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# S100A9 protein aggregates boost hippocampal glutamate modifying monoaminergic neurochemistry: a glutamate antibody sensitive outcome on Alzheimer-like memory decline

Marina A. Gruden[1], Tatiana V. Davydova[2], Vladimir S. Kudrin[3], Chao Wang[4], Victor B. Narkevich[3], Ludmilla A. Morozova-Roche[4], Robert D. E. Sewell*[5]

[1]P. K. Anokhin Research Institute of Normal Physiology, Moscow**,** 125315 Russia.

[2]Research Institute of General Pathology and Pathophysiology, Moscow**,** 125315 Russia.

[3]V. V. Zakusov Research Institute of Pharmacology, Moscow, 125315 Russia.

[4]Department of Medical Biochemistry and Biophysics, Umeå University, Umeå, SE-90187, Sweden.

[5]Cardiff School of Pharmacy and Pharmaceutical Sciences, Cardiff University, Cardiff, CF10 3NB, U.K.

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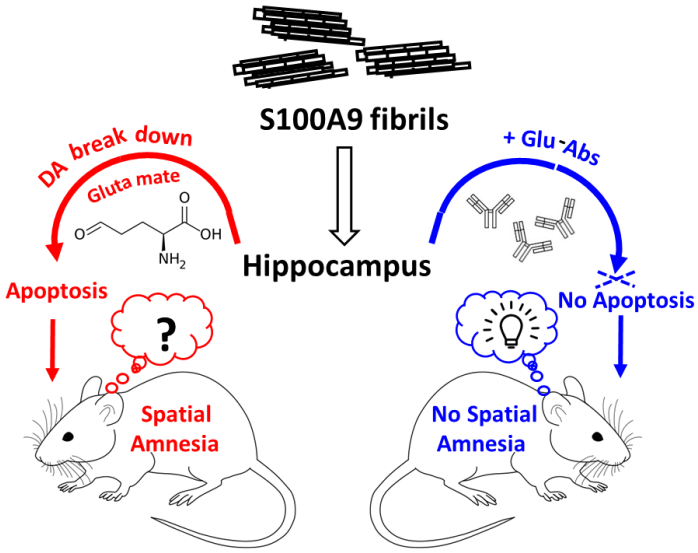
## ABSTRACT

Alzheimer's disease (AD) involves dementia conceivably arising from integrated inflammatory processes, amyloidogenesis and neuronal apoptosis. Glutamate can also cause neuronal death via excitotoxicity and this is similarly implicated in some neurological diseases. The aim was to examine treatment with *in vitro* generated pro-inflammatory protein S100A9 aggregate species alone or with glutamate antibodies (Glu-Abs) on Morris water maze (MWM) spatial learning and memory performance in 12-month old mice. Amino acid and monoamine cerebral neurotransmitter metabolic changes were concurrently monitored. Initially, S100A9 fibrils were morphologically verified by atomic force microscopy and Thioflavin T assay. They were then administered intranasally alone or with Glu-Abs for 14 days followed by a 5-day MWM protocol before hippocampal and prefrontal cortical neurochemical analysis. S100A9 aggregates evoked spatial amnesia which correlated with disrupted glutamate and dopaminergic neurochemistry. Hippocampal glutamate release, elevation of DOPAC and HVA, as well as DOPAC/DA and HVA/DA ratios were subsequently reduced by Glu-Abs which simultaneously prevented the spatial memory deficit. The present outcomes emphasized the pathogenic nature of S100A9 fibrillar aggregates in causing spatial memory amnesia associated with enhanced hippocampal glutamate release and DA-ergic disruption in the aging brain. This finding might be exploited during dementia management through a neuroprotective strategy.

## INTRODUCTION

Spatial memory deficits have been suggested as specific pathophysiological clinical indicators of Alzheimer's disease (AD) dementia[1] and also as markers in AD animal models[2]. However, the altered molecular mechanisms of AD-like memory impairment remain inconclusive. It has been reported that in AD neurodegenerative conditions, there are contributory processes such as neuroinflammation, oxidative stress and amyloidogenesis which incite memory deficits[3]. The amyloid concept primarily assumes that proteins which undergo aberrant physiological folding, generate oligomeric and fibrillar species capable of inducing a decline in synaptic plasticity, programmed cell death (apoptosis) and perturbation of neuronal networks thereby instigating cognitive dysfunction[4]. Recent evidence indicates that the "amyloid cascade hypothesis" alone cannot completely account for the neuronal damage observed in AD, as demonstrated both by autopsy and imaging studies[5]. Furthermore, neuroinflammation is implicated in this neurodegenerative disease, although debate is ongoing concerning its precise role[6]. Whilst the memory system is affected during neurodegeneration, a growing number of studies have focused on the early identification of integrative molecular processes underlying cognitive insufficiency[1]. Thus, we have investigated a combination of neuroinflammatory signals and misfolded protein assemblies with respect to altered neurochemistry[7] which may lead to memory failure in AD. Regarding the amyloid-neuroinflammatory cascade, there is one candidate, the pro-inflammatory calcium binding protein S100A9, which has been reported to be elevated in several inflammatory conditions, including AD[5,8]. Moreover, due to its inherent amyloidogenicity, S100A9 has a propensity to amyloid plaque formation along with β amyloid (Aβ) peptide. Accordingly, an increased Aβ aggregate load in AD, accompanied by S100A9 inclusion, substantiates a potential link between amyloidogenesis and inflammation-related neurodegeneration. *In vitro*, S100A9 forms neurotoxic linear and annular amyloid structures resembling Aβ protofilaments[9]. Thus, S100A9 amyloid cytotoxicity and native S100A9 pro-inflammatory signaling may be exacerbated by its co-aggregation with Aβ. In addition, S100A9 has been observed in both hippocampal and cortical neurons in AD dementia and non-demented aging[5]. Recently, we have described outcomes of the dual pro-inflammatory and amyloidogenic properties of S100A9 in the passive avoidance memory task conducted alongside neurochemical assays in cortical and hippocampal structures in aged mice. In consequence, *in vitro* generated S100A9 oligomers and fibrils both displayed amnesic activity which correlated with disrupted prefrontal cortical and hippocampal dopaminergic adaptations. Additionally, it was confirmed that intranasal administration of S100A9 aggregates was devoid of any anxiety-like behavioral upshot or any motor deficits in an open-field environment[7]. These results provide insight into a novel pathogenetic mechanism underlying

amnesia in a fear-aggravated memory task based on amyloidogenesis of a pro-inflammatory factor in turn leading to disrupted brain neurochemistry. The data further suggests that amyloid species of S100A9 create deleterious effects principally on the dopaminergic system and this finding might be exploited during dementia management through a neuroprotective strategy.

While the amyloid cascade and its involvement in amnesia is under extensive study, the activation of specific neurochemical circuits still needs scrutiny. As long ago as 1984, the concentrations of free neurotransmitter amino acids (taurine, glutamate and GABA) were reported to be lowered in post mortem temporal cortex from sufferers of Alzheimer-type dementia[10]. However, it has also been reported that excessive release of glutamate is a key contributor to neuronal damage in several neurological diseases[11]. Glutamate is a ubiquitous excitatory neurotransmitter in the mammalian CNS and it plays an important role both in physiological and pathological brain function[12]. It has also been reported that glutamate can regulate molecular and cellular processes such as neurogenesis, neurite outgrowth, synaptogenesis and apoptosis[13]. Moreover, it has been established that some of the most important brain functions, including learning and memory depend on the release of synaptic glutamate[12]. Elevated extracellular glutamate levels can cause neuronal death and this phenomenon, termed “excitotoxicity”, is involved in many neurological diseases where there is disruption of CNS normal activity[14].

Glutamate neurotoxicity arises from glutamate binding to NMDA receptors (NMDARs) and other receptor subtypes and this process depends on neuronal $Ca^{2+}$ overloading[15]. Additionally, essential molecules participating in NMDAR signalling at different subcellular locations have been proposed as crucial in activating pathways leading to neuroprotection versus neurodestruction[12]. It has also been established that glutamate is not only dependent on calcium homeostasis, but also on mitochondrial function[16]. Characteristically, in AD-like neurodegeneration, Aβ compromises neurons in the magnocellular nucleus basalis via an excitotoxic pathway entailing astroglial depolarization, extracellular glutamate accumulation, NMDA receptor activation and a subsequent intracellular $Ca^{2+}$ overload leading to cell death[17]. Deficits in glutamate neurotransmission and mitochondrial function have been detected in the frontal cortex and hippocampus of aged 3×Tg-Alzheimer's disease mice and it was suggested that impairment of mitochondrial bioenergetics might sustain failure in energy-requiring glutamatergic transmission[16].

Mechanisms of brain cell protection against glutamate toxicity are notionally useful against neurodegenerative conditions and immunoprotection is one practical approach. Previously, we unveiled antiamnestic efficacy of glutamate antibodies (Glu-Abs) following administration of the neurotoxic amyloidogenic fragment-$A\beta_{25-35}$ into the nucleus basalis of Meynert which led to

murine long-term memory impairment. Hence, a single intranasal treatment with Glu-Abs 1 h after neurotoxic damage in these animals restored learning capacity in the conditioned passive avoidance paradigm. In addition, in these experimental conditions, Glu-Abs reduced caspase-3 activity in the prefrontal cortex and hippocampus reflecting a decrement in apoptotic signal[18]. Consequently, the aim of this study was primarily to investigate the effects of intranasal treatment with *in vitro* generated S100A9 fibrils alone and in combination with Glu-Abs on performance in the Morris water maze spatial learning and memory paradigm in aged mice. Concomitantly, hippocampal and prefrontal cortical monoamine and amino acid cerebral neurotransmitter metabolic changes were measured.

## RESULTS AND DISCUSSION

### *Characterization of S100A9 fibrillar aggregates*

S100A9 fibrillar structures were developed after 24h of incubation under the protocol conditions. S100A9 fibrils were characterized by curved and coiled morphology as shown in the AFM height image (Fig. 1A).

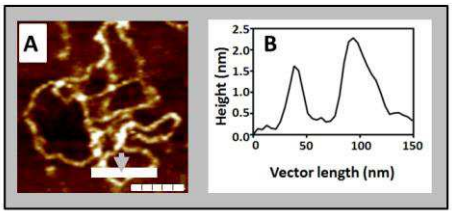



***Figure 1.*** ***In vitro characterization of S100A9 amyloid fibrils***

A. AFM height image of S100A9 amyloid fibrils formed after 24 h of incubation.

B. AFM cross-sectional analysis of representative fibril.

Lower white scale bar = 100 nm and AFM cross-section of amyloid fibrils is indicated by grey arrowed bar in A.

They reached a few hundred nanometers in length and due to their curved nature they formed encircled structures (Fig. 1A). Some oligomeric species, shown as round-shaped structures in the AFM image, were also present in the sample, but only in a minor quantity. S100A9 fibrils were thin with 1.5 nm to 2 nm AFM heights as shown in the AFM cross-section in Fig. 1B. The fibrillar sample was also characterized by ca. 5.6-fold increase in Thioflavin-T fluorescence and by reactivity with A11 antibodies[19] which further confirmed their amyloid character.

***Morris water maze amnestic effect of intranasal S100A9 fibrils reversed by glutamate antibodies in aged mice.***

Daily intranasal treatment with saline combined with MWM training induced long-term spatial memory formation in 12-month old mice. This was reflected by a steady decline in mean escape latency (the time to locate the platform) from 120.0 ± 0.0 s on the first training day to 57.9 ± 16.6 s on the last test day (Fig. 2).

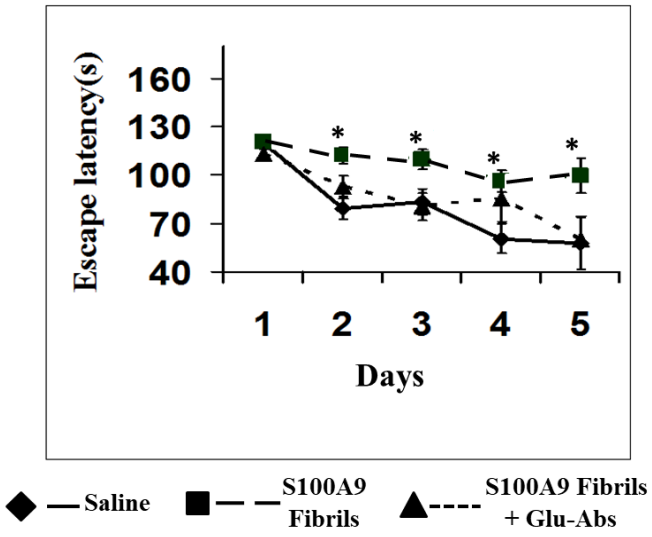



***Figure 2.*** *Behavioral effects of intranasal dosing with S100A9 fibrillar species in the presence or absence of Glu-Ab co-treatment in 12-month old C57Bl/6 mice on escape latency (s) in the MWM paradigm involving 4 training days (protocal days 15-18) and testing on the 5th day (protocol day 19) in the absence of the platform.*

*P< 0.05 compared with control.

In contrast, administration of S100A9 fibrils over 14 days significantly (P<0.005) impaired memory formation and produced an amnestic effect manifested by an increased escape latency on all days in comparison with the saline control treated group (P<0.05). An intranasal combination of glutamate antibodies plus daily dosing with S100A9 aggregates reversed the memory deficit produced by S100A9 fibrillar species to a level that was comparable to the saline treated control group during the training period and the test day (Fig. 2 and 3).

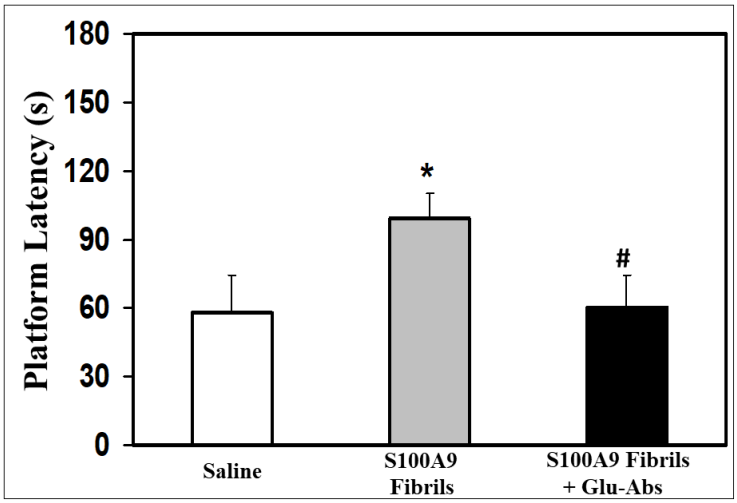



***Figure 3.*** *Behavioral effects of intranasal dosing with S100A9 fibrillar species in the presence or absence of Glu-Ab co-treatment in 12-month old C57Bl/6 mice on platform latency (s) in the MWM paradigm testing on protocol day 19.*

*P< 0.05 compared with control; #P< 0.05 compared with the S100A9 fibril treatment group

Thus, S100A9 fibrils significantly impaired acquisition of MWM spatial memory performance thereby producing amnesia. Interestingly, similar to their passive avoidance test outcomes, all animals from the S100A9 aggregate treatment group exhibited amnestic behavior expressed as an increase in their escape latency in the water maze paradigm. These mice exhibited platform access learning inability throughout the protocol and displayed slower latencies on the test day following platform removal. Behavioral analysis did not reveal any differences between water maze parameters such as distance traveled or swim speed in the S100A9 fibrillar aggregate treated group. These data confirm that the physical activity of aged mice was not altered during intranasal administration of S100A9 aggregates implicating cognitive rather than motor processes[7]. This

conclusion is reinforced by the fact that the S100A9 gene is significantly upregulated not only in the AD brain but also in AD animal models[5,20]. In addition, experiments have shown that knockdown of S100A9 expression improves cognitive function in Tg2576 mice (an AD model) and these animals exhibit a reduced amyloid plaque burden. In this context, a new transgenic animal model of AD was established by crossbreeding Tg2576 mice with S100A9 knockout mice. Furthermore, the resultant S100A9KO/Tg2576 mice displayed increased spatial reference memory in the MWM and Y-maze tasks as well as decreased Aβ neuropathology and elevated anti-inflammatory as well as reduced inflammatory markers. Overall, such findings signified that S100A9 is involved in the neurodegeneration and cognitive deficits in Tg2576 mice[20].

***Morris water maze distance travelled and swim speed parameters of aged mice treated with S100A9 fibrils in the presence and absence of glutamate antibodies.***

In animals administered daily intranasal S100A9 fibrils, S100A9 fibrils plus Glu-Abs or saline vehicle, there were no significant group mean differences in distance travelled or swim speed throughout the entire 4 days of MWM acquisition training followed by testing on the next day (data not shown). In the 1980s, it was reported in the context of senescence that aged rats exhibited impaired water maze performance[21], motor incoordination, downgraded locomotor activity and exploratory behavior[22]. The current results accord with previous "open field" and "passive avoidance" findings which verified that neither locomotor activity nor emotionality (anxiety-like behavior) was perturbed at the end of 14-day intranasal S100A9 aggregate daily dosing[7].

The ensuing question was to unveil the specific molecular processes initiating the amnesia observed in our study. Previously, cellular mechanisms were postulated via the amyloid hypothesis that misfolded proteins generate toxic oligomeric species capable of inducing a decline in synaptic plasticity, disordered neuronal function and cell death[23] instigating cognitive dysfunction. Moreover, spatial memory in the Morris water maze is a hippocampal-dependent phenomenon[24] and damage to this neuroanatomical structure and its connections is conducive to amnesia[25]. In relation to this deduction, hippocampal-prefrontal cortical circuitry has been postulated as an integrative structural center for spatial memory establishment[26].

***Activity of intranasal S100A9 fibril treatment in the presence or absence of glutamate antibody intranasal co-treatment on hippocampal and prefrontal cortical concentrations of free neurotransmitter amino acids (aspartate, glutamate, glycine taurine and GABA) in aged mice.***

In saline control treated animals after the MWM protocol, the following concentrations of free neurotransmitter amino acids were identified in hippocampal samples: aspartate (1.97±0.21 μM/g tissue), glutamate (5.35±0.10 μM/g tissue), glycine (0.61±0.05 μM/g tissue), taurine (5.18±0.46 μM/g tissue) and GABA (1.38±0.19 μM/g tissue). In the prefrontal cortex after the MWM protocol, the following levels were documented: aspartate (3.56±0.85 μM/g tissue), glutamate (11.19±1.72 μM/g tissue), glycine (0.65±0.18 μM/g tissue), taurine (11.61±1.70 μM/g tissue) and GABA (2.30±0.34 μM/g tissue). There were no significant mean differences between groups either in hippocampal, or prefrontal cortical aspartate, glycine, taurine or GABA levels. However, it was notable that hippocampal but not prefrontal cortical glutamate concentrations were augmented ($P<0.05$) compared to those in the saline control group although this elevated level was significantly decreased by co-administration of Glu-Abs to a level that was not different from controls (Figs. 4 and 8).

In addition to monoaminergic disruption, S100A9 fibrillar structures also augmented hippocampal glutamate thus promoting the likelihood of excitotoxicity and a deficit in performance of the navigation task (Figs. 2, 4 and 8). Glutamate is stored in synaptic vesicles by an uptake system that is dependent on the proton electrochemical gradient. Along with inflammatory signals from S100A9 species, disturbance of the glutamatergic system can activate fast-acting excitatory ionotropic receptors and slower-acting metabotropic receptors.

These actions may well stimulate $Na^+$-dependent glutamate transporters located on neuronal and glial cell membranes to rapidly terminate glutamate activity and maintain its extracellular concentration below excitotoxic levels[27]. Accumulating evidence suggests that mitochondrial dysfunction might be a primary event in glutamate excitotoxicity[28]. In fact, during ATP production, mitochondria also produce reactive oxygen/nitrogen species (ROS/RNS) as a product of cell respiration which can damage neurons promoting the release of glutamate. Taking into consideration that ROS appears to be one of the key contributory factors in disturbing mitochondrial respiration, these molecular species are thought to be extensively involved in functional changes in the brain during aging [29]. Along with these facts, it is widely accepted that alterations in mitochondrial function are actively engaged in a range of neurodegenerative diseases, including AD[30]. It has even been hypothesized that deficits in these organelles may be the source of AD progression itself during aging[31] and along with glutamate neurotoxicity, the phenomenon is magnified.

The amnestic effects of S100A9 fibrils on spatial memory in the MWM arising from an increased hippocampal glutamate release and a DA-ergic decrement prompts the possibility that protection against glutamate toxicity may be a prospective therapeutic strategy. It has been shown that immune protection is effective for brain defense and the organism itself instigates this type of mechanistic protection in AD-like brain damage[32,33]. On this topic, we have also demonstrated an *in vivo* effectiveness for generated Glu-Abs in reducing $A\beta_{25-35}$ peptide amnesia through a decrease in caspase-3 activity[18]. Daily application of S100A9 fibrillar species, in combination with Glu-Abs, resulted in a shortening of escape and platform latencies signifying an abolition of memory deficit through glutamate-Glu-Ab binding. This was supported by the Glu-Ab-induced decline in hippocampal glutamate release to a level comparable to controls and the fall in HVA (Figs 2, 4 and 5). In light of this outcome, we recently identified impaired passive avoidance learning after chronic intranasal administration of pro-inflammatory S100A9 fibrillar protein structures in aged mice. Moreover, combined treatment with S100A9 fibrils and glutamate antibodies in these animals was followed by an increase in locomotor activity in the open-field test[34].

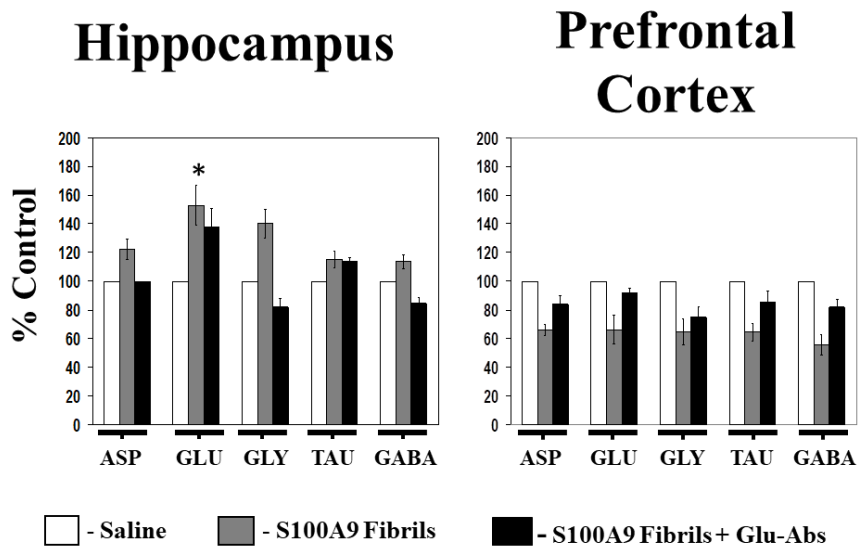



***Figure 4.*** *Amino acid neurotransmitter levels measured in the hippocampus and prefrontal cortex of 12-month old C57Bl/6 mice following intranasal dosing with S100A9 fibrillar species in the presence of Glu-Ab co-treatment.*

Animal groups (n=14) were intranasally administered saline, S100A9 fibrils or S100A9 fibrils plus Glu-Abs daily for 14 days and amino acid levels were measured as % of control.
In saline control or Glu-Abs treated animals after the MWM protocol, the following free neurotransmitter amino acids were identified in hippocampal and prefrontal cortical samples: aspartate, glutamate, glycine, taurine and GABA.

*P< 0.05 compared with control.

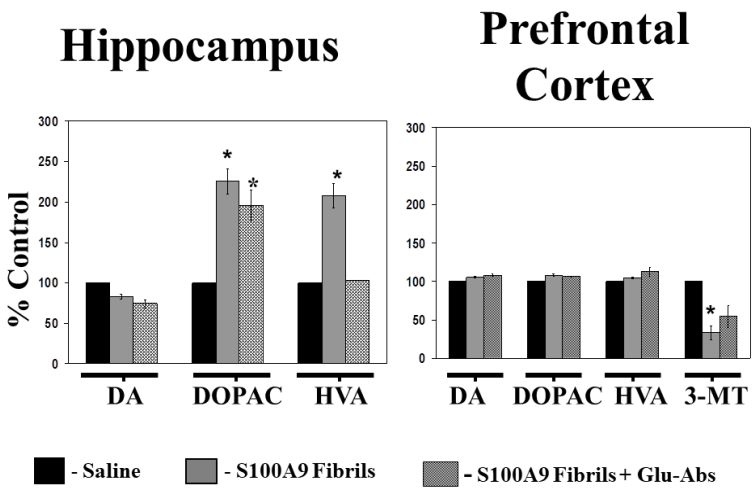



***Figure 5.*** *Hippocampal and prefrontal cortical levels (expressed as % control) of DA, HVA and DOPAC and prefrontal cortical 3-MT levels measured in 12-month old C57Bl/6 mice following intranasal dosing with S100A9 species in the presence or absence of Glu-Ab co-treatment.*

Animal groups (n=14) were intranasally administered saline, S100A9 fibrils or S100A9 fibrils plus Glu-Abs daily for 14 days and hippocampal DA, DOPAC and HVA levels were measured as % of control.

*P< 0.05 compared to control.

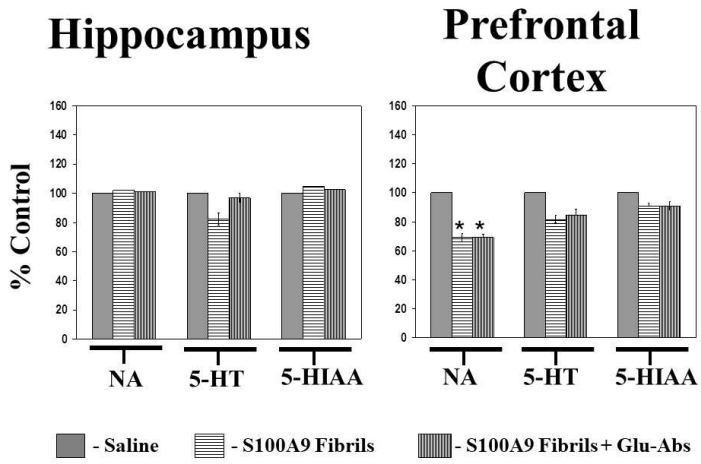



***Figure 6.*** *Hippocampal and prefrontal cortical levels (expressed as % control) of NA, 5-HT and 5-HIAA measured in 12-month old C57Bl/6 mice following intranasal dosing with S100A9 fibrillar species in the presence or absence of Glu-Ab co-treatment.*
Animal groups (n=14) were intranasally administered saline, S100A9 fibrils or S100A9 fibrils plus Glu-Abs daily for 14 days and hippocampal NA, 5-HT and 5-HIAA levels were measured as % of control.

*P< 0.05 compared to control.

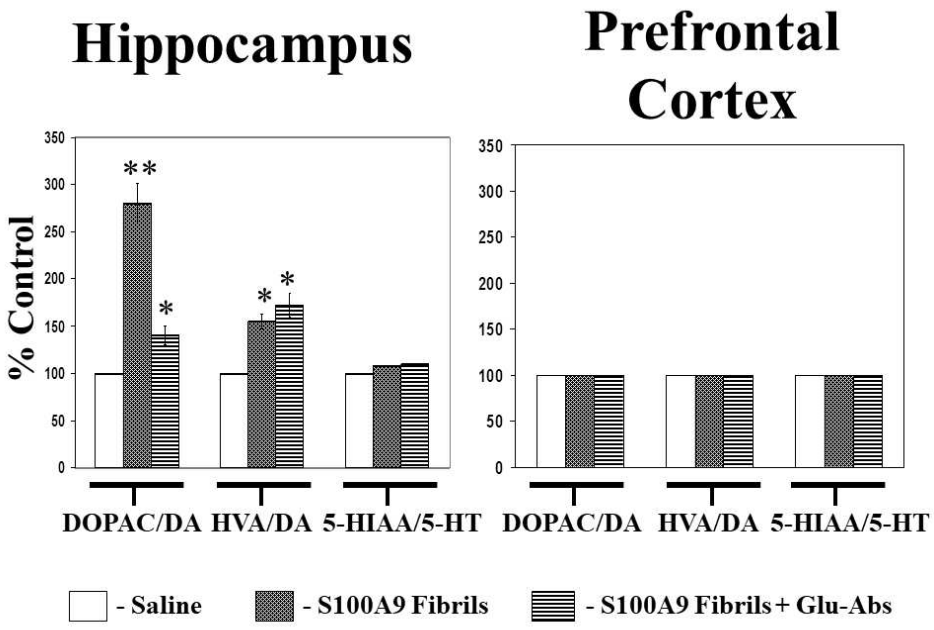



***Figure 7.*** *Hippocampal and prefrontal cortical DOPAC/DA, HVA/DA and 5-HIAA/5-HT ratios (calculated as % of control) in 12-month old C57Bl/6 mice following intranasal dosing with S100A9 fibrillar species in the presence or absence of Glu-Ab co-treatment.*

Animal groups (n=14) were intranasally administered saline, S100A9 fibrils or S100A9 fibrils plus Glu-Abs daily for 14 days and hippocampal and prefrontal cortical DOPAC/DA, HVA/DA and 5-HIAA/5-HT ratios were expressed (control values = 100%).

***Activity of intranasal S100A9 fibril administration in the presence or absence of glutamate antibody intranasal co-treatment on hippocampal and prefrontal cortical concentrations of dopamine and its metabolites in aged mice.***

In saline control treated animals after the MWM protocol, the following concentrations of DA and its metabolites were identified in hippocampal samples: DA (0.79±0.06 nM/g tissue), DOPAC (0.27±0.01 nM/g tissue) and HVA (0.33±0.02 nM/g tissue). In the prefrontal cortex after the MWM protocol, the following levels were noted: DA (11.44±1.82nM/g tissue), DOPAC (0.89±0.04 nM/g tissue), HVA (1.67±0.03 nM/g tissue) and 3-MT (0.57±0.04 nM/g tissue). Although hippocampal and prefrontal cortical DA concentrations remained unchanged following intranasal administration of S100A9 fibrils, after the MWM protocol hippocampal but not cortical levels of DOPAC and HVA were increased ($P<0.05$). However, only the HVA effect was reversed by concomitant treatment with Glu-Abs. By way of contrast, although 3-MT levels were below the detection limits of the assay in the hippocampus, prefrontal cortical 3-MT concentrations were effectively reduced by S100A9 fibrillar administration ($P<0.05$) after the MWM protocol. In fact,

this decline remained unaltered by combined dosing with S100A9 fibrils plus Glu-Abs (Figs 5 and 8).

The present evidence indicates that S100A9 fibrillar aggregates modified hippocampal DA metabolism not only raising DOPAC and HVA concentrations but also escalating the consequent DOPAC/DA and HVA/DA metabolic marker ratios (Figs. 5, 7 and 8). Co-treatment with Glu-Abs on the other hand, reversed these dopaminergic metabolic changes. In the case of the amnesia caused by S100A9 fibrils, it may be suggested that these misfolded aggregates incited inflammation[35] and interfered with DA metabolism thereby contributing to memory impairment[36]. It is also interesting to note, that in the prefrontal cortex, as opposed to the hippocampus, S100A9 fibrils influenced only the NA-ergic system causing a 20% fall in NA concentration (Fig. 6 and 8). In regard to this result, the monoaminergic system overall is implicated in cognitive processes through an influence on cortical and subcortical regions[37].

Extensive neuropathological studies have established a compelling link between abnormalities in structure and function of subcortical monoaminergic systems and AD pathophysiology. The main neuronal and glial cell populations of these systems (locus coeruleus, raphe nuclei, and the tuberomamillary nucleus) undergo degeneration in AD thus depriving hippocampal and cortical neurons of their critical modulatory influence. The widespread distribution of these monoaminergic networks is one of the main difficulties in analyzing their functions and interactions[38]. To address this complexity in relation to the present results, it might be assumed that amyloid structures specifically destroy monoaminergic systems which escalate brain adaptive processes during cognitive failure.

***Activity of intranasal S100A9 fibril treatment in the presence or absence of glutamate antibody intranasal co-treatment on hippocampal and prefrontal cortical concentrations of NA, 5-HT and 5-HIAA in aged mice.***

In saline control treated animals after the MWM protocol, the following hippocampal concentrations of NA (4.16±0.63 nM/g tissue), 5-HT (7.10±0.98 nM/g tissue) and 5-HIAA (3.81 ±0.67 nM/g tissue) were detected. Control animals also exhibited the following prefrontal cortical concentrations of NA (3.14±0.54 nM/g tissue), 5-HT, (4.73±0.85 nM/g tissue), 5-HIAA (1.41±0.3 nM/g tissue).

Only prefrontal cortical NA concentrations were decreased ($P<0.05$) by S100A9 fibrillar treatment and this decrement was unaffected by glutamate antibody co-administration. In the case of the

hippocampal or prefrontal cortical concentrations of 5-HT or 5-HIAA and hippocampal NA, there were no significant variations induced by Glu-Ab treatment in comparison with the saline control treated group after MWM training and testing (Figs. 6 and 8)

***Activity of intranasal S100A9 fibril treatment in the presence or absence of glutamate antibody intranasal co-treatment on hippocampal and prefrontal cortical DOPAC/DA, HVA/DA or 5-HIAA/5-HT ratios in aged mice.***

In saline control treated animals after the MWM protocol, the following metabolite/neurotransmitter ratios were calculated from the hippocampal samples: DOPAC/DA = 0.82, HVA/DA = 0.43 and 5-HIAA/5-HT = 0.52. In the prefrontal cortex samples after the MWM protocol the following control metabolite/neurotransmitter ratios were derived: DOPAC/DA = 0.07, HVA/DA = 0.14 and 5-HIAA/5-HT = 0.30. Daily intranasal inoculation with S100A9 fibrils boosted the hippocampal DOPAC/DA ratio ($P<0.01$) when measured after the MWM protocol and this was very noticeably reversed by concomitant Glu-Ab administration. Likewise, the HVA/DA ratio was raised by administration of S100A9 fibrils in the hippocampus ($P<0.05$) but this increase was brought down by Glu-Ab co-treatment. There were no changes in hippocampal or prefrontal cortical 5-HIAA/5-HT ratios, neither was there any alteration in cortical DOPAC/DA nor HVA/DA ratios in response to S100A9 fibrils in the presence or absence of Glu-Abs (Fig. 7 and 8)

The AD-like amnesia provoked by S100A9 fibril administration noted here potentially stems from neuroinflammation and protein neuroaggregation linked to aberrant neurochemistry[36]**.** Earlier, we have shown that fibrillar S100A9 treatment induced passive avoidance memory retention deficits in 87.3% of aged animals and this correlated with an enhancement of DA turnover in the prefrontal cortex as well as an increased DA level and its metabolites in the hippocampus[7]. It has also been reported previously that different degrees of DA dysfunction can occur during AD progression[39]. There have been indications of a distinctive DA role along with its receptors in forming long-lasting memories. For example, activation of the prefrontal cortical, striatal, and hippocampal dopamine DA1- family of receptors (D1- but not D5-like receptors) is necessary for normal spatial information processing[40]. In addition, reduced DA transporter (DAT) expression in the caudate putamen, hippocampus and frontal cortex has been described during human brain aging[41] and DAT has also been hypothesized as a possible target of amyloid insult. Thus, an influence of S100A9 aggregates on DAT function cannot be excluded as one of the elements of DA disrupted function in spatial memory[42].

## Conclusion

During neurodegenerative conditions where memory loss may occur, there are contributory cascade-dependent phenomena such as neuroinflammation, oxidative stress and amyloidogenesis which induce memory impairment. Recent findings established that hippocampal inflammatory processes contribute to spatial memory deficits[43]. Analogously, the pro-inflammatory and amyloidogenic properties of S100A9 protein have been explored in a fear aggravated memory task (passive avoidance) alongside neurochemical assays in the prefrontal cortex and hippocampus of aged mice[7]. The novel outcome of the current study has emphasized the pathogenic nature of S100A9 fibrillar aggregates in causing spatial memory amnesia in the water maze paradigm which is associated with enhanced hippocampal glutamate release and DA-ergic disruption in the aging brain (Graphic Table). Moreover, it might be hypothesized that a treatment for AD could be based on application of Glu-Abs to prevent the central glutamate neurotoxicity induced by misfolded protein species.

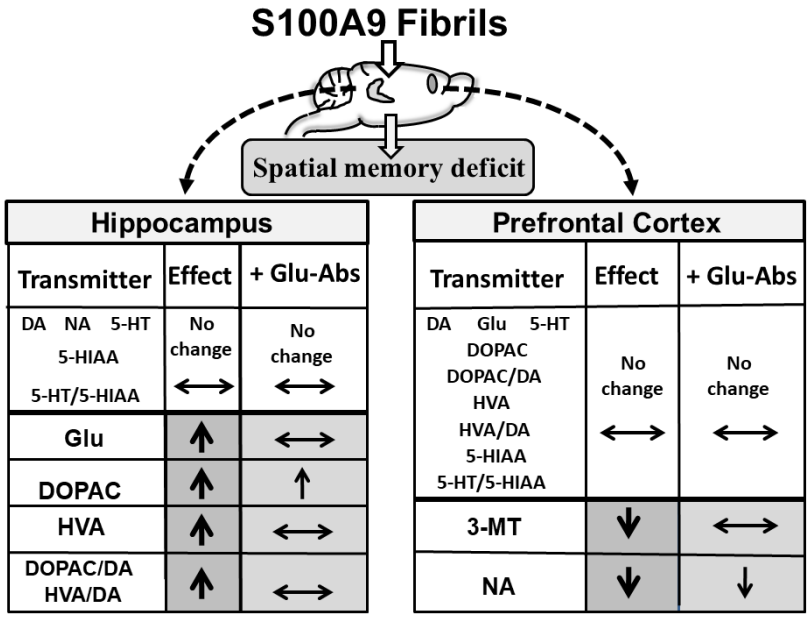



***Figure 8.*** *Summary showing the significant hippocampal and prefrontal cortical neurochemical outcomes of 14-day intranasal administration of S100A9 fibrils in 12-month old C57Bl/6 mice on protocol day 20.*

Upward arrow = increase; downward arrow = decrease; horizontal double headed arrow = no change.

## MATERIALS AND METHODS

### *Subjects*

Adult male C57Bl/6 mice aged 12-months and weighing 31.2±1.1g, were used throughout. The animals were group housed on a 12:12 light-dark cycle at a constant temperature of 21$^{o}$C and 50% humidity with access to food and water *ad libitum*. All experimental procedures were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996); the UK Animals Scientific Procedures Act 1986 and associated guidelines; the European Communities Council Directive of 24 November 1986 (86/609/EEC) for care and use of laboratory animals. They were also approved by the Animal Care and Use Committee of the P. K. Anokhin Research Institute of Normal Physiology.

### *Procedures and dosing protocol*

Experiments were performed between 10.00-15.00 hours and mice were divided into three groups (n = 14 per group) which underwent the protocol chronology shown in Fig. 9. Group (1; naïve control) was administered saline vehicle intranasally (i.n) in alternate nostrils daily in a total volume of 8 µL/animal daily (i.e. 4 µL/nostril using a Hamilton syringe) over a total dosing period of 14-days. Group (2) was administered a solution of S100A9 fibrillar aggregates (15.0 µg in 8 µL = 0.48 mg/kg) using the same 14-day dosing schedule. Group (3) was co-administered S100A9 fibrillar aggregates (15.0 µg in 8 µL = 0.48 mg/kg) in one nostril nasal side and antibodies to glutamate (7.8 µg in 8 µL = 0.25 mg/kg) to the other nostril another side using the 14-day dosing schedule. At the end the 14-day protocol (i.e. on day 15), animal groups 1-3, underwent a modified behavioral protocol[7] [13] involving four days of acquisition training (Fig. 1). The next day, after behavioral testing (day 5), mice were killed and neurochemical analysis of the hippocampus and prefrontal cortex was performed (n = 12 per group). All behavioral tests and neurochemical analyses were performed under blind conditions.

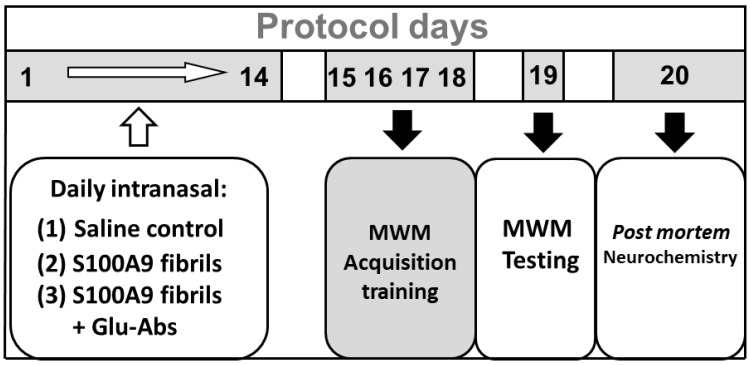



***Figure 9.*** *Scheme showing the chronology of the intranasal S100A9 dosing protocol, MWM acquisition training and testing then the post mortem hippocampal and prefrontal cortical sampling period in 12-month old C57Bl/6 mice prior to neurochemical analysis.*

***Production of S100A9 protein***

S100A9 was expressed in E. coli and purified as described previously [44]. Its concentration was determined by using $\varepsilon 280 = 0.53\ (mg/ml)^{-1}\ cm^{-1}$.

***Production of S100A9 fibrillar aggregates***

In order to avoid the presence of the preformed S100A9 aggregates in solution, the protein was initially dissolved in 10 mM PBS buffer, pH 7.4, subjected to 15 min sonication, then to 15 min centrifugation at 14,000 rpm in a minicentrifuge (Eppendorf Centrifuge 5417R) and the supernatant collected from the upper layer was filtered through a 0.22 um filter (Millex). The final solution was incubated in at a 2.0 mg/ml concentration in 10 mM PBS buffer, pH 7.4 at 37 $^{o}$C, using continuous agitation at 600 rpm (Eppendorf Thermomixer Compact). Amyloid S100A9 fibrils were produced after 24h of incubation. The fibrillar sample was stored at +4 °C prior to administration. The morphological parameters of stored fibrils were compared with freshly produced structures using AFM imaging and they were confirmed to be essentially unchanged.

***Immunological methods***

***Synthesis of glutamate/BSA immunogenic conjugate.***

The synthesis of the glutamate conjugate with bovine serum albumin (BSA, Sigma- Aldrich,USA) was performed according to a modified protocol[45]. Glutamate (L-glutamic acid monosodium salt monohydrate, Sigma-Aldrich,USA) 10 mg in 1.0 ml of distilled water was mixed with 1.0 ml of 3 M acetate buffer (pH 7.8) containing 30 mg of BSA. The reaction was started by adding 1.0 ml of 5% glutaraldehyde and it lasted for 3 min at room temperature. Glutaraldehyde as a crosslinking reagent was chosen for its high yield of coupling with the L-amino group of lysyl residues in proteins. An orange-yellow color and stability at pH ≈ 7.0 indicated that the coupling reaction was complete; 1.0 ml of a sodium borohydride solution (10 mM) (Merck, USA) was added to saturate the double bonds. After reduction, the mixture turned from orange-yellow to translucent. The solution was then dialyzed at +4.0 $^{o}$C and the precipitate was removed by centrifugation. The weight of 1.0 ml of the lyophilized conjugate were determined, allowing molar ratios to be calculated as Glu/BSA = 7.0.

***Immunization and production of antibodies to glutamate.***

Three white chinchilla rabbits (6 months old, weighing 2.6-2.7 kg) were housed in individual cages, fed a premeasured pelleted diet ration once daily with constant access to water. Animals were immunized using immunogenic glutamate conjugate with BSA according a standard 70-day immunization serum production protocol[45]. The first booster injection contained 1.0 mg of glutamate/BSA conjugate emulsified in 1.0 ml of 0.15 M NaCl and 1.0 ml of complete Freund's adjuvant (Difco). Using the resultant 2.0 ml of mixture, 10 subcutaneous sites and 10 intramuscular sites were utilized for immunisation. Over the next 14, 28, 42, 56 and 70 days, similar booster injections were repeated using incomplete Freund's adjuvant. Production bleeds were performed on days 28, 42, 56, 70 days and 10 ml of blood was taken with subsequent separation of serum. The γ-globulin fraction was produced from serum after ammonium sulfate precipitation, dealization and affinity chromatographic purification on BSA/CNBr-activated Sepharose™ 4B sorbent before lyophilization. The purified anti Glu/BSA γ-globulin fraction was then analysed by ELISA using Glu/BSA conjugate as antigen for Glu/Abs titres and estimations were determined as 1: 1200 ± 1: 100.

***Fluorescence assay***

The thioflavin T (ThT) binding assay was performed using a modification of LeVine's method[46]. Thioflavin T fluorescence was measured by a Jasco FP-6500 spectrofluorometer (Jasco, Japan), using excitation at 440 nm and collecting the emission between 450–550 nm, with excitation and emission slits set at a 5 nm width.

***Atomic force microscopy (AFM) assay***

Atomic force microscopy (AFM) imaging was carried out using a BioScope Catalyst AFM (Bruker) in the peak force mode in air at a resonance frequency of ca. 70 kHz and a resolution of 256 x 256 pixels; scan sizes ranged from 0.5 to 10 μm. Amyloid samples were deposited on the surface of freshly cleaved mica (Ted Pella) for 15 min, washed 3 times with 100 μl deionized water and dried at room temperature and then subjected to AFM analysis (Fig. 2).

***Behavioral test***

***Morris water maze (MWM) test***

Cognitive function was evaluated by a modified protocol of the Morris water maze protocol (Fig. 1) as previously described by Yu et al.,[47]. The water maze consisted of a grey colored circular pool (140 cm in diameter and 60 cm in height) filled to a depth of 40 cm with water rendered opaque by the addition of a small quantity of powdered milk[48]. The temperature of the water was maintained at 22.0 ± 1.0 $^{o}$C and the pool was divided into four quadrants. A transparent circular escape platform (11 cm in diameter, 40 cm in height) was located in one quadrant of the pool 2.0 cms beneath the water surface and hidden from animal view. The platform had a rough surface which facilitated animal access onto the platform once its presence was detected. The maze was positioned in a well-lit room with several posters and other distal visual stimuli on the walls to provide external spatial cues. All groups of mice were trained to spatially locate the hidden platform on 4 consecutive days. Each day, they received four consecutive training trials during which the hidden platform was kept in a constant location. Every trial was commenced by carefully placing each animal into the water facing the wall of the pool at one of three random start positions avoiding the quadrant including the platform. Animals were allowed 60 s to find the platform and in instances of platform location failure within this period, mice were placed on the platform for 10 s, and the latency was recorded as 60 s. Behavioral parameters including escape

latency (time to find the platform), distance traveled and swimming speed were analyzed by an EthoVision video tracking system version 8 (Noldus Information Technology,Netherlands). On the 15$^{th}$ protocol day, the hidden platform was removed from the water maze, and mice were allowed to swim freely for 60 s; the number of times animals crossed the target platform were recorded. Throughout, the observers were blind to the experimental conditions. After behavioral experiments, animals were killed and brain structures (hippocampus and prefrontal cortex) were dissected on ice (4 $^{o}$C) and immediately stored in liquid nitrogen for subsequent neurochemical analysis.

***Neurochemical assays***

***Neurochemical determination of hippocampal and prefrontal cortical content of neurotransmitter amino acids (aspartate, glutamate, glycine taurine and GABA) in aged mice.***

The determination of amino acid (aspartate, glutamate, glycine taurine and GABA) content in the hippocampus and prefrontal cortex of aged mice was performed according to a modified method of Pearson et al.,[49]. Since neurotransmitter amino acids are weak chromophores, it was necessary to modify them for stable detection by addition of o-phthalaldehyde (OPA) to form fluorescent complexes. Cerebral structures were homogenized in 0.1 N perchloric acid (1:20) with 0.5 μM 3,4-dihydroxybenzoic acid as internal standard and centrifuged (10,000g x 10 min, 4 $^{0}$C; Eppendorf 5415 R, Germany). In order to achieve derivatization, 25 μl of 0.1M borate buffer (pH 9.5) and 10 μl of OPA was added to 25 μl of tissue supernatant. The samples were incubated (20 min at room temperature) and 20 μl of each sample was subjected to analysis in an Agilent 1100 chromatograph with a fluorescent detector and wavelengths of excitation and emission set at 230 and 392 nm, respectively (Agilent Technologies, USA) using a HYPERSIL ODS column (4.6×250 mm, 5 μm). The eluent phase consisted of 0.06 M $NaH_2PO_4$ x $H_2O$, 0.0032 M $Na_2HPO_4$, 0.025 mM EDTA and 1.24 mM $CH_3OH$ (pH=5.6) and the flow rate was 1.5 ml/min. A standard sample consisted of 0.1 μM/ml in 0.1N $HClO_4$ of GABA, aspartate, glutamate, taurine and glycine (Sigma-Aldrich, USA) of each amino acid.

***Neurochemical determination of the tissue content of DA, 5-HT and their metabolites (DOPAC, HVA and 5-HIAA) as well as NA in mouse brain structures by high performance liquid chromatography with electrochemical detection (HPLC/ED)***

The procedure for neurochemical determination of tissue content of DA, 5-HT and their metabolites (DOPAC, HVA and 5-HIAA) as well as NA in the mouse hippocampus and prefrontal

cortex was performed and analyzed by high performance liquid chromatography with electrochemical detection[7].

***Statistics***

Statistica 7.0 software was used for statistical analysis. The distribution of behavioral data did not conform to a normal distribution (Lilliefors test, $P<0.01$) and thus, univariate nonparametric analysis of variance Kruskal-Wallis test (H-criterion) with by post-hoc analysis by the Mann-Whitney U test was performed. Data are presented as mean ± s.e.m. The critical level of statistical significance in the test for the null hypothesis was accepted at $P<0.05$.

## AUTHOR INFORMATION


### Corresponding Author

*Robert D. E. Sewell. Email: sewell@cardiff.ac.uk


### Author Contributions

M.A.G., L.A.M.-R., and R.D.E.S. designed the project and prepared the manuscript. T.V.D., V.S.K., C.W., and V.B.N., performed the experiments. All authors contributed to analysis of the experimental data.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### Notes

The authors declare no competing financial interest.

## ABREVIATIONS

Aβ, β amyloid peptide; AD, Alzheimer's disease; AFM, atomic force microscopy; CNS, Central nervous system; DA, dopamine; DAT, dopamine transporter, DOPAC, 3,4-dihydroxyphenylacetic acid; GABA, γ-aminobutyric acid; Glu-Abs. glutamate antibodies; HVA, homovanillic acid; 5-HIAA, 5-hydroxyindoleacetic acid; HPLC/ED, high performance liquid chromatography with electrochemical detection; 5-HT, 5-hydroxytryptamine; i.n., intranasal; OPA, o-phthalaldehyde; MWM, Morris water maze; NMDA, *N*-methyl-D-aspartate; NA, noradrenaline; ROS/RNS, reactive oxygen/nitrogen species.

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