could restore memory impairment after weakly reinforced training, i.e. training on a diluted aversant (Glucose or Insulin). Group sizes of 15–16, $*p < 0.01$ for effect of

Aβ. From Gibbs et al., 2009.




Serum β-Hydroxybutyrate, mM
1.2
1
0.8
0.6
0.4
0.2
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-20
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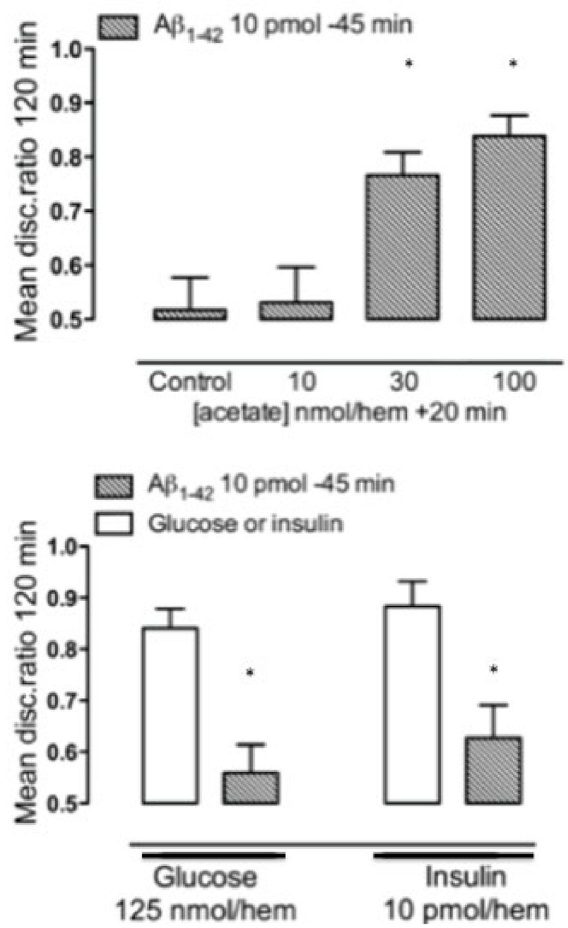
5
10
Paragraph recall change scores


Aβ1-42 10 pmol -45 min
Mean disc.ratio 120 min
1.0
0.9
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Control
10
30
100
[acetate] nmol/hem +20 min
Aβ1-42 10 pmol -45 min
Glucose or insulin
Mean disc.ratio 120 min
1.0
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Glucose
125 nmol/hem
Insulin
10 pmol/hem