Cloning and Characterisation of *GRIPE*, a Novel Interacting Partner of *E12* During Brain Development

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A thesis submitted in total fulfilment of the requirements for the degree of Doctor of Philosophy (PhD)

October, 2002

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Scientific Abstract

The mammalian cerebral cortex is a remarkable product of brain evolution, and is the structure that most distinctively delineates the human species from others (Northcutt and Kaas, 1995; Rakic, 1988). Neurons in the adult brain are organised into cytoarchitectonic areas, defined by distinct biochemical, morphological and physiological characteristics (Rakic 1988). Remarkably, this complex structure is generated from a simple neuroepithelium.

What are the signalling mechanisms that direct neuron formation and subsequent functional-parcellation of the cerebral cortex? Key to the study of this process is an understanding of neuronal fate determination. Available evidence demonstrates an intrinsic programming potential by neuronal progenitors within subdomains of the developing cerebral cortex that is instructive for proper corticogenesis. These regional domains are demarcated by expression of certain transcription factors, including members of the Helix-Loop-Helix (HLH) family of proteins.

The HLH family of transcription factors are key contributors to a wide array of developmental processes, including neurogenesis and haematopoiesis. These factors are thought to exert their regulatory influences by binding to cognate promoter-DNA sequences as dimers. While studies in mice have convincingly demonstrated that neurogenic HLH proteins such as NeuroD (Lee et al., 1995; Miyata et al., 1999; Liu et al., 2000) and Mash1 (Casarosa et al., 1999) are intimately involved in neuronal fate determination and terminal differentiation, the role of the ubiquitously expressed HLH protein, E12, in mammalian neurogenesis remains ambiguous. Originally discovered as an important regulator of lymphopoiesis, expression studies revealed its widespread expression in proliferative zones of multiple nascent organs of the embryo, including the developing cerebral cortex; implying a role for E12 in development.
of the nervous system. Since the function of $E12$ is, in part, coded by its capacity for protein dimerisation, a search was undertaken for binding partners in developing mouse brain, and using a yeast 2-hybrid assay.

Yeast 2-hybrid prey libraries were constructed using complementary DNA (cDNA) isolated from embryonic mouse forebrain tissue at early (embryonic day e11.5) and peak (e15.5) stages of neurogenesis. Screening of these libraries for binding partners to an E12 bait resulted in cloning of HLH factors, such as $Mash1$, $NSCL$ and $Id2$. Importantly, a novel binding partner, named $GRIPE$, was cloned as a novel GAP Related Interacting Protein to $E12$. $GRIPE$ binds to the HLH region of E12, and may require E12 for nuclear import. Furthermore, GRIPE may negatively regulate E12-dependent target gene transcription. High levels of $GRIPE$ and $E12$ mRNA were coincidently detected during embryogenesis, but only $GRIPE$ mRNA levels remained high in adult brain, particularly in neurons of the cortex and hippocampus. These observations were reconfirmed through an in vitro model of neurogenesis. Taken together, these results indicate that GRIPE is a novel protein whose dimerisation with E12 has important consequences for cells undergoing neuronal differentiation. A model is proposed to suggest how neurogenic HLH proteins that dimerise to E12 may promote signalling cascades driving early neuroblast differentiation.
Lay Abstract

The mammalian forebrain is a remarkable product of evolution, and is the structure that most distinctively delineates the human species from others. Comprising of two main cell types, neurons and glia, this organ is structurally and functionally subdivided into regions for coordinating the senses such as sight, smell, taste and hearing. Remarkably, this complex structure is generated from an outwardly simple sheet of embryonic brain cells, which exhibits no peculiar physical attributes early in development.

What then are the mechanisms that direct brain formation in the embryo? Over the last 100 years, scientists working with model organisms such as fruit flies, frogs and mice have discovered that development of the nervous system involves a precise co-ordination of signals received by embryonic cells, followed by interpretation of these signals through mechanisms within the cell that lead to acquisition of a brain-cell identity. Thus, one facet of the study of brain development is to understand “within-cell” signalling mechanisms, through identification of specific genes that program brain development.

Genes are functional products encoded by our deoxyribonucleic acid (DNA) complement, otherwise known as our genome. These products of the genome orchestrate the necessary activities in every cell of the body, such as energy production, water and electrolyte balance, and cell division. In immature cells of the developing embryonic brain, certain genes function as regulatory switches that trigger or silence the activities of other genes, a function crucial to endowing a brain-cell identity. These genes are known as transcription factors, and can influence the developmental outcome of embryonic cells. Examples of transcription factors include members of the Helix-Loop-Helix (HLH) gene family.
The HLH family of transcription factors comprise members that can behave as “master regulatory switches”, directing embryonic cells to become the functional units of adult organs such as blood, brain, muscle or pancreas. While the function of brain-specific HLH factors, such as *NeuroD*, is readily examined through existing molecular biological approaches, the role of the HLH transcription factor *E12* in brain development is not clear. Although this gene is “switched on” in the immature brain cell and “switched off” in the adult neuron, its function in the early steps of neuron formation remains ambiguous. Since part of the function of *E12* is dependent upon its interaction with other genes, a search was conducted for key partners to this transcription factor in developing brain, and using the mouse as a model organism to study mammalian development.

This thesis details a search for genes that interact with *E12* in developing mouse forebrain. This has led to identification of several HLH genes known to orchestrate brain development, such as *Mash1* and *Id2*. Most importantly, a novel gene named “GRIPE” that interacts with *E12*, has been cloned. By virtue of its presumed function, the acronym was conceived for this GAP-Related Interacting Partner to *E12*.

The evidence presented in this thesis demonstrates that GRIPE is found in the same embryonic brain cells that express *E12*, and their combined function may be important for triggering or silencing the activities of other genes during brain development. Further, while *E12* is “switched off” in mature neurons of the adult brain, GRIPE is still found in these, suggesting a role for this novel gene in mature brain cells that is independent of *E12*. Finally, an evaluation of the genomes of the fly, mouse and human has revealed that the functions of GRIPE and *E12* may constitute an ancient genetic circuit that is conserved during evolution of the nervous systems of these complex organisms. Taken together, this thesis presents vital new data that clarifies the contribution of *E12* to brain development, and furthers the understanding of the functions of HLH transcription factors in mammalian brain.
Declaration

I declare that the work presented in this thesis is my own work except where due acknowledgment has been made in the appropriate body of text. This work has not been submitted, either in whole or in part, for the award of a degree at this university or any other institution of higher education. This thesis is less than 100,000 words in length exclusive of tables, bibliographies and appendices, and complies with the stipulations set out for the degree of Doctor of Philosophy by the University of Melbourne.

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The University of Melbourne.
Acknowledgements

“And science is difficult. Any commonsensical view of the world does not fit with science. Science is peculiar and it is not natural.” (Lewis Wolpert, University College, London)

First and foremost, I want to acknowledge the Heng family, as well as the “extended” Chan family for undying love and recreation, be it gastronomical, social, musical or “mahjong-ical”.

To my partner, Cheryl, whose unconditional love and support has been the most vital reagent in all my experiments.

To my parents, Kevin and Ping, whose collective foresight has given rise to opportunities otherwise unavailable to us children.

To my brothers, Siong and Elvin - two beacons of inspiration and boundless courage.

This work would not have been possible without continual generosity of time and resources from my supervisor, Associate Professor Seong-Seng Tan. I sincerely thank you for your tutelage.

To my colleagues in the Brain Development Group at the Howard Florey Institute: Jenny Gunnersen, Vicki Hammond, Tony Hannan, Qian Sang, Violeta Spirkoska, Christopher Job, Helen Valcanis, Leanne Godinho, Elisa Hill, Frank Weissenborn, Cheryl Augustine, Mary Kim, Bronwym Kenoshole, Wee Ming Boon, Irene Koukoulas. Thank you, Chris, for your friendship and impeccable clown skills.

Finally, I would like to extend a general “thank you” to all colleagues, past and present, who have assisted with experimental protocols, reagents and equipment.
Publications

Papers published in International Scientific Journals


**Heng, J. I.** and Tan, S-S. (2003). The role of Class I HLH genes in neural development – have they been overlooked? *Bioessays. (In press)*

Publications

Papers published in Conference Proceedings of International and National Scientific Societies

Poster presentations:


Oral presentations:

**Heng J. I. T.** and Tan, S-S.  Identification and expression of *GRIPE*, a novel interacting partner to the transcription factor *E12*.  *Liana Colvill and Ayse Berke Travel Award for the top oral presentation of 2001*, Howard Florey Institute, Melbourne, Australia, June 2002.

### Abbreviations

<table>
<thead>
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<th>Description</th>
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<tbody>
<tr>
<td>18S rRNA</td>
<td>18S ribosomal RNA</td>
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<tr>
<td>Å</td>
<td>angstrom</td>
</tr>
<tr>
<td>AMV</td>
<td>avian myeloblastosis virus</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>ara-c</td>
<td>cytosine arabinoside</td>
</tr>
<tr>
<td>β-gal</td>
<td>β-galactosidase</td>
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<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>C. elegans</td>
<td>Caenorhabditis elegans</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>calcium chloride</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin dependent kinase</td>
</tr>
<tr>
<td>CDKI</td>
<td>Cyclin dependent kinase inhibitor</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CE/Da</td>
<td>homologue of daughterless in C. elegans</td>
</tr>
<tr>
<td>ChIP</td>
<td>chromatin immunoprecipitation assay</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CO-IP</td>
<td>coimmunoprecipitation</td>
</tr>
<tr>
<td>cRNA</td>
<td>complementary RNA</td>
</tr>
<tr>
<td>Cx</td>
<td>cortex</td>
</tr>
<tr>
<td>D. melanogaster</td>
<td>Drosophila melanogaster</td>
</tr>
<tr>
<td>dATP</td>
<td>deoxyadenosine triphosphate</td>
</tr>
<tr>
<td>DBD</td>
<td>DNA-binding domain</td>
</tr>
<tr>
<td>dCTP</td>
<td>deoxycytosine triphosphate</td>
</tr>
<tr>
<td>DDW</td>
<td>distilled deionised water</td>
</tr>
<tr>
<td>dGTP</td>
<td>deoxyguanosine triphosphate</td>
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<tr>
<td>DIG</td>
<td>digoxigenin</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified essential medium</td>
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<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<td>deoxyribonuclease</td>
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<tr>
<td>dNTP</td>
<td>deoxynucleotide triphosphate</td>
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<td>dsDNA</td>
<td>double stranded DNA</td>
</tr>
<tr>
<td>dTTP</td>
<td>deoxythymidine triphosphate</td>
</tr>
<tr>
<td>E. coli</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>E2-2</td>
<td>also known as ME2</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene diamine tetra acetate</td>
</tr>
<tr>
<td>EGFP</td>
<td>Enhanced green fluorescent protein</td>
</tr>
<tr>
<td>EST</td>
<td>Expressed Sequence Tags</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
</tr>
<tr>
<td>GAD67</td>
<td>glutamic acid decarboxylase, 67 kDa isoform</td>
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<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
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<tr>
<td>GAPDH</td>
<td>glycerdehyde 6-phosphate dehydrogenase</td>
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<td>GDP</td>
<td>guanine diphosphate</td>
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<td>GE</td>
<td>ganglioside eminence</td>
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<tr>
<td>GEF</td>
<td>guanine nucleotide exchange factor</td>
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<tr>
<td>GR-1</td>
<td>GRIPE-related 1</td>
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<tr>
<td>GRIPE</td>
<td>GAP related interacting partner to E12</td>
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<tr>
<td>GST</td>
<td>glutathione S-transferase</td>
</tr>
<tr>
<td>GTPase</td>
<td>guanine triphosphate phosphatase</td>
</tr>
<tr>
<td>H. sapiens</td>
<td>Homo sapiens</td>
</tr>
<tr>
<td>HAT</td>
<td>histone acetyl transferase</td>
</tr>
<tr>
<td>HEB</td>
<td>also known as ME1</td>
</tr>
<tr>
<td>HEK293T</td>
<td>human embryonic kidney 293T cell line</td>
</tr>
<tr>
<td>HLH</td>
<td>Helix-loop-helix</td>
</tr>
<tr>
<td>ID</td>
<td>inhibitor of dimerisation</td>
</tr>
<tr>
<td>kDa</td>
<td>kiloDaltons</td>
</tr>
<tr>
<td>M. musculus</td>
<td>Mus musculus</td>
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<tr>
<td>Mash1</td>
<td>Mammalian achaete-scute homologue-1</td>
</tr>
<tr>
<td>Math1</td>
<td>Mammalian atonal homologue-1</td>
</tr>
<tr>
<td>MCK</td>
<td>muscle creatine kinase</td>
</tr>
<tr>
<td>µg</td>
<td>microgram</td>
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<tr>
<td>MgCl₂</td>
<td>magnesium chloride</td>
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<tr>
<td>min</td>
<td>minutes</td>
</tr>
<tr>
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<td>microlitre</td>
</tr>
<tr>
<td>µM</td>
<td>micro molar</td>
</tr>
<tr>
<td>mM</td>
<td>milli molar</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>NCBI</td>
<td>National Center for Biotechnology Information</td>
</tr>
<tr>
<td>Ngn1</td>
<td>neurogenin 1</td>
</tr>
<tr>
<td>Ngn2</td>
<td>neurogenin 2</td>
</tr>
<tr>
<td>NSCL</td>
<td>Neuronal Stem Cell Ligand</td>
</tr>
<tr>
<td>ONPG</td>
<td>o-Nitrophenyl β-D-galactopyranoside</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PNS</td>
<td>peripheral nervous system</td>
</tr>
<tr>
<td>pRB</td>
<td>Retinoblastoma protein</td>
</tr>
<tr>
<td>RA</td>
<td>all-trans retinoic acid</td>
</tr>
<tr>
<td>Rap</td>
<td>Ras-associated protein</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNase</td>
<td>ribonuclease</td>
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<tr>
<td>RT-PCR</td>
<td>reverse transcriptase-polymerase chain reaction</td>
</tr>
<tr>
<td>SAGE</td>
<td>serial analysis of gene expression</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SSC</td>
<td>sodium citrate, sodium chloride buffer</td>
</tr>
<tr>
<td>TAD</td>
<td>transcriptional activation domain</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-acetate buffer containing EDTA</td>
</tr>
<tr>
<td>Taq</td>
<td>Thermus aquaticus DNA polymerase</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline containing Tween 20</td>
</tr>
<tr>
<td>TH</td>
<td>tyrosine hydroxylase</td>
</tr>
<tr>
<td>Ubc9/UBCE2A</td>
<td>ubiquitin conjugating enzyme to E2A</td>
</tr>
<tr>
<td>UTP</td>
<td>uridine triphosphate</td>
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<tr>
<td>X-gal</td>
<td>5-bromo-4-chloro-3-indolyl β-D-galactopyranoside</td>
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Author/s:
Heng, Julian Ik Tsen

Title:
Cloning and characterisation of gripe: a novel interacting partner of e12 during brain development

Date:
2002-10

Citation:

Publication Status:
Unpublished

Persistent Link:
http://hdl.handle.net/11343/39471

File Description:
Front

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