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AGLAEA

Development of novel animal models of glutamatergic central nervous system disorders using *in-vivo* shRNA and transgenic approaches.

FP6 - Specific Targeted Research Projects (STREP)

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RE	Restricted to a group specified by the consortium (including the Commission Services)	
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Approvals

	First name & Name	Organisation	Date	Visa
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1 Executive summary of project results

Glutamate is the most abundant excitatory transmitter in the brain. However, few animal models exist to explore the potential of drugs that modulate glutamate transmission. Altered glutamate transmission is involved in numerous psychiatric diseases, resulting in the need for models to characterize the effects of hypo- and hyper-glutamatergic states involved in the onset and development of psychiatric diseases.

AGLAEA aimed to develop and characterize models of selective, partial knockdown of specific components of the brain glutamatergic system in mice: **glutamate membrane transporter (EAAT2/GLT-1) and the vesicular membrane transporter (VGLUT1)**. These models are believed to provide better comprehension of the implication of glutamate signaling in diseases such as schizophrenia, anxiety and cognitive disorders.

In order to selectively turn off specific components of the glutamatergic pathways, both siRNA and shRNA approaches were attempted for both molecular targets.

Main findings and achievements of the project AGLAEA were:

- In vitro studies identified suitable siRNA and shRNA constructs for the glutamate membrane transporter (GLT-1/EAAT2) and the vesicular membrane transporter (VGLUT1) respectively. Beyond the initial plan and foreseen deliverables, in vitro system was developed for assessing the functional consequence on proteins knock-down. It was possible to show the consequence of altering the gene expression and its repercussions on protein level and function of one glutamate transporter.

- Initial in vivo studies showed poor brain penetration following icv administration of TurboGFP-encoding lentiviral particles, indicating the shRNA approach required localised administration (in contrast to siRNA that will be applied by icv infusion). In general both siRNA and shRNA were tolerated and had no effect on gross behavioral measures.

- Behavioral studies indicated that disruption of GLT1 transporter resulted in an anxiogenic behavioral profile, as expected given the role of this transporter in maintaining extracellular glutamate at physiologic levels. Interestingly, benzodiazepine was able to reverse the anxiogenic phenotype. At this stage, the conclusion is that a model exists although there is some work still to do to guarantee robustness and reproducibility for its use in routine for the testing of new compounds.

- Also behavioral studies indicated that shRNA directed at VGLUT1 impaired specific aspects of cognition (after bilateral intra-hippocampal injections), as expected from the role of this transporter in the control of glutamate release. Interestingly it was shown that one antagonist of 5-HT_6 receptor was able to reverse cognitive deficits.

- Results gained with shRNA-VGLUT1 demonstrated that the initial plan to produce an inducible transgenic mouse showing partial loss of VGLUT1 expression as a model of the cognitive deficits was a valid goal. Despite a dedicated strategy and efforts, the consortium did not deliver the transgenic mice but did progress till the stage of the constructions of some lentiviruses encoding shRNA.

- The consortium managed to produce germline transmitting C57/Bl6 mES cell line for generation of animal models using in behavioural studies. Mice with C57/BL6 background are much more amenable to behavioural studies than the 129 SV line, for which ES cell lines are already

available. Mycoplasma free cell clones were characterized in vitro and tested in vivo for germline transmission potential.

2 Contractors involved

The AGLAEA consortium brought together high level research centres, with complementary expertises and approaches: Three high tech SMEs: **ADDEX Pharmaceuticals** (psychopharmacology and behaviour), **BIOTALENTUM** (transgenic mice), **BRAINS ON LINE** (neurochemistry) and two academic groups: **RUG** (neurochemistry), **UNOTT** (molecular biology, neuropharmacology and behaviour) together with management provided by **ALMA**.

Full names of **partners** are given below:

- ADDEX Pharmaceuticals France SAS will appear as ADDEX (FR)
- BioTalentum Ltd will appear as BIOTALENTUM (HU)
- University of Nottingham will appear as UNOTT (UK)
- University of Groningen will appear as RuG (NL)
- Brains-On-line will appear as BOL (NL)
- ALMA Consulting Group will appear as ALMA (FR)

3 Scientific approach

Modulation of glutamate signalling levels is associated with many psychiatric disorders. For example, schizophrenia has been linked clearly to a hypo-glutamatergic state (Carlsson et al., 1999), and the symptoms of schizophrenia can be mimicked by antagonists of NMDA glutamate receptors such as PCP and ketamine (Krystal, et al., 2003). Glutamate has also been implicated in learning and memory (Lynch 1998) and hypo-glutamatergic states result in impaired memory (Krystal et al., 2005).

Reliable and practical models of altered brain glutamate function through traditional approaches, such as pharmacological blockade of single receptors, give only a limited picture of glutamate function (Geyer and Ellenbroek, 2003). Also, receptor-subtype selective and brain-penetrant compounds are often not available for glutamate receptors. Constitutive gene knockout (KO) or transgenic approaches in rodents do offer some advantages when addressing a target for which there are no selective pharmacological agents. However, development of knockout rodents is expensive and time consuming, and gene KO is often associated with developmental alterations. Inducible and conditional knockout technologies have helped to alleviate this problem; however, the gene is nearly completely suppressed when the induction occurs. Because glutamate is a ubiquitous excitatory transmitter, full knockout of a specific glutamate receptor or transporter often yields a phenotype incompatible with behavioural testing (Mohn et al., 1999).

Indeed, in the post-genomic era increasing importance is given to understanding gene-function relationship. knockout (KO) or transgenic approaches represent a useful tool in functional genetic studies, however with important down-sides.

RNA interference has recently emerged as an interesting alternative, offering the advantage of transient and reversible manipulation of gene expression in adults at the transcriptional level. Genes involved in vital functions may thereby become accessible to more subtle manipulations.

To achieve its ambitious objectives the AGLAEA work programme has been split into 4 scientific work packages (WP):

- 1 work package (WP1) is dedicated to the in vitro development and validation of the si/shRNAs: the overall objective of WP1 is to produce non-toxic siRNAs/shRNAs with sufficient efficacy to be used in the in vivo infusion system.
- 1 work package (WP2) is dedicated to the in vivo infusion of the si/sh RNA for the knockdown of the glutamate transporters: WP2 will perform initial in vivo infusions of the siRNAs/shRNAs developed in WP1, in order to determine in vivo their efficacy, time course information and regional effects using behavioural tests, histological investigation and measurement of the target protein levels.
- 1 work package (WP3) is dedicated to the functional assessment of the generated knockdowns: the overall objective of WP3 is to characterize hypo- and hyper-glutamatergic states in live animals.
- 1 work package (WP4) is dedicated to the generation and characterisation of the transgenic mice: WP4 will generate transgenic animals with inducible alterations of glutamate components and it will characterize them as potential experimental models.

In addition to the scientific WPs, WP5 - **Management, Exploitation and Dissemination** will deal with project management, consortium animation and the management of knowledge and intellectual property, in order to establish the best exploitation and dissemination strategies for the consortium.

4 Work performed and achievements

WP1 – siRNA/shRNA development and in vitro validation

1- State-of-the art at project start

There is extensive evidence for glutamate disruptions in numerous human psychiatric diseases, including schizophrenia, anxiety disorders and cognitive disorders (e.g. Alzheimer's disease). In spite of this, very few models exist to explore the potential of drugs that modulate glutamate transmission to treat these diseases. Novel models would provide a powerful tool for pharmaceutical companies and the scientific community to identify and validate targets, and for the development and testing of new drugs. In addition, these models would also benefit researchers investigating the neurobiological and neurochemical bases of psychiatric disorders.

Full knockout of glutamate function by targeting either the transmitter or its receptors often yields a phenotype incompatible with behavioural testing. By using the technique of RNA interference, AGLAEA will generate models in which 1) a hyperglutmatergic state is induced by partial knockdown of the glutamate reuptake transporter EAAT2/GLT-1, to model anxiety disorders, or 2) a hypoglutamatergic state is produced by partial knockdown of the vesicular glutamate transporter VGLUT1 in order to reduce synaptic glutamate release, and model aspects of schizophrenia.

2- Project objectives

The overall aim of the project was to use RNA interference techniques to partially knockdown EAAT2/GLT-1 or VGLUT1 and create novel *in vivo* models of relevant hyper- or hypoglutamatergic states, respectively. The first stage in this process was to use an *in vitro* system to identify suitable shRNA constructs to progress to *in vivo* administration.

3- Project achievements

In vitro studies in a mouse neuroblastoma cell line successfully identified suitable shRNA constructs to progress to *in vivo* targeting of EAAT2/GLT-1 and VGLUT1. These findings were presented at the British Pharmacological Society winter meeting (Kurian *et al*, 2007) and the Society for Neuroscience annual meeting (Kurian *et al*, 2008).

WP2 – In vivo siRNA and shRNA knockdown of glutamate transporters

1- State-of-the art at project start

Although siRNA has recently been used to alter gene expression in adult mouse brain (Thakker et al 2004, Thakker et al 2005) the knockdown produced by this approach is only maintained during the relatively short infusion period (1-2 weeks). In contrast, a single intracerebral injection of a shRNA-encoding lentiviral vector should produce permanent knockdown in the targeted brain region. Prior to the start of the AGLAEA project neither non-viral (siRNA) nor viral (shRNA) delivery techniques had been used to influence glutamate transporter expression *in vitro* or *in vivo*.

2- Project objectives

The aim of WP2 was to perform initial *in vivo* studies with the chosen RNAi strategies; lentiviral shRNA delivery for VGLUT1, and non-viral siRNA delivery for GLT1-EAAT2, to determine the effect of the selected constructs on gross behavioural measures.

3- Project achievements

Preliminary *in vivo* studies evaluated the chosen RNAi strategies:

- Lentiviral shRNA delivery for VGLUT1 was well tolerated; injected mice maintained normal body weight and did not exhibit any gross physiological or behavioural abnormalities which would make them unsuitable for subsequent more detailed behavioural assessment. These findings were presented at the British Association for Psychopharmacology Summer meeting (King et al, 2009).

- For the second transporter, the validation of a surgical procedure for Alzet micro-osmotic pump implantation to deliver (icv) siRNA targeting GLT1 was a prerequisite. Notably, body weight was not significantly affected by the treatment. Gross evaluation of the infused mice in the Irwin test revealed no adverse physiological or behavioral effects and finally the assessment of motor coordination in the rotarod revealed no difference between groups.

WP3 – Functional assessment of the siRNA/shRNA-mediated knockdowns

1- State-of-the art at project start

RNA interference (RNAi) is a naturally occurring mechanism for regulation of gene expression in plants and animals (Fire et al., 1998). Experimental intracellular introduction of short sequences of double-stranded RNA (short hairpin, or short interference RNA) that are homologous to an endogenous gene results in sequence-specific target knockdown. This technique has been used extensively *in vitro,* and has been adapted for use in mammals *in vivo* (Dorn et al., 2004). Within the last years, siRNA (Hommel et al., 2003; Bahi et al., 2004; Bahi et al., 2005) and shRNA (Thakker et al., 2004; Thakker et al., 2005) have been used successfully to suppress gene expression in specific brain regions in rodents.

2- Project objectives

The aim of WP3 was to perform detailed behavioural assessments (in the same si/shRNA treated animals as WP2 wherever possible) in rodent tests of cognition and anxiety, and to combine these with assessments of neurotransmitter release and target protein levels.

Specific siRNAs have been selected in order to knockdown targets that would alter the amount of glutamate release and uptake rather than altering individual glutamate receptors. The vesicular glutamate transporter 1 (VGLUT1) has been shown to be decreased in the brains of schizophrenic subjects (Eastwood & Harrison, 2005) and was chosen to decrease synaptic release of glutamate. In contrast, synaptic levels of glutamate could be increased by knocking down the primary brain glutamate transporter EAAT2/GLT-1, which is decreased in the brains of patients with ALS (Rothstein et al., 1995) and in mouse models of HD (Behrens et al., 2002).

In order to selectively turn off specific components of the glutamatergic pathways, both siRNA and shRNA approaches were used. The effect of altered glutamate signalling were characterised using relevant behavioural, neurochemical and immunohistochemical tests including further evaluation by functional magnetic resonance imaging (fMRI) and microdialysis/microsensor methodology.

3- Project achievements

- Lentiviral delivery of a shRNA vector targeting VGLUT1 into mouse hippocampus produced significant memory impairments in hippocampal dependents tasks (Novel Object Discrimination and Morris water maze) without producing any confounding adverse effects (King et al, 2009). The parallel in vivo microdialysis and microsensor studies revealed altered patterns of glutamate release (attenuation of the glutamate efflux) in VGLUT1 knockdown mice, consistent with the induction of a hypoglutamatergic state (Qin 2010). These findings were presented at British Association for Psychopharmacology Summer meeting 2009. Interestingly, the 5-HT_6 receptor

antagonist SB-399885 reversed the NOD deficit when tested at single dose in mice receiving bilateral injections of VGLUT1 targeting lentiviral virions (King et al. submitted for British Association for Psychopharmacology Summer meeting 2010).

- EAAT2/GLT-1: Behavioural studies indicated that disruption of GLT1/EAAT2 using the siRNA technique results in a focused anxiogenic behavioural profile, which was successfully reversed by a drug from the class of benzodiazepine commonly used to treat anxiety in patients. However, to the best of our knowledge, this hyperglutamatergic model has shown immune response in tissues exposed to siRNA which must be taken into account for further analyses and use. There was no evidence of a similar immune response in the hypoglutamatergic model (King et al submitted for British Association for Psychopharmacology Summer meeting 2010).

WP4 – Generation and characterization of transgenic mice

1- State-of-the art at project start

Creation of reliable and practical models of altered brain glutamate function has been difficult (Mohn et al., 1999). Glutamate is the most abundant excitatory transmitter in the brain and interacts with numerous receptors and transporters responsible for both fast synaptic transmission and modulatory functions. Thus, traditional approaches, such as pharmacological blockade of single receptors, give only a limited picture of glutamate function (Geyer and Ellenbroek, 2003). Also, receptor-subtype selective, brain penetrant compounds are often not available. Constitutive gene knockout or transgenic approaches do offer some advantages, however, development of knockout (KO) models is expensive and time consuming, and gene KO is often associated with developmental alterations. Inducible and conditional knockout technologies have helped to alleviate this problem; however, the gene is nearly completely suppressed when the induction occurs. Because glutamate is a ubiquitous excitatory transmitter, full knockout of a specific glutamate receptor or transporter often yields a phenotype incompatible with behavioural testing.

2- Project objectives

The present project is aimed at developing models of selective, partial knockdown of specific components of the brain glutamatergic system that results in hypo- and hyper-glutamatergic states yielding animals amenable to behavioural investigation. The results drive the creation of specific transgenic lines with targeted knockdown of components of the brain glutamate system yielding mice that can be studied over long periods. The overall objective in this work package was the creation of transgenic animals with inducible alterations of glutamate transmission and their characterisation as potential preclinical models of mental disorders.

3- Project achievements

Generation of the planned conditional mouse model, in which modulation of glutamate signalling can be induced at different stages of development, is a complex work. Modification/delayed start of this part and some technical difficulties had also impacts on the delivery timing. The overall objectives have been partially achieved: generation of C57Bl6 ES cell line has been successfully completed, however, the production and characterization of transgenic mice need to be postponed beyond the project's end. The consortium is actively discussing the steps needed to complete the work and implement the overall goals after the end of the AGLAEA project.

WP5 – Management

1- Project objectives

- Achieve the project's objectives by efficiently using the planned resources
- Fulfil contractual requirements towards the EC
- Develop and facilitate efficient communication between partners and thus optimise indirect benefits of the project

2- Project achievements

The resources of the project were efficiently managed thanks to a regular financial and technical follow up done by the Coordinator with ALMA's support. All the contractual deliverables were sent to the EC respecting the schedule. The generation of the transgenic vector required the reallocation of tasks and budget within the consortium and a consequent change in the contract that was amended and approved by the EC. To ensure an efficient communication, a public web site (http://www.aglaea.com) was designed and an intranet web platform was developed (https://www.myndsphere.com).

WP5 Exploitation and Dissemination

1- Project objectives

- Management of knowledge and intellectual property and establishment of exploitation and dissemination strategies

2- Project achievements

A public web site was created and regularly updated: www.aglaea.com. The statistics revealed that it was visited by internet users from 34 different countries among which there are Europe, Canada, USA, India and Australia.

A brochure with the AGLAEA results can be downloaded from the web site and it was distributed to the main conferences/congresses of the field by all the partners.

- 8 abstracts were presented to internationally renowned conferences and congresses.
- Five publications are in preparation to disseminate the major results of the project.
- The consortium had regular meetings to discuss about the exploitation strategy. At the moment, only the C57Bl6 ES cell line can be exploited.

5 Conclusions and openings

The AGLAEA project aimed to develop and characterize models of selective, partial knockdown of specific components of the brain glutamatergic system in mice: glutamate membrane transporter (EAAT2/GLT-1) and the vesicular membrane transporter (VGLUT1). These models are believed to provide better comprehension of the implication of glutamate signalling in diseases such as schizophrenia, anxiety and cognitive disorders and are useful for drug discovery and development and study of disease mechanisms.

The results presented in this report are important and as such they should help science to investigate the neurobiological and neurochemical bases of those diseases: short and mid-term and Help Society and Health Community to provide patients with better treatment: long-term.

Despite the non achievement of all the deliverables all information generated have paved the ways for very interesting follow-up as this will survive the end of the collaboration due soon but also are expected to impact positively for anxiety, psychotic states, autism, cognitive deficits, etc.

Fundamentally the results open the way for an extension of in vivo siRNA/shRNA methods and application to further specific disease states.

Despite the fact that the transgenic cell line was not generated by the end of this collaborative effort, it is foreseen that interesting and promising follow-up studies may emerge in the near future which may impact positively on the outcome of research on the neurobiological and neurochemical bases of psychiatric disorders.

These results hold the promises for high impact publications and already high visibility during important international meetings.

All members of the consortium active in the AGLAEA project are grateful to EEC for allowing exploration of basic Science that will feedback to help Public Health and especially Mental Health.