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Targeting *Homer* genes using adeno-associated viral vector: lessons learned from behavioural and neurochemical studies

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Abstract

Over a decade of in-vitro data support a critical role for members of the Homer family of postsynaptic scaffolding proteins in regulating the functional architecture of glutamate synapses. Earlier studies of *Homer* knockout mice indicated a necessary role for *Homer* gene products in normal mesocorticolimbic glutamate transmission and behaviours associated therewith. The advent of adeno-associated viral vectors carrying cDNA for, or short hairpin RNA against, specific Homer isoforms enabled the site-directed targeting of Homers to neurons in the brain. This approach has allowed our groups to address developmental issues associated with conventional knockout mice, to confirm active roles for distinct Homer isoforms in regulating glutamate transmission *in vivo*, as well as in mediating a variety of behavioural processes. This review summarizes the existing data derived from our studies using adeno-associated viral vector-mediated neuronal targeting of *Homer* in rodents, implicating this family of proteins in drug and alcohol addiction, learning/memory and emotional processing.

Keywords

adeno-associated viral vector; addiction; anxiety; glutamate; hippocampus; Homer proteins; knockout; memory; mouse; nucleus accumbens; RNA interference

Molecular biology of Homer proteins

In mammals, the Homer family of proteins is encoded by three independent genes (*Homer1–3*) (Brakeman *et al.*, 1997; Kato *et al.*, 1998; Xiao *et al.*, 1998; Foa *et al.*, 2005). In humans, *Homer1*, *Homer2* and *Homer3* are localized to chromosomes 5, 15 and 19, respectively (Xiao *et al.*, 1998). As their original description in the late 1990s, 21 Homer mRNAs have been isolated from rat, mouse and human brain; however, the proteins for some of these mRNAs are yet to be detected in mammalian brain tissue (Berke *et al.*, 1998; Soloviev *et al.*, 2000; Saito *et al.*, 2002). The mammalian genes encoding Homer proteins have open-reading frames that spread over 10 exons (Fig. 1), the transcription of which results in both constitutively expressed, as well as immediate early gene (IEG) products (Brakeman *et al.*,

1997; Kato *et al.*, 1998; Bottai *et al.*, 2002; Klugmann *et al.*, 2005b). The 5' untranslated region (UTR) and the translational initiation codon ATG are contained in exon 1 and upstream of this initiation codon lie a number of transcription factor-binding sites, including: specific promoter 1, activator protein 1, GATA, octamer recognition site, enhancer box element and cyclic adenosine monophosphate response element (CRE) (Bottai *et al.*, 2002), indicating that *Homer* gene transcription can be regulated by the activation of IEGs, mitogen-activated protein kinase cascade, as well as by CRE-binding protein.

With the exception of the recently characterized Homer1g (Klugmann *et al.*, 2005b), exons 2–5 of the *Homer* genes encode an enabled/vasodilator-stimulated phosphoprotein homology 1 (EVH1) domain (Gertler *et al.*, 1996; Sun *et al.*, 1998; Beneken *et al.*, 2000), which exhibits a RxxxxxGLGF sequence that is common to most PDZ (postsynaptic density 95/*Drosophila* discs large tumour suppressor gene/*Zona occludens*-1) domains mediating protein–protein interactions (Kim and Sheng, 2004). The EVH1 domain exhibits a high degree of similarity across *Homer* isoforms and is essential for *Homer* interactions with a proline-rich sequence (PPSPF) displayed by proteins regulating neuronal morphology, synaptic architecture and glutamate receptor signalling/intracellular calcium dynamics, including the mGluR1a and mGluR5 subtypes of group 1 metabotropic glutamate receptors (mGluRs) (Brakeman *et al.*, 1997; Kato *et al.*, 1998; Tu *et al.*, 1998; Roche *et al.*, 1999; Ango *et al.*, 2000; Soloviev *et al.*, 2000), the *N*-methyl-D-aspartate (NMDA) glutamate receptor scaffolding protein Shank (Naisbitt *et al.*, 1999; Tu *et al.*, 1999; Shiraishi *et al.*, 2003), the inositol-1,4,5-triphosphate (IP3) receptor, a downstream mediator of group 1 mGluR signalling (Tu *et al.*, 1998; Shin *et al.*, 2003; Yuan *et al.*, 2003; Nakamura *et al.*, 2004; Hwang *et al.*, 2005; Sala *et al.*, 2005), F-actin (Shiraishi *et al.*, 1999; Sala *et al.*, 2001; Usui *et al.*, 2003; Inoue *et al.*, 2004; Kuriu *et al.*, 2006; Ajima *et al.*, 2007), phosphoinositide 3 kinase enhancer-long (Rong *et al.*, 2003), ryanodine receptors (Feng *et al.*, 2002, 2008; Westhoff *et al.*, 2003; Hwang *et al.*, 2005; Huang *et al.*, 2007), transient receptor potential canonical-1 and 3 ion channels (Yuan *et al.*, 2003; Kim *et al.*, 2006) and most recently diacylglycerol lipase- α (Jung *et al.*, 2007). As illustrated in Fig. 1, exons 6–10 encode the carboxy tail of a majority of *Homer* proteins that consists of a coiled-coil (CC) domain, 2 leucine zipper motifs and the 3' UTR (Kato *et al.*, 1998; Xiao *et al.*, 1998; Soloviev *et al.*, 2000). In contrast to the high (about 80%) sequence homology within exons 2–5 of the three *Homer* genes, exons 6–10 exhibit low homology (about 20–30%) (Xiao *et al.*, 1998; Soloviev *et al.*, 2000). Through interactions via their CC/leucine zipper motifs (Kato *et al.*, 1998; Sun *et al.*, 1998; Tu *et al.*, 1998; Xiao *et al.*, 1998; Soloviev *et al.*, 2000; Hayashi *et al.*, 2006), *Homer* proteins form tetramers, oriented in parallel, and this structure confers slower turn-over rates and greater efficiency of protein localization to dendritic spines (Hayashi *et al.*, 2006).

Although alternative transcript splicing in regions downstream from exon 5 and premature termination of gene transcription has been reported for all three *Homer* genes, this phenomenon has been best described for the *Homer1* gene (Brakeman *et al.*, 1997; Xiao *et al.*, 1998; Soloviev *et al.*, 2000; Bottai *et al.*, 2002). The premature termination of *Homer1* transcription results in truncated or 'short' *Homer* isoforms that lack the CC and leucine zipper motifs necessary to multimerize (Fig. 1), of which Homer1a and ania-3 have been identified in brain (Brakeman *et al.*, 1997; Berke *et al.*, 1998; Soloviev *et al.*, 2000).

Although the precise mechanisms underlying the premature termination of *Homer1* transcription are unknown, a transcriptional stop codon lies within intron 5 (Bottai *et al.*, 2002) and it appears that *Homer1a* mRNA terminates approximately 4.4 kb into intron 5, whereas ania-3 is generated by alternative transcript splicing from exon 5 into an intron 5 sequence that lies approximately 5.7 kb downstream of the polyadenylation site for *Homer1a* mRNA (Bottai *et al.*, 2002). Although not likely involved in premature termination of transcription, in both the cases of *Homer1a* and ania-3, the mRNA contains several AUUUA repeats at their 3' UTRs (Bottai *et al.*, 2002). This feature may be responsible for destabilizing interactions with ribosomal translational machinery and contribute to the characteristic IEG-like, fast decay of mRNA transduction exhibited by these two *Homer* isoforms (Bottai *et al.*, 2002).

As there is no pharmacological tool currently available for studying *Homer* function, the majority of in-vivo studies have relied on complementary gene knockout (KO) and virus-mediated gene delivery approaches. This review describes the use of recombinant adeno-associated viral vector (AAV) technology for investigating *Homer* protein function *in vivo* and summarizes the current status of our knowledge regarding the various roles played by members of the *Homer* protein family in regulating brain and behaviour as derived using this complementary strategy.

Adeno-associated viral vector as a genetic tool to study *Homer* proteins

Being capable of easily and efficiently interfering with central nervous system (CNS), gene expression is essential for defining the roles of genes in the many functionally distinct brain regions. Although traditionally the generation of transgenic rodents has been the system of choice for this functional genomics approach, viral vectors have become increasingly popular to transfer genes in the CNS (Janson *et al.*, 2001). Viral vectors can be complementary to conventional mouse transgenics created by gene targeting using homologous recombination in embryonic stem cells or pronucleus injection, but one of their most striking advantages is that they are not limited to mice alone. Virus-based somatic gene transfer can be used in species in which embryonic stem cell lines are not available. Indeed, viral vectors have been instrumental in rats and primates, in which the physiology is well established and behavioural paradigms are well defined (Jacob, 1999). Although both are stand-alone technologies, the introduction of viral vectors to existing transgenic lines is providing novel tools for fine dissection of gene function (Kaspar *et al.*, 2002).

Germ line transgenic technologies offer targeted and/or inducible mutagenesis of virtually any gene, but are laborious, slow, expensive and limited to mice. Spatio-temporal control is possible but hard to achieve. In comparison, viral vectors are potentially effective in all mammals, fast, specific, inexpensive and allow easy spatio-temporal control. Somatic gene delivery, however, requires invasive surgery, brain-wide expression is difficult to achieve, and the genetic material is limited to packaging size. Viral vectors are derived from common human viral pathogens and exploit the natural ability of viruses to infect mammalian cells. Genetically modified to carry a transgene cassette and deposit it within a cell, recombinant vectors are engineered for a single round of host cell infection by the removal of viral genes involved in replication.

More than 10 different vector types are in use now. Simple viruses, such as adenovirus or adeno-associated virus, consist of genetic material encased within a protective protein coat called a capsid. In contrast, enveloped virions, including retrovirus, lentivirus and herpes simplex virus, bear capsids surrounded by a lipid bilayer envelope (Davidson and Breakefield, 2003). They all differ with regard to their genetic material, size and natural tropism (target cell specificity). On account of the specific binding to cell-surface receptor elements on the target cells, and internalization properties of proteins on the virus capsid or envelope, some brain cells are intrinsically more susceptible to infection with certain vectors. An ideal viral vector for gene delivery to the CNS should have the following characteristics (Somia and Verma, 2000): (i) infection of nondividing cells because neurons are postmitotic; (ii) large packaging size capacity to be able to transfer large cDNAs; (iii) nonimmunogenicity to avoid a neutralizing immune response that would result in reduced transgene expression; (iv) tissue specificity to minimize side effects owing to off-target transduction; (v) sustained or regulated transgene expression for potentially lifelong replacement of a nonfunctional gene; (vi) simple production for generation of high titre vector stocks; and (vii) episomal nuclear localization to avoid random mutagenesis or position effects. Currently, no vector that meets all these criteria is found but the recombinant AAV comes closest.

Adeno-associated viral vector biology

Wild-type AAV (wtAAV) is a nonpathogenic member of *Parvoviridae* and shows a broad tropism among mammalian cells (Wu *et al.*, 2006). Natural replication deficiency and interdependence on additional viruses, usually adenovirus or herpes virus, classify AAV as a dependo-virus. Members of this family of small animal viruses have a single-stranded DNA genome that shares a similar genomic organization. All AAVs have a genome of about 4.7 kb, which is flanked by two palindromic hairpin sequences known as inverted terminal repeats (ITRs). wtAAV encodes four replication and three capsid proteins from two open-reading frames by efficiently combining differential splicing and alternative start codon usage. The viral genome is encased by a capsid that consists of 60 components arranged in isocahedral symmetry. These components, referred to as viral proteins 1–3, are represented in the capsid at a ratio of 1 : 1 : 18. AAVs are formally grouped into serotypes. By definition, a serotype is a newly described virus that does not efficiently cross-react with neutralizing antibodies specific to all characterized serotypes. AAV serotype 2 (AAV2) was the first infectious clone established in the laboratory (Samulski *et al.*, 1982), which made this serotype the best studied and the most commonly used one to date. As the hallmark paper by Kaplitt *et al.* (1994) showed safe and efficient AAV2-mediated CNS gene transfer, AAVs have become increasingly popular in CNS gene delivery applications because of their lack of pathogenicity, neurotropism and ability to establish sustained transgene expression with very little tendency to integrate into the genome of the host cell (Schnepp *et al.*, 2005).

Adeno-associated viral vector tropism in the central nervous system

Different AAV serotypes target diverse cell types in the CNS because they enter cells via distinct cell surface receptors (Burger *et al.*, 2005). Typically, nonenveloped viruses bind cell surface glycosaminoglycan receptors. Secondary interactions of viral capsid proteins with coreceptors seem to be pivotal for internalization. Heparan sulphate proteoglycan is the

primary receptor for AAV2 (Summerford and Samulski, 1998), whereas human fibroblast growth factor receptor 1 (Qing *et al.*, 1999), $\alpha V\beta 5$ integrin (Summerford *et al.*, 1999) and hepatocyte growth factor (Kashiwakura *et al.*, 2005) act as coreceptors. AAV2 was the first AAV to be used in the brain and its pattern of transduction has been characterized in full detail (Xu *et al.*, 2001; Burger *et al.*, 2004). The membrane-bound heparan sulphate proteoglycan receptor of AAV2 is present throughout the brain and on the surface of neurons and glia (Fuxe *et al.*, 1994). In recent years, several AAV serotypes have revealed distinct patterns of transduction within the CNS. In general, AAV1 exhibits higher transduction frequencies than AAV2 in all CNS regions tested (Wang *et al.*, 2003; Burger *et al.*, 2004).

To combine the beneficial properties of both parental serotypes, we modified our AAV protocol (During *et al.*, 2003) to produce mosaic AAV1/2 vectors (Hauck *et al.*, 2003; Klugmann *et al.*, 2005a, b, 2006). These vectors could still be purified using heparin columns, and the neuronal tropism *in vivo* was enhanced because of the AAV2-specific and AAV1-specific properties, respectively. Assessment of the transduction profiles of AAV2 and AAV1/2 *in vivo* showed that AAV1/2 was superior over AAV2 in terms of viral spread and efficacy (Richichi *et al.*, 2004), with a strictly neuronal tropism (Klugmann *et al.*, 2005a; Franich *et al.*, 2008). Consequently, AAV1/2 Homer vectors were used in all studies described in this review.

Despite the different pathways for cell entry used by distinct AAV, all serotypes tested in CNS applications to date preferentially target to neurons. Hence, a promoter that drives neuron-specific expression is not necessarily required and in the light of the tight packaging size limit, the promoter of choice for CNS delivery should be strong, small and constitutively active.

Design of expression cassettes

AAVs lack any viral coding sequences because the latter have been replaced with an expression cassette for the gene of interest (Samulski *et al.*, 1987), that is, a Homer cDNA, or a reporter gene (Fig. 2). The ITRs, which flank the wtAAV genome and are critical in the process of excising it in the presence of a helper virus, however, need to be presented in the AAV expression cassette. Despite a weak intrinsic promoter activity of the ITR (Flotte *et al.*, 1993), the minimum requirements of an AAV expression cassette are a promoter, a transgene and a polyadenylation site, flanked by ITRs.

Critical to the success of AAV as a gene transfer vector into the brain is the design of the expression cassette, which once delivered by the AAV, maintains control over the level and duration of transgene expression within the tissue. The choice of promoter often depends upon a compromise between the levels of expression required, the target cell type and the size of the promoter that can be accommodated by the cassette without overstressing the 4.7-kb AAV packaging limit (Dong *et al.*, 1996). This packaging limit can be considered the largest disadvantage of the AAV system, as cell type-specific regulation of transgene expression is usually achieved by large *cis*-acting elements, which cannot be accommodated within the small AAV particles. Therefore, it might not be possible to restrict AAV-mediated expression to specific subsets of neuronal cells through promoter targeting. The 1.7-kb cytomegalovirus-enhancer/chicken β -actin (CBA) promoter has been shown to facilitate a

high level of AAV-mediated gene expression in many brain areas (Doll *et al.*, 1996; Kaemmerer *et al.*, 2000; Klein *et al.*, 2002, 2008; Broekman *et al.*, 2006). In our expression cassettes, we have used a shorter 1.1 kb CBA promoter composed of 266 bp of the cytomegalovirus immediate early enhancer and a 410-bp sequence containing exon 1 of CBA, a hybrid CBA/rabbit β -globin intron and the 5' end of a rabbit β -globin exon. A multiple cloning site allows insertion of a cDNA of choice (Fitzsimons *et al.*, 2002). The woodchuck posttranscriptional regulatory element is a *cis*-acting RNA element originating from the woodchuck hepatitis B virus, which facilitates cytoplasmic accumulation and stability of RNA (Donello *et al.*, 1998). Insertion of woodchuck posttranscriptional regulatory element into an AAV cassette between the transgene and the polyadenylation signal produced 5–10-fold higher expression levels *in vivo* (Loeb *et al.*, 1999; Paterna *et al.*, 2000; Xu *et al.*, 2001; Klein *et al.*, 2002). Our AAV expression cassette is flanked by AAV2 ITRs that are known to cross-package efficiently. Using this strong expression cassette packaged in high-titre, high-capacity AAVs results in transduction of over 2×10^5 neurons *in vivo* per microliter of vector (Xu *et al.*, 2001).

To combine the powerful RNA interference technology with the versatile AAV system, we added to our standard expression cassette the reporter gene encoding green fluorescent protein, and the mouse U6 RNA polymerase III (U6) promoter upstream of the CBA promoter, respectively (Fig. 2). A set of unique enzyme recognition sites at the 3' end of the U6 promoter allows insertion or the simple replacement of short hairpin RNA (shRNA) cassettes. We have earlier used this technology to specifically silence Homer 1a in the mouse (Tappe *et al.*, 2006) and other genes in the rat (Franich *et al.*, 2008). We have identified target sequences producing robust knockdown *in vitro* and an approximately 50% protein knockdown *in vivo* (Fig. 3) and are now using this strategy to silence the expression of CC-Homers in both mice and rats.

Homer proteins regulate the functional architecture of glutamate synapses

Within the postsynaptic density (PSD), CC-Homer proteins colocalize with both group 1 mGluR/IP3R clusters, as well as F-actin/Shank/PSD-95/guanylate kinase-associated protein/NMDA receptor clusters (Fig. 4) (Tu *et al.*, 1998, 1999; Xiao *et al.*, 1998; Shiraishi *et al.*, 2003; Usui *et al.*, 2003) and Homer proteins are critical for regulating not only dendrite morphology, but also the recruitment and coclustering of glutamate receptors to the PSD during neuronal development (Shiraishi *et al.*, 2003, 2004). Cotransfection of developing hippocampal neurons with Homer1b and/or the NMDA receptor-associated scaffolding protein Shank1b causes an enlargement in dendritic spine size and recruits to the PSD mGluR1/5, IP3Rs, the NR2b subunit of the NMDA receptor, as well as a number of other scaffolding and signaling molecules associated with glutamate receptors, including guanylate kinase-associated protein, PSD-95, F-actin and various proteins related to the endoplasmic reticulum cisterna (Sala *et al.*, 2001, 2003, 2005; Shiraishi *et al.*, 2003, 2004; Usui *et al.*, 2003). In cultured cells, neuronal depolarization or calcium entry via voltage-gated calcium channels induce the translocation of CC-Homers to spines and enhances Homer-NMDA coclustering (Okabe *et al.*, 2001), whereas the application of glutamate and NMDA receptor stimulation reduces CC-Homer-NMDA coclustering within the spine (Okabe *et al.*, 2001). These *in-vitro* data indicate a dynamic interplay between the kinetics of

calcium entry, Homer protein localization to the PSD and consequent effects upon neuronal morphology.

Through their EVH1 domains, Homers bind directly to a PPxxFr sequence located on the C-terminus of the group 1 mGluR subtypes mGluR1 and mGluR5, the activation of which leads to the stimulation of phospholipase C, the generation of IP3 and the subsequent mobilization of calcium from internal stores (Conn and Pin, 1997). Homers also interact with a proline-rich sequence located on IP3Rs (Tu *et al.*, 1998) and form mGluR/Homer/IP3R clusters in the perisynapse (Tu *et al.*, 1998; Sala *et al.*, 2005). These interactions place Homer proteins in a position to regulate the efficiency of IP3-mediated signalling upon group 1 mGluR stimulation. However, the results of in-vitro studies *vis-à-vis* the effects of CC-Homer and IEG-Homer expression upon group 1 mGluR trafficking and function in mature cells are mixed at best. A number of studies indicate that CC-Homers are necessary for the redistribution of group 1 mGluRs from the soma to distal dendrites (Tadokoro *et al.*, 1999; Ango *et al.*, 2000; Ciruela *et al.*, 2000; Kammermeier, 2006) and showed that CC-Homer1 isoforms are necessary for the plasma membrane localization of group 1 mGluRs, the accumulation of IP3 upon their activation, the capacity of group 1 mGluR stimulation to induce phosphoinositide 3 kinase activation and to inhibit Big K⁺ channel and calcium channel activity (Ciruela *et al.*, 2000; Kammermeier *et al.*, 2000; Rong *et al.*, 2003; Sala *et al.*, 2005; Kammermeier and Worley, 2007; Ronesi and Huber, 2008), whereas overexpression of the IEG-Homer1a decreases the size of PSD-95 and NMDA clusters, reduces α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) plasma-membrane expression, reduces both NMDA-mediated and AMPA-mediated synaptic currents and disrupts the efficiency of group 1 mGluR-mediated inhibition of ion channel activity (Sala *et al.*, 2003; Kammermeier, 2006; Kammermeier and Worley, 2007). Other investigations of both non-neuronal and neuronal cells, however, indicate that CC-Homers inhibit group 1 mGluR trafficking to the plasma membrane/receptor function and facilitate their retention in the endoplasmic reticulum (Ciruela *et al.*, 1999; Ango *et al.*, 2001, 2002; Coutinho *et al.*, 2001), whereas Homer1a exerts a dominant negative effect and enables group 1 mGluR and NMDA receptor trafficking to the cell surface, the formation of IP3 upon group 1 mGluR stimulation and constitutive group 1 mGluR activity (Ciruela *et al.*, 1999; Ango *et al.*, 2001, 2002).

The former in-vitro data are more or less consistent with what little is known regarding Homer-mediated regulation of glutamate receptor expression and function *in vivo* as derived from studies of *Homer* KO mice. Both *Homer1* and *Homer2* KO mice exhibit a blunted capacity of the group 1 mGluR agonist dihydroxyphenylglycol to elicit a rise in extracellular levels of glutamate within the nucleus accumbens (NAC) (Szumlinski *et al.*, 2004; Williams *et al.*, 2007), an effect attributed in earlier studies in the activation of mGluR1a subtype of the receptor (Swanson *et al.*, 2001). Although *Homer1* KO mice do not exhibit reduced levels of mGluR1 or mGluR5 (Williams *et al.*, 2007), *Homer2* KO mice exhibit an approximately 25% reduction in NAC mGluR1 expression (Szumlinski *et al.*, 2004), but this does not reflect a reduction in the amount of receptor protein localized to the plasma membrane (Szumlinski *et al.*, 2005b). With respect to ionotropic glutamate receptors, neither *Homer1* nor *Homer2* deletion affects the total protein expression of AMPA or NMDA receptors within striatal or frontal cortical regions (Szumlinski *et al.*, 2004; Williams *et al.*,

2007); however, *Homer2* deletion reduces the plasma membrane expression of the NR2a and NR2b subunits of the NMDA receptor within the NAC, but does not affect the plasma membrane expression of GluR1 (Szumlinski *et al.*, 2005b). The functional consequences of Homer-mediated regulation of NAC iGluR expression have yet to be elucidated *in vivo*, although the data are consistent with a preliminary report of an approximately 50% reduction in NMDA-mediated, but not AMPA-mediated, whole-cell current in hippocampal cultures derived from *Homer2* KO mice (Smothers *et al.*, 2003). However, as with any study of constitutive gene KO mice, it remains to be determined whether or not the observed abnormalities in glutamate receptor expression/function exhibited by *Homer* KOs reflect developmental compensations secondary to gene deletion or active roles for their gene products as indicated by the results of the in-vitro studies above. Moreover, if *Homer* gene products are active regulators of glutamate receptor function *in vivo*, it remains to be determined which precise gene products are responsible for this regulation. To this end, an AAV strategy has been applied to the hippocampus of rats to examine the effects of Homer1a, Homer1c and Homer1g overexpression upon protein levels of glutamate receptors and other proteins implicated in synaptic plasticity (Klugmann *et al.*, 2005b). This study showed that overexpression of none of these proteins affected mGluR1/5 levels; however, overexpression of all three Homer isoforms elevated hippocampal levels of the transcription factor CRE-binding protein, whereas Homer1a overexpression reduced IP3R and Homer1c overexpression reduced NR1 subunit expression (Klugmann *et al.*, 2005b). The latter observation is not consistent with the available data from Homer KO and antisense oligonucleotide studies (Ghasemzadeh *et al.*, 2003; Szumlinski *et al.*, 2004, 2005b), and further transgenic studies are necessary to fully comprehend the potentially diverse role of different Homer isoforms in regulating glutamate receptor function *in vivo*.

Homer proteins actively regulate mesocorticolimbic extracellular glutamate levels

AAV-mediated neuronal transduction with *Homer* cDNA has proven indispensable for elucidating the precise role for different Homer isoforms in regulating extracellular levels of glutamate within the prefrontal cortex (PFC) and the NAC – two brain regions exhibiting functional abnormalities in animal models of various neuropsychiatric disorders, including addiction/alcoholism, schizophrenia, depression and anxiety disorders. When assessed using various in-vitro microdialysis techniques, both *Homer1* and *Homer2* KO mice exhibit an approximately 50% reduction in NAC basal extracellular levels of glutamate (Baker *et al.*, 2003; Szumlinski *et al.*, 2006). Although an intra-NAC infusion of AAVs carrying *Homer1a*, *Homer1c* or *Homer2b* does not alter basal glutamate content in wild-type (WT) mice or experimentally naive rats (Szumlinski *et al.*, 2004, 2006), AAV-Homer2b normalizes glutamate levels in Homer2 KO mice (Szumlinski *et al.*, 2004) and both AAV-Homer1c and AAV-Homer2b reverse the drop in NAC basal glutamate content observed during withdrawal from repeated cocaine administration (Szumlinski *et al.*, 2006). Together, these data support active and/or necessary roles for Homer1c and Homer2b in maintaining basal NAC glutamate content. Within the PFC, *Homer1* deletion elevates glutamate content by approximately 50% (Szumlinski *et al.*, 2004, 2005a; Lominac *et al.*, 2005) and AAV-mediated transfection of PFC neurons with *Homer1c*, but not *Homer1a*, restores PFC basal

glutamate levels to that of WT controls, implicating the Homer1c CC-isoform in regulating extracellular glutamate levels within this region (Lominac *et al.*, 2005). Our earlier studies of *Homer2* KO mice failed to indicate a necessary role for *Homer2* gene products in maintaining PFC basal glutamate content (Szumlinski *et al.*, 2004). Recent AAV studies, however, indicated that PFC Homer2b overexpression enhances, whereas shRNA-mediated Homer2b knockdown modestly reduces PFC glutamate content (Ary, Klugmann and Szumlinski, unpublished observations). Thus, Homer2b does actively regulate PFC glutamate levels, and these AAV data highlight the importance of employing complementary transgenic strategies when attempting to ascertain the functional importance of specific proteins.

At present, it is not clear how manipulations of Homer protein expression lead to such pronounced changes in basal glutamate content within the PFC and NAC. Although evidence to date supports a potential role for Homer regulation of the sodium-independent cystineglutamate antiporter system Xc- (Szumlinski *et al.*, 2004), an anti-porter system localized primarily (although not exclusively) to glial cells (Sato *et al.*, 2002). Indeed, some evidence indicate that certain Homer isoforms are localized to glial cells (Dietrich *et al.*, 2007; Weerth *et al.*, 2007), where they regulate aspects of calcium signalling (Weerth *et al.*, 2007); however, amino acid sequence analyses of Xc- have failed to indicate a Homer-binding site (unpublished data). Thus, it is not likely that a direct Homer–Xc- interaction contributes to the regulation of extracellular glutamate levels. Other possibilities include Homer regulation of presynaptically localized group 1 mGluRs or NMDA receptors; however, no evidence to date supports the presynaptic localization of Homer proteins. Alternatively, through interactions with postsynaptically localized group 1 mGluRs or NMDA receptors, Homers might affect retrograde messenger systems including nitric oxide (via NMDA receptor activation) or 2-arachidonoylglycerol, an endocannabinoid whose production is regulated by group 1 mGluR activation in a Homer-dependent manner as illustrated in Fig. 4 (Jung *et al.*, 2007).

Coiled-coil Homer1 and Homer2 proteins are active regulators of cocaine sensitivity

Repeated cocaine exposure elicits numerous alterations in mesocorticolimbic glutamate function that include a reduction in NAC basal glutamate content (Baker *et al.*, 2003; Szumlinski *et al.*, 2006), a sensitized NAC and PFC glutamate response to a cocaine challenge injection (Pierce *et al.*, 1996, 1998; McFarland *et al.*, 2003; Williams and Steketee, 2004; Szumlinski *et al.*, 2006) and complex alterations in both the function and expression of iGluRs, mGluRs and glutamate transporters within a variety of mesocorticolimbic sites (Wolf, 1998; Carlezon and Nestler, 2002; Everitt and Wolf, 2002; Kalivas *et al.*, 2005; Steketee, 2005; Wang *et al.*, 2006). As Homer isoforms regulate both presynaptic and postsynaptic aspects of glutamatergic signaling within the NAC and PFC (de Bartolomeis and Iasevoli, 2003; Szumlinski *et al.*, 2008b), this protein family is a likely molecular candidate mediating the cocaine-induced glutamatergic abnormalities contributing to the addictive and psychomotor-activating properties of this drug (Szumlinski *et al.*, 2008b). Consistent with this notion, a single nucleotide polymorphism in *Homer1* is associated with

cocaine addiction (Dahl *et al.*, 2005). Although post-mortem studies of cocaine addicts are yet to be conducted to examine for changes in Homer mRNA/protein expression, several studies using laboratory animals indicate that both IEG and CC-Homer protein expression is regulated within forebrain structures, including the PFC, NAC and hippocampus by cocaine experience. The mRNA and protein for IEG Homer isoforms is upregulated within 1–3 h in various brain structures after acute cocaine (Ghasemzadeh *et al.*, 2006; Zhang *et al.*, 2007); however, this rise is transient, dissipating within 6–12 h after acute injection (Brakeman *et al.*, 1997; Zhang *et al.*, 2007), shows tolerance with repeated cocaine administration (Ghasemzadeh *et al.*, 2006) and thus, is not likely to contribute directly to the chronic nature of addiction. In contrast, delayed changes in CC-Homer expression are produced within the NAC, PFC and hippocampus after both repeated noncontingent cocaine administration (Swanson *et al.*, 2001; Ary and Szumlinski, 2007), as well as intravenous cocaine self-administration (Ary *et al.*, 2006; Obara *et al.*, 2007). Within the NAC, Homer1b/c and Homer2a/b isoforms are downregulated in both cocaine-injected and self-administering animals (Swanson *et al.*, 2001; Ary and Szumlinski, 2007; Obara *et al.*, 2007) and, at least in the case of cocaine-injected mice and rats, the reduction in NAC CC-Homer expression is accompanied by lowered levels of mGluR1/5 and NR2 expression (Swanson *et al.*, 2001; Ary and Szumlinski, 2007). Through a combination of gene KO and AAV approaches, we have shown that the cocaine-induced reduction in either Homer1c or Homer2b expression within the NAC is both necessary and sufficient to elicit behavioural and neurochemical sensitization to cocaine. *Homer1* and/or *Homer2* KO mice exhibit increased sensitivity to the locomotor activating, reinforcing and conditioned rewarding properties of cocaine, a behavioural phenotype accompanied by a ‘presensitized’ NAC glutamate response to the drug (Szumlinski *et al.*, 2004). Although AAV-Homer2 overexpression does not alter behavioural or neurochemical sensitivity to cocaine (Szumlinski *et al.*, 2004, 2006), restoration of Homer2b levels to the NAC of *Homer2* KO mice reverses their ‘presensitized’ phenotype (Szumlinski *et al.*, 2004). Moreover, preventing the cocaine-induced reduction in NAC CC-Homer levels through an intra-NAC infusion of either AAV-Homer1c or AAV-Homer2b prevents the expression of both behavioural and glutamate sensitization in cocaine-injected rats, whereas AAV-Homer1a infusion is without effect (Szumlinski *et al.*, 2006). Together, these data point to a cocaine-induced reduction in NAC CC-Homer1/2 expression as a critical molecular adaptation actively regulating an individual’s sensitivity to both the rewarding and psychomotor-activating properties of cocaine.

In contrast to the NAC, long-term withdrawal from either repeated cocaine injections or cocaine self-administration does not alter Homer1b/c expression within the PFC (Swanson *et al.*, 2001; Ary *et al.*, 2006; Ary and Szumlinski, 2007). The cocaine ‘presensitized’ phenotype of *Homer1* KO mice is, however, accompanied by a blunted capacity of cocaine to elevate PFC extracellular glutamate levels (Lominac *et al.*, 2005; Szumlinski *et al.*, 2005a), which presumably reflects reduced release from glutamatergic afferents to this region, as *Homer1* KO mice also exhibit significantly blunted high K⁺-stimulated glutamate release in the PFC (Williams *et al.*, 2007). To ascertain which Homer1 isoform in the PFC might contribute to the cocaine behavioural and glutamate phenotype of *Homer1* KO mice, we again used an AAV approach and showed that an intra-PFC infusion of AAV-Homer1c, but not AAV-Homer1a, could reverse the genotypic difference in acute cocaine-induced

locomotion and elevations in PFC glutamate (Lominac *et al.*, 2005). Although overexpression of neither IEG nor CC-Homer expression in WT/drug-naive animals affects cocaine's capacity to elevate NAC glutamate (Szumlinski *et al.*, 2004, 2006), intra-PFC infusions of AAV-Homer1a blunts, whereas AAV-Homer1c enhances, cocaine-induced increases in PFC glutamate levels in WT mice (Lominac *et al.*, 2005). From these latter data, we hypothesized that cocaine-induced imbalances between IEG and CC-Homer expression may contribute to the enduring abnormalities in PFC function observed in cocaine addicts (Szumlinski *et al.*, 2008b). Converging data from recent immunoblotting and AAV studies, however, point also to imbalances between PFC CC-Homer1 and CC-Homer2 expression in regulating behavioural and neurochemical sensitivity to cocaine. First, long-term withdrawal from both experimenter cocaine and self-administered cocaine elevates PFC Homer2a/b expression in rodents (Ary *et al.*, 2006; Ary and Szumlinski, 2007). Second, although *Homer2* deletion does not alter cocaine-stimulated PFC glutamate release (Szumlinski *et al.*, 2004), mimicking the cocaine-induced rise in PFC Homer2 levels via the local infusion of AAV-Homer2b blunts, whereas reducing Homer2b expression via an AAV carrying an shRNA against *Homer2b* enhances, the capacity of acute cocaine to elevate PFC glutamate levels in C57BL/6J mice (Fig. 5a). Intriguingly, the effect of PFC Homer2 overexpression upon the PFC glutamate response to acute cocaine is similar to that reported earlier for Homer1 deletion (Lominac *et al.*, 2005; Szumlinski *et al.*, 2005a), as well as that produced by PFC Homer1a overexpression in C57BL/6J-Sv129 hybrid WT mice (Lominac *et al.*, 2005). Conversely, the effect of PFC shRNA-Homer2 upon the PFC glutamate response to cocaine is similar to that reported earlier for AAV-Homer1c in WT mice (Lominac *et al.*, 2005). Thus, it would appear that the CC products of the *Homer1* and *Homer2* genes play opposing roles in regulating cocaine-stimulated glutamate release in the PFC. Finally, an intra-PFC infusion of AAV-Homer2b enhances, whereas shRNA-Homer2b does not affect, the magnitude of a cocaine-conditioned place preference (Fig. 5b). Combined with the data from *Homer2* KO mice (Szumlinski *et al.*, 2004), these preliminary data indicate that the cocaine-induced increase in PFC Homer2 expression is sufficient, but not necessary, to exhibit heightened drug-seeking behaviour and pose an important role for a relative increase in CC-Homer2 versus CC-Homer1 levels within the PFC in the neuropathology of cocaine addiction.

Coiled-coil Homer2 proteins are active regulators of alcohol-induced neuroplasticity

Alcohol is a drug of abuse that inhibits iGluR and group 1 mGluR (mGluR5) receptor function (Lovinger, 1996; Minami *et al.*, 1998; Woodward, 2000) and many of the acute behavioural effects of alcohol are related to the inhibition of glutamate receptor signaling within the mesocorticolimbic and extended amygdala circuits (Koob, 2003; Krystal *et al.*, 2003; Carpenter-Hyland *et al.*, 2004; Carpenter-Hyland and Chandler, 2007). Alcohol produces biphasic acute effects upon extra-cellular glutamate within the forebrain (Dahchour *et al.*, 1994; Moghaddam and Bolinao, 1994; Lominac *et al.*, 2006; Szumlinski *et al.*, 2007; Kapasova and Szumlinski, 2008), elevates basal glutamate content in the NAC (Dahchour *et al.*, 1998; Dahchour and De Witte, 2000; Melendez *et al.*, 2005; Szumlinski *et al.*, 2007; Kapasova and Szumlinski, 2008) and sensitizes the capacity of alcohol to elevate NAC

extracellular glutamate levels (Dahchour and De Witte, 2003a, b; Szumlinski *et al.*, 2005b, 2007, 2008a; Lominac *et al.*, 2006; Kapasova and Szumlinski, 2008). Moreover, repeated alcohol administration produces an enduring upregulation in both iGluR and mGluR expression, function as well as increased glutamate receptor trafficking to, and clustering within, the plasma membrane (Smothers *et al.*, 1997; Chen *et al.*, 1999; Tarelo-Acuna *et al.*, 2000; Carpenter-Hyland *et al.*, 2004; Carpenter-Hyland and Chandler, 2006, 2007; Hendricson *et al.*, 2007; Szumlinski *et al.*, 2007; Zhou *et al.*, 2007). Given the roles for Homer proteins in regulating these aspects of glutamate transmission, it is perhaps not surprising that alcohol also regulates the expression of Homer proteins as assessed by studies of laboratory animals. Alcohol-experienced rodents exhibit a pronounced (2.0–2.5-fold increase) and selective upregulation in Homer2a/b protein expression within the NAC after various alcohol treatment regimens. The alcohol-induced rise in NAC Homer2 levels produced by chronic (3 months) alcohol consumption under continuous access conditions is persistent, enduring for at least 2 months after the last drinking session (Szumlinski *et al.*, 2008a). As observed in our cocaine studies (Ary *et al.*, 2006; Ary and Szumlinski, 2007; Obara *et al.*, 2007), the alcohol-induced rise in Homer2a/b levels is accompanied by elevations in the total protein expression of group 1 mGluRs and/or NR2 subunits (Szumlinski *et al.*, 2008a; Cozzoli *et al.*, 2008; Obara *et al.*, 2008). These data have led to the overarching hypothesis that an alcohol-induced increase in NAC Homer/glutamate receptor expression is a neuroadaptation-mediating excessive alcohol intake. Although it remains to be determined whether or not Homer expression is altered in post-mortem brains of alcohol-dependent humans, in support of this hypothesis, basal Homer2a/b expression in the shell subregion of the NAC, but not in the core subregion or dorsal striatum, of the alcohol-preferring C57BL/6J mouse is approximately double than that observed in the alcohol-avoiding DBA2/J mouse (Goulding *et al.*, 2008).

Continuing this hypothesis, *Homer2* KO mice exhibit an alcohol-avoiding and intolerant behavioural and neurochemical phenotype that is characterized by: alcohol-conditioned place aversion, shifts to the right and down in the dose–response functions for alcohol preference and intake, increased alcohol-induced sedation, a lack of tolerance to the locomotor-inhibiting effects of alcohol upon repeated alcohol administration and a lack of alcohol-induced dopamine and glutamate sensitization in the NAC (Szumlinski *et al.*, 2005b). We used our AAV approach to confirm an active role for NAC Homer2b expression in mediating the alcohol phenotype of KO mice and showed a complete phenotype reversal (Szumlinski *et al.*, 2005b). More recently, we have used our AAV approach to mimic the alcohol-induced increase in NAC Homer2b expression in C57BL/6J mice and showed an enhancement of alcohol preference under free-access conditions in the home cage, alcohol-conditioned place-preference, lever-pressing for alcohol, response-contingent alcohol consumption and alcohol preference in the home cage (Szumlinski *et al.*, 2005b, 2008a). Moreover, NAC Homer2 overexpression in C57BL/6J mice facilitated the development of tolerance to the locomotor-impairing effects of alcohol and augmented both acute and sensitized glutamate response to the drug (Szumlinski *et al.*, 2008a). Although studies are on-going to assess the effects of NAC Homer2 overexpression and underexpression upon alcohol drinking and preference in high-drinking versus low-drinking inbred mouse strains, our preliminary data support active roles for strain differences in NAC Homer2 expression in

mediating also genetic variance in alcohol consumption (Goulding *et al.*, 2008). Collectively, the immunoblotting and behavioural genetic studies to date implicate an upregulation in NAC Homer2 expression as an important cellular adaptation to alcohol facilitating alcohol-induced changes in behaviour, including alcohol drinking. Whether or not alcohol-induced changes in Homer2a/b and glutamate receptor expression within other extended amygdala structures, such as the central nucleus of the amygdala, also (Obara *et al.*, 2008) contribute to genetic variance in alcoholism-related behaviours is a topic of current investigation in our laboratories.

Immediate early gene and coiled-coil Homer isoforms actively regulate certain forms of learning and memory

Behavioural screens of adolescent and adult *Homer1* and *Homer2* KO mice revealed striking differences in the ‘undrugged’ phenotype of these two mutant strains (Szumlinski *et al.*, 2008b). *Homer1* KO mice exhibit deficits in sensorimotor and cognitive processing that are not observed in *Homer2* KO mice. *Homer1* KO mice exhibit pronounced learning deficits during the acquisition phase of both Morris water and radial arm mazes, indicating poor reference and working memory in these mice (Szumlinski *et al.*, 2004, 2005a; Lominac *et al.*, 2005; Jaubert *et al.*, 2007). Normal working memory, at least as assessed by a water version of the radial arm maze, requires Homer1c in the PFC as AAV-mediated restoration of Homer1c, but not Homer1a, reverses radial arm maze deficits in *Homer1* KO mice (Lominac *et al.*, 2005). The learning deficits produced by *Homer1* deletion may stem from abnormalities in attentional processing as *Homer1* KO mice are poor at locating platforms upon maze rotation (Jaubert *et al.*, 2007). Moreover, when tested as adolescents or young adults, *Homer1* KO mice show deficits in prepulse inhibition of acoustic startle (Lominac *et al.*, 2005; Szumlinski *et al.*, 2005a), although this deficit may be age-dependent as another study failed to detect genotypic differences when mice were tested as older adults (Jaubert *et al.*, 2007). The deficits in prepulse inhibition exhibited by *Homer1* KO mice can also be reversed by AAV-mediated restoration of Homer1c to the PFC (Lominac *et al.*, 2005), or by systemic administration of the typical antipsychotic haloperidol (Szumlinski *et al.*, 2005a). Whether or not antipsychotic drugs may exert their ‘therapeutic’ effects via increasing PFC levels of CC-Homers remains to be determined. The majority of evidence, however, indicate little regulation of PFC IEG Homer expression by antipsychotic drug treatment (Szumlinski and Kippin, 2007), posing the regulation of CC-Homers by antipsychotic treatments as a potential mechanism contributing to their therapeutic effects.

AAV-Homer-based genetic complementation studies are yet to be performed in hippocampus of *Homer1* KO mice to assay the relative role for IEG versus CC-Homer isoforms in regulating hippocampus-dependent cognitive function. When overexpressed in the hippocampus of rats, however, Homer1g enhances, whereas Homer1c does not affect Morris water maze performance (Klugmann *et al.*, 2005b), although a recent report by Brouillette *et al.* (2007) reported that hippocampal Homer1c overexpression abolishes the memory-improving effects of gamma-amino-butyric acid (GABA) B receptor antagonists in rats pretreated with the amnesic scopolamine. With regard to IEG Homers, hippocampal Homer1a overexpression impairs Morris water maze performance (Klugmann *et al.*, 2005b).

and abolishes the memory improvement produced by GABA B receptor antagonism in scopolamine-treated rats (Brouillette *et al.*, 2007). Such data are consistent with the results of a recent studies showing that the transgenic overexpression of Homer1a within mouse forebrain impairs CA3-CA1 long-term potentiation, as well as the acquisition of a spatial working memory task (Celikel *et al.*, 2007). Moreover, these data are consistent with the recent demonstration that AAV-mediated hippocampal Homer1a overexpression produces a spatial working memory deficit akin to that exhibited by Homer1a overexpressing transgenic mice (Celikel *et al.*, 2007). However, it should be noted that although hippocampal Homer1a overexpression impairs the acquisition of spatial working memory tasks, it does not seem to impair long-term spatial reference memory (Klugmann *et al.*, 2005b; Celikel *et al.*, 2007). The role of Homer2b in regulating behavioural indices of hippocampus function remains to be determined. However, IEG and CC-Homer1 isoforms both play active, but distinct, roles in regulating specific aspects of attention, learning and memory, with the majority of data indicating that the induction of IEG Homer1 isoforms impairs, whereas the overexpression of CC-Homer1 facilitates, attentional and cognitive processing.

Immediate early gene Homer1 isoforms regulate stress and emotional behaviour

In addition to abnormalities in cognitive and attentional processing, deletion of *Homer1*, but not of *Homer2*, enhances the expression of certain anxiety-related and depression-related behaviours in mice. In comparison with WT controls, *Homer1* KO mice exhibit (i) increased reactivity to handling when experimentally naive; (ii) decreased latencies to interact with novel objects and reduced time spent in contact with novel objects; (iii) increased locomotor hyperactivity in response to novel environments and reduced habituation to a familiar environment; and (iv) decreased latencies to float and increased incidences of floating behaviour in a forced swim test (Szumlinski *et al.*, 2004, 2005a; Lominac *et al.*, 2005). Moreover, increased aggression has also been observed in Homer1 heterozygous mice, relative to WT and KO animals (Jaubert *et al.*, 2007). The 'hyperemotional' behavioural phenotype produced by *Homer1* deletion in mice seems to result from an inability to induce Homer1a in the PFC as AAV-mediated restoration of *Homer1a* to the PFC of KO mice reverses genotypic differences in a forced swim test and in locomotor hyperactivity in response to familiar environments (Lominac *et al.*, 2005). In contrast, PFC restoration of Homer1c in *Homer1* KO mice enhances genotypic differences in both paradigms (Lominac *et al.*, 2005), a finding consistent with a reduction in elevated-plus maze exploration by rats overexpressing another CC-Homer1 isoform, Homer1g, within hippocampus (Klugmann *et al.*, 2005b). Again, the behavioural phenotype produced by AAV-mediated alterations in Homer2b expression within discrete brain regions is yet to be assessed, although studies are on-going in our laboratories in this regard. The data to date, however, pose active and distinct roles for IEG and CC-Homer1 isoforms in behavioural responding to repeated stressors, whereas the induction of IEG Homer1 isoforms within cortical structures facilitates the ability to cope with stress, the overexpression of CC-Homer1 isoforms leads to behavioural debilitation. Consistent, in part, with this suggestion, prenatally stressed weanling rats exhibit enduring elevations in PFC levels of both Homer1a and Homer1b/c (Ary *et al.*, 2007). When combined with the behavioural genetic data, we suggest that a

balance in PFC levels of IEG and CC-Homer isoforms is critical for maintaining normal executive functioning and that disruption in this balance, be it by environmental (i.e., prenatal stress or drug experience) or genetic factors (i.e. polymorphisms in the gene or epigenetic regulation of gene expression), reduces executive processing and behavioural inhibition.

Conclusion/outlook

We have shown that AAV is an outstanding tool for addressing the role of Homer proteins in complex behaviours. The combination of state-of-the-art technologies such as this viral system, stereotaxic brain surgery and mouse germ line transgenics allows for exact spatio-temporal control of overexpression or knockdown, and hence dissection of gene function of specific Homer isoforms with high anatomical resolution. Are there new genetic tools on the horizon? The CRE recombinase is part of a site-specific recombination system derived from the bacteriophage P1. Two short sequences (loxP sites) are engineered to flank the target DNA. Activation of the CRE recombinase enzyme catalyses recombination between the loxP sites, leading to excision of the intervening sequences. AAV-mediated delivery of CRE provides another valuable approach to alter the mouse genome (Allison *et al.*, 2006; Monory *et al.*, 2006; Adesnik *et al.*, 2008), and this strategy will complement the existing battery of tools to dissect the role of Homer proteins *in vivo* as soon as conditional Homer mutants become available. A greater question concerns the feasibility of using AAV or other viral strategies for the treatment of chronic neuropsychiatric conditions such as addictive, psychotic and affective disorders. Although viral-mediated gene delivery approaches are being explored clinically for the treatment of single gene disorders such as cystic fibrosis (for reviews, see Rosenecker *et al.*, 2006; White and Ponnazhagan, 2006), many neuropsychiatric disorders are likely polygenetic in origin, involve gene–gene and gene–environment interactions and may result from cytogenetic (chromosomal) abnormalities, independent of specific nucleotide polymorphisms or groups of polymorphisms (aka haplotypes) (for reviews, see Kennedy *et al.*, 2003; Pickard *et al.*, 2005; Venkitaramani and Lombroso, 2007). As AAV or other currently available viral-mediated gene delivery strategies cannot target chromosomal abnormalities or haplotypes, they will not likely serve well as ‘geno-therapeutic’ interventions for complex neuropsychiatric diseases. Nevertheless, AAV and other viral-mediated gene delivery approaches serve as important and useful experimental tools for delineating the functional significance of specific gene products (or abnormal gene variants) and their application in preclinical research will continue to increase our understanding of the relevance of specific genes for the manifestation of normal and abnormal brain and behaviour.

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Fig. 1. Splice isoforms of the *Homer 1* gene. Above, structure of the rodent *Homer 1* gene comprising 10 exons. Below, transcripts of the *Homer 1* gene (adapted from Klugmann *et al.*, 2005a, b). Boxes represent exons, horizontal lines introns. An arrow at the end of exon 1 marks the common translation start of all isoforms. Shading of boxes: white, noncoding; dark grey, enabled/vasodilator-stimulated phosphoprotein homology-1 domain encoding; light grey, coiled-coil-domain encoding; hatched, Homer 1a-specific exon 5'; black, Homer 1c-specific exon 6 encoding 12 additional residues. Note: only Homer 1a, 1b, 1c mRNAs have been shown to be translated.

Fig. 2.

Adeno-associated viral vector (AAV) expression cassettes. Above, strong constitutive expression of hemagglutinine (HA)-tagged Homer proteins is ensured by the chicken β -actin (CBA) promoter and the woodchuck hepatitis postregulatory element (WPRE) followed by the bovine growth hormone (bGH) polyadenylation signal. The entire expression cassette is flanked by AAV2 inverted terminal repeats (ITRs). Below, Homer mRNA isoforms are targeted by RNA interference-mediated silencing. Knockdown and simultaneous visualization of transduced cells can be achieved by bicistronic expression of short hairpin RNAs (driven by the mouse RNAPIII U6 promoter) and humanized renilla GFP (hrGFP, driven by CBA promoter).

Fig. 3.

In-vitro and in-vivo verification of adeno-associated viral vector (AAV)-mediated Homer protein knockdown. (a) Representative immunoblots illustrating the effects of cotransfection of human embryonic kidney cells with AAVs carrying cDNA for Homer1c (H1c) and Homer2b (H2b) with two different short hairpin RNAs (shRNAs) against these cDNAs (for Homer1c: sh_H1c#1 & sh_H1c#2; for Homer2b: sh_H2b#1 & sh_H2b#2). As depicted, AAV-mediated delivery of our shRNA constructs produced robust and specific protein knockdown *in vitro*. (b) Representative immunoblot (probed with an antibody against Homer2) from the nucleus accumbens of mice infused with 0.5 μ l of the four different shRNAs or the scrambled control. Mice were killed at 1 week after infusion and immunoblotting revealed an approximately 50% reduction in total protein expression of Homer2a/b. Note that there is no off-target effect on Homer 1c, and that sh_RNA infusion does not affect the optical density of the nonspecific bands. (c) Summary of the effects of an intra-accumbens infusion of the two sh_Homer2b constructs upon Homer2a/b protein expression at 1 and 3 weeks postinfusion. These data indicate that maximal knockdown (~50%) occurs at 1 week after infusion. The data are expressed at the mean percent change ($\pm$ SEM) of the average optical density of Homer2a/b from mice infused with scrambled control or AAVs carrying shRNA against Homer1c. $n = 2$ for each of the five AAVs.

Fig. 4.

Schematic representation of some well-characterized intracellular interactions of short and long Homer isoforms. Glutamate release activates Homer-associated ionotropic *N*-methyl-D-aspartate receptors, as well as group 1 mGluRs. Receptor stimulation then instigates diacylglycerol (DAG)-mediated, calcium (Ca^{2+})-mediated, as well as phosphoinositide 3 kinase PI3K-mediated signaling, which are all dependent upon coiled-coil (CC)-Homer expression. The activation of these intracellular pathways, in turn, stimulate a number of third and fourth messenger pathways, as well as affect presynaptic neurotransmitter release. Through multimerization, CC-Homer proteins enhance the coclustering of these glutamate-signaling mechanisms and facilitate their integration within the postsynaptic neuron. The induction of immediate early gene Homers upon synaptic activation interrupts these Homer-protein interactions and deconstructs the postsynaptic density – an effect that produces a temporary reduction in glutamate signaling efficiency but enables protein trafficking and synaptic rearrangement.

Fig. 5.

Both Homer1 and Homer2 gene products actively regulate the prefrontal cortex (PFC) glutamate response to acute cocaine. (a) Striking parallels in the cocaine-induced rise in PFC extracellular glutamate levels are revealed when the effects of adeno-associated viral vector (AAV)-mediated overexpression of Homer1a and Homer1c within the PFC of WT C57BL/6J-Sv129 hybrid mice (adapted from Szumlinski *et al.*, 2005a, b) are compared with those produced by AAV-mediated overexpression and underexpression of Homer2b within the PFC of C57BL/6J mice. Overexpression of Homer1a or Homer2b markedly blunt the

capacity of cocaine to elevate PFC glutamate, whereas overexpression of Homer1c or underexpression of Homer2b potentiate cocaine-induced glutamate release within the PFC. The data represent the mean $\pm$ SEM of the area under the curve for the percentage of change from the average baseline values during a 3-h microdialysis session. * $P < 0.05$ versus control virus (GFP or empty); + $P < 0.05$ versus AAV-Homer1a or AAV-Homer2b. (b) Mimicking a cocaine-induced rise in PFC Homer2 expression using AAV-Homer2b enhances the magnitude of a cocaine-conditioned place preference, whereas short hairpin RNA (shRNA)-mediated knockdown of Homer2b is without effect. The data represent the mean $\pm$ SEM difference in the time spent on the cocaine-paired compartment before and after pairing four times with 15 mg/kg cocaine. * $P < 0.05$ versus empty; + $P < 0.05$ versus shRNA-Homer2b. Samples sizes are indicated in parentheses.