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**Differential activity by polymorphic variants of a remote enhancer that supports galanin expression in the hypothalamus and amygdala; implications for obesity, depression and alcoholism.**

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**Key words;** Galanin, transcription, enhancer, paraventricular nucleus, comparative genomics, polymorphisms, differential gene regulation, primary cell culture, magnetofection, luciferase, transgenic mouse, amygdala, alcoholism, depression, obesity.

**Abstract;** The precise expression of the galanin gene (GAL) in the paraventricular nucleus (PVN) and in the amygdala of higher vertebrates suggests the involvement of highly conserved but as yet unidentified regulatory sequences that are critical to allow the galanin gene to control alcohol and fat intake and modulate mood. We used comparative genomics to identify a remote and highly conserved sequence that lay 42kb 5' of the human GAL transcriptional start site that we called GAL5.1. GAL5.1 activated promoter activity in neurones of the PVN, arcuate nucleus and amygdala that also expressed the galanin peptide. Analysis in SHSY-5Y neuroblastoma cells demonstrated that GAL5.1 acted as an enhancer of promoter activity after PKC activation. GAL5.1 contained two polymorphisms; rs2513280(C/G) and rs2513281(A/G), that only occurred in two allelic combinations (GG or CA) where the dominant GG allele occurred in 70-83% of the human population. Recreation of these alleles in reporter constructs and subsequent magnetofection into primary rat hypothalamic neurones showed that the CA allele was 40% less active than the GG allele. Based on these observations, and considering the known roles of galanin in modulating alcohol and fat intake, we propose the hypothesis that the weaker CA allele may affect food and alcohol intake preference. Further analysis of this novel polymorphic enhancer may allow us to understand how allele specific differences in the regulation of the GAL gene may play a role in alcoholism, depression and obesity.

## **Introduction;**

The neuropeptide, galanin, is a thirty amino acid neuropeptide that is expressed in a number of discrete regions of the brain that include the paraventricular nucleus (PVN), the arcuate nucleus of the hypothalamus and the amygdala (Ceccatelli *et al*, 1992; Cortes *et al*, 1990; Kaplan *et al*, 1988; Miller *et al*, 1993a; Planas *et al*, 1994b).

Expression of galanin in the PVN has been shown to positively influence fat intake and preference in rodents (Adams *et al*, 2008; Karatayev *et al*, 2009a Kyrkouli, 1986 #7472; Karatayev *et al*, 2009b; Leibowitz, 2005, 2007; Leibowitz and Kim, 1992; Poritsanos *et al*, 2009). Furthermore, recent studies in humans have shown the presence of polymorphisms strongly associated with elevated triglyceride levels in the blood (Plaisier *et al*, 2009).

Expression of galanin in the PVN also modulates ingestion of ethanol (Karatayev *et al*, 2009b; Karatayev *et al*, 2009c; Leibowitz *et al*, 2003) and a possible involvement of the GAL gene in susceptibility to alcoholism was suggested by an association study carried out on Finnish and Plains-Indian populations (Belfer *et al*, 2006). Links between the expression of galanin in the hypothalamus and modulation of the drug reward system of the mesolimbic dopaminergic system have prompted an interest in galanin and addictive behaviours (Picciotto *et al*). Although the mechanisms are unclear, injection of galanin into the PVN increases dopamine release from

the nucleus accumbens which is the centre of the brain responsible for addictive behaviour (Rada *et al*, 1998). Thus, it is possible that mis-expression of galanin in the PVN may contribute to the rewarding effects of alcohol.

Targeted deletions of the galanin gene and pharmacological manipulation of its receptors have also been shown to influence anxiety and mood related behavior in mice (Barr *et al*, 2006; Holmes *et al*, 2003; Holmes and Picciotto, 2006; Kuteeva *et al*, 2008a; Kuteeva *et al*, 2007; Kuteeva *et al*, 2008b). Furthermore, polymorphisms in and around the GAL gene have been associated with panic disorder and anxiety in female patients (Unschuld *et al*, 2008; Unschuld *et al*, 2010). As a result of its expression in the amygdala (Miller *et al*, 1993b; Planas *et al*, 1994a) and its known role in mood modulation (Crawley *et al*, 2002; Hobson *et al*, 2006; Karlsson and Holmes, 2006; Madaan and Wilson, 2009; Paschos *et al*, 2009; Rotzinger *et al*, 2009) galanin and its three receptors have received a great deal of attention by researchers attempting to understand the role of the galaninergic system in chronic anxiety and depression and to develop novel therapies. Indeed, a recent GWAS analysis demonstrated evidence for an association between polymorphisms close to the Galanin gene locus and major depressive disorder (Wray *et al*, 2010).

Identifying and characterising the regulatory sequences responsible for delimiting the expression in the PVN and amygdala, and how these

sequences may have been altered by human variation, is a major scientific priority for those interested in the role of galanin in the control of alcohol intake, fat intake and mood modulation. For example, one SNP (rs948854) was discovered 2kb from the GAL locus and was associated with panic disorder in women (Unschuld *et al*, 2008; Unschuld *et al*, 2010). However, what effects this polymorphism had on the activity of the GAL promoter was not reported. Further analysis of a 20kb region flanking the GAL gene succeeded in identifying a regulatory region capable of being induced following sensory nerve axotomy. However, no activity was reported in any other region of the brain associated with the expression of the galanin gene (Bacon *et al*, 2007; Rokaeus and Waschek, 1998). These studies suggest that the sequences essential to PVN and amygdala specific expression of the GAL gene lie more than 20kb away from the GAL transcriptional start site.

The very precise expression pattern of the galanin gene seen in the PVN in a number of different species including rats, mice (Ceccatelli *et al*, 1989), sheep (Barker-Gibb and Clarke, 1996) and birds (Azumaya and Tsutsui, 1996) suggests that the regulatory systems responsible are critical for the proper function of galanin and have not changed for hundreds of millions of years. We therefore used comparative genomics and transgenic analysis to explore the location of remote, polymorphic and highly conserved regulatory regions that may be responsible for driving the expression of the galanin gene in these regions of the brain. We also used transfection of primary hypothalamic neuron cultures with reporter

genes to determine whether common allelic variations of these enhancers showed significant differences in their activities in these cells. Considering the important roles played by galanin gene expression in the PVN and the amygdala these studies may provide a unique opportunity to understand the cellular systems that control galanin regulated appetite, mood and alcohol intake and how human variation in these systems may change their activity and contribute to obesity, mood disorders and alcoholism.

## **Materials and Methods**

### **Bioinformatics and Sequence Analysis**

Evolutionary conservation was identified by genomic comparison using the ECR Browser (Ovcharenko *et al*, 2004) and the UCSC genome browser (Kent *et al*, 2002). Prediction of the effects of SNPs on transcription factor–DNA binding was carried out using the newly developed RegSNP (<http://viis.abdn.ac.uk/regsnp/Home.aspx>) (Davidson *et al*, submitted).

### **Plasmid Construction**

The GAL5.1 region was amplified from human placental DNA using a high fidelity polymerase (Roche; Expand HI FI kit), and the following oligonucleotide primers: GAL5.1\_F; TGC TCC TGG ATG GAG AGA AG, GAL5.1\_R; CTC CAT CAG AGA CGC CAA AC. After confirmation of correct amplification by DNA sequencing the PCR product was restriction digested with ApaI and SspI and cloned into pGEM-5Z (Promega) that had been

restriction digested with ApaI and EcoRV to produce pGAL5.1GEM5. The GAL5.1-h $\beta$ g-lacZ construct containing the human beta-globin promoter (h $\beta$ g) and the beta-galactosidase reporter gene (LacZ) was created by removing the GAL5.1 element from the pGAL5.1GEM5 plasmid and inserting it into the p1230 plasmid (a kind gift from Robert Krumlauf) by restriction digest with SalI and ApaI. Allelic variants of the GAL5.1 region were created in pGAL5.1GEM5 by site directed mutagenesis using a Quick Change II kit (Stratagene) using the following primer pairs: rs2513280 Forward Primer GTG GTA ATT AAA GTA ATG TCC TGT GCT CAA ATT GCT TGT GC, Reverse Primer GCA CAA GCA ATT TGA GCA CAG GAC ATT ACT TTA ATT ACC AC; rs2513281 Forward Primer ATG ACT GTG TAA CGT TCG TCA CCT TTA TTT TTG TAT CTG TAA ATG C, Reverse Primer GCA TTT ACA GAT ACA AAA ATA AAG GTG ACG AAC GTT ACA CAG TCA T. Luciferase constructs were created by inserting the different allelic variants of the GAL5.1 element from pGAL5.1GEM5 into the SmaI and NotI restriction sites of the pTAL-Luc plasmid (Clontech, abbreviated to pLuc) to form pGAL5.1(GG)-Luc, pGAL5.1(GA)-Luc, pGAL5.1(CG)-Luc and pGAL5.1(CA)-Luc (Figure 3A).

### **Transgenic mouse production.**

Following linearization and removal of the plasmid backbone the GAL5.1-h $\beta$ g-lacZ construct DNA was microinjected into 1-cell C57/BL6xCBA F1 mouse embryos at a concentration of 2–4 ng/ $\mu$ l as

previously described (Nagy, 2003). Surviving embryos were oviduct transferred into pseudopregnant CD1 host mothers.

### **Analysis of LacZ gene expression in transgenic lines.**

Six week old F1 transgenic mice from lines 18 and 21 were humanely sacrificed by lethal injection of euthatol according to current UK Home Office guidelines. Following 4 hours of fixation in 4% paraformaldehyde in phosphate buffered saline (PFA-PBS) brain sections were then stained using 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal) solution for 4–12 h as previously described (Nagy, 2003). X-gal stained tissues were then prepared for vibratome sectioning as previously described (Davidson *et al*, 2006b). 50 micron sections were then cut on a Vibratome series 1000. Sections were mounted on glass slides and photographed under light field illumination.

### **In situ Hybridisation;**

Radioactive *in-situ* hybridisation was carried out on 10 micron thick sections of six week old mouse brain as previously described (Mercer *et al*, 1996).

### **Immunofluorescence**

Brain tissues derived from 6 week old GAL5.1-h $\beta$ g-LacZ transgenic mice (Line 21) were fixed in 4% PFA-PBS for 4 hours and prepared for microtome sectioning by allowing tissue to equilibrate in 30% sucrose in PBS at 4°C. 60 micron sections were then cut on a Microtome (American

Optical Company) and maintained as free floating sections within Netwells (Sigma) in a 12 well plate. Free floating sections were washed with antibody buffer (phosphate buffered saline containing 0.1% Triton-X100 and 0.3 M NaCl) and allowed to incubate overnight at 4°C with Rabbit anti-Galanin 1/1000 (Millipore; Peninsula Labs) and Chicken anti-beta-galactosidase 1/300 (Abcam) primary antibodies diluted in antibody buffer. Secondary antibody used were donkey anti-rabbit (Invitrogen; Alexa 488) and donkey anti-chicken (Invitrogen; Alexa 594) in antibody buffer overnight at 4°C. Sections were then mounted on glass slides with and sealed with VECTASHIELD mounting medium with DAPI (Vector labs). Immunofluorescence was visualised and images of 1 micron optical sections obtained using a Zeiss 510 laser scanning confocal microscope.

### **Cell Culture and luciferase reporter gene assays**

In order to recover sufficient cells the whole hypothalamus region, as previously defined (Paxinos, 1997), was dissected from 1-3 day old rat neonates into Neurobasal-A Medium (Invitrogen) and treated with 0.05% trypsin/EDTA for 15 minutes. Trypsinisation was stopped by the addition of trypsin inhibitor followed by replacement into fresh Neurobasal-A medium. Tissues were triturated with a fire polished Pasteur pipette before being filtered through a cell strainer to create a single cell suspension that was cultured in Neurobasal-A medium with B27 additive and glutamax (invitrogen). These cells were transfected with plasmid DNA using Neuromag according to manufacturer's instructions (OZ

bioscience). Briefly, Neuromag reagent was added to a plasmid-media preparation and incubated at room temperature for 15 minutes allowing 250 ng of plasmid DNA in the media per 150,000 cells to be transfected. The DNA-Media-Neuromag solution was then added to previously prepared single cell cultures in 24 well plates and incubated on a magnetic plate for 15 minutes in a cell culture incubator at 37°C 5% CO<sub>2</sub>.

SHSY-5Y neuroblastoma cells were cultured as previously described (Gillies *et al*, 2010) and magnetofected as described above.

Luciferase activity of plasmid constructs were measured using dual luciferase assay kits (Promega) using lysates from cell cultures transfected with the GAL5.1 luciferase constructs. Cell cultures were lysed and the proteins stabilised using the passive lysis buffer as per the manufacturer's instructions (Promega). The dual luciferase assay analysis was carried out on a glomax 96 microplate luminometer (Promega) using 20 µl of cell lysate per well of a white 96 well plate.

## **Results;**

**Identification, isolation and cloning of GAL5.1.** We have previously shown that comparative genomic analysis is an effective method of detecting functional gene regulatory regions (Davidson *et al*, 2006a, b; Mackenzie *et al*, 2004a; MacKenzie *et al*, 1997; MacKenzie and Quinn, 2004b; Miller *et al*, 2007; Miller *et al*, 2008; Shanley *et al*, 2010). We carried out comparative analysis of 100kb of the genome surrounding the human GAL locus (Figure 1A and B) using the monkey, dog, mouse,

rat, opossum and chicken genomes and succeeded in identifying an area of homology that was located 42kb 5' of the GAL transcriptional start site that we called GAL5.1. This sequence had been conserved since bird-mammal divergence reflecting 310 million years of conservation.

**GAL5.1 supports gene expression in the amygdala and hypothalamus in regions that also express galanin.**

We used high fidelity PCR to isolate the GAL5.1 sequence and cloned it into a LacZ reporter plasmid driven by the previously characterised h $\beta$ globin promoter (Yee and Rigby, 1993) to form GAL5.1-h $\beta$ g-LacZ. Two transgenic mouse lines (18 and 21) were generated using the GAL5.1-h $\beta$ g-LacZ construct (See figure 2A and B and 3A,) and both lines were analysed in parallel for the expression of the  $\beta$ -galactosidase (LacZ) marker. Brain and spinal cord were dissected from individuals from each line and incubated overnight in X-gal stain. We also examined the expression of the endogenous galanin gene using radioactive in-situ hybridisation and noticed a remarkable correlation in the expression of the transgene with the distribution of GAL mRNA (Figure 2A-C and E-G). In the hypothalamus there was strong expression of  $\beta$ -gal in areas which included the PVN, the ventromedial hypothalamic nucleus, the arcuate nucleus in both of the GAL5.1-LacZ transgenic lines generated (Figure 2 A-C and E-G). In addition, we observed activity of both the transgene and the galanin gene within the tuberal nucleus (Figure 2E-G) as previously reported (Evans *et al*, 1993). Expression was also seen in the medial and

central amygdala (Figure 2 A-C and E-G). These observations were confirmed at the cellular level using fluorescent immunohistochemistry on 10 micron sections through the amygdala and hypothalamus of GAL5.1-h $\beta$ g-LacZ transgenic mouse using antibodies against galanin and  $\beta$ -galactosidase that were analysed using 1  $\mu$ m optical sections using a confocal microscopic (Figures 2D and H). Although there were a number of non-neuronal areas of transgene activity in areas such as the vasculature of the pancreas this expression was not consistently observed in both lines. This was also true of expression of LacZ observed in a subpopulation of cells in the dorsal root ganglia where galanin is known to be expressed. However, this expression was only observed in a few individuals of one line (21) so may have represented an insertional effect.

**GAL5.1 can enhance promoter activity following activation of PKA pathways.** We next sought to determine whether the GAL5.1 sequence could act as an enhancer of promoter activity thus acting as a classical enhancer sequence. In addition, we further explored the identity of the cellular transduction systems that influenced the activity of the GAL5.1 sequence. We transfected SHSY-5Y neuroblastoma cells (Scott *et al*, 1986) with either the pTAL-Luc vector (abbreviated to pLuc) or pGAL5.1(GG)-Luc plasmid and cultured these cells in the absence or presence of agonists of the PKA (forskolin), PKC (phorbol ester PMA) or MAPkinase (angiotensinII) pathways. None of the agonists used had any effect on the herpes simplex virus thymidine kinase (HSV-TK) promoter contained within pLuc (Figure 3B). However, in the absence of any

agonists the GAL5.1 sequence also demonstrated no significant ability to further enhance promoter activity in these cells (Figure 3B). In addition, the use of angiotensinII or forskolin also had no significant effect. However, addition of PMA induced a significant enhancement in the ability of GAL5.1 to induce promoter activity (Figure 3B). These studies suggest that GAL5.1 is an enhancer sequence that responds to activation of the PKC signalling cascade.

### **Bioinformatic analysis of allelic variants of GAL5.1.**

Because of the possibility that galanin mis-regulation may play a role in obesity, alcoholism or mood disorders we explored whether there were polymorphisms of the GAL5.1 enhancer in the human population that might alter its activity. Using the dbSNP database and the UCSC browser we identified two human polymorphisms within the GAL5.1 enhancer; rs2513280 (G/C) and rs2513281 (G/A) that occur in GAL5.1 sequence (Figure 1B, C and D). Multiple species alignments show that in all vertebrate species studied the G allele is conserved at the non-human equivalents of both the rs2513280 and rs2513281 loci (Figure 1C and D). The conservation of a G residue through evolution at both these sites reflects the allelic distribution in the human population whereby 70-83% of alleles, at both these loci, are G residues. However, a significant proportion of the European (16.7%), African (26.2 and 20%) and Asian (29.2%) populations maintain C and an A alleles at the rs2513280 and rs2513281 loci respectively. Heatmap analysis using Hapmap shows that

these alleles are very closely linked (LOD=14.97,  $D'=1$ ) to the degree that there is little evidence in any population of genotypes other than GG or CA. However, linkage disequilibrium with other functional SNPs in and around the galanin locus, that have been previously associated with depressive illness, food intake and alcoholism, such as rs2156464 (Wray *et al*, 2010), rs948854 and rs4432027 (Unschuld *et al*, 2008; Unschuld *et al*, 2010) rs694066 (Ruano *et al*, 2006) and rs3136540 (Belfer *et al*, 2006) was not detected.

In order to predict the effects of these polymorphisms on the activity of the GAL5.1 enhancer we developed a novel algorithm, called RegSNP that predicts the effects of human polymorphisms on the binding sites of all the know transcription factor binding matrices contained within the Transfac database (Davidson *et al*, Submitted). RegSNP holds the binding matrices of all of the transcription factors contained in the Transfac data base. Using regSNP we were able to predict the ability of a number of transcription factors, known to be expressed in the hypothalamus, to bind the CA allele. In the case of rs2513280 the predicted decrease in the binding of c-ETS-1 was interesting as c-ETS-1 is expressed strongly in the PVN (Figure 2A inset from Allen brain atlas). In rs2513281 the change from a G to an A residue decreased the predicted affinity of the thyroid receptor (T3R), PXR, TCF and WT1. However, the A allele at this locus was predicted to increase the ability to bind AP-1 and the kruppel like transcription factors.

**The dominant GG allele of GAL5.1 demonstrates significantly higher activity than the CA activity in hypothalamic neurones.**

It has been suggested that changes in the regulation of gene expression, due to polymorphic variation, is a major contributing factor in disease susceptibility. Because the GAL gene plays such an important role in the PVN and the amygdala we explored whether the polymorphic variants we had discovered affected the activity of the GAL5.1 enhancer in hypothalamic neurones. We used site directed mutagenesis to recreate known human polymorphic variants of the GAL5.1 enhancer and cloned these variants into a luciferase reporter construct (Figure 3A). These constructs were transfected into primary neonate rat hypothalamic neurones together with a renilla luciferase control plasmid using neuromag magnetic transfection technology. After 24 hours cells were lysed and lysates assayed using a dual luciferase assay. Significant differences were observed in the ability of these different polymorphic variants of GAL5.1 to drive luciferase expression in these primary hypothalamic neurones (Figure 3C). The Gal5.1(GG)-luc construct that contains the GG allele, which has been evolutionary conserved and represents the major haplotype in human populations, showed the highest level of luciferase expression. Relative to the Gal5.1(GG)-luc construct luciferase activity from GAL5.1(GA)-Luc was reduced by 60%, whilst the GAL5.1(CG)-Luc and GAL5.1(CA)-Luc demonstrated a 35% and 40% decrease in activity respectively (Figure 3C).

## **Discussion.**

Although neuropeptides in the hypothalamus have been widely studied in the context of obesity (Lu *et al*, 2007), alcoholism and to a lesser extent, in the amygdala and mood disorders (Sergeyev *et al*, 2005), there is little evidence that mutations or polymorphisms in the coding sequences of the genes that encode these neuropeptides play a major role in conferring susceptibility to these disorders. However, evidence suggests that polymorphic variation of regions of the genome that control the expression of genes, at the level of transcription, splicing or translation, may be major contributory factors in conferring disease susceptibility (Wray, 2007). This was confirmed following the results of a recent meta-analysis of data from multiple GWA studies that concluded that 88% of disease associated "hits" occur within the non-coding genome (Hindorff *et al*, 2009; Singleton *et al*, 2010 ). The high prevalence of disease causing SNPs in the non-coding genome, when combined with the comparative lack of understanding of the non-coding genome in gene regulation and a relative inability to efficiently analyse this uncharted part of the genome has led to much concern. Thus, in addition to determining the mechanisms that control the expression of galanin in the hypothalamus and the amygdala the current study sought to demonstrate the combined use of comparative genomics, transgenic analysis and primary cell analysis to identify, characterise and assess the effects of human variation on the

regulatory regions that maintain the expression of the galanin gene in these critical parts of the brain.

The high degree of conservation of the patterns of expression of the galanin gene in the hypothalamus and amygdala suggests that the mode of action of galanin depends on its expression in very specific groups of cells. Thus, the evidence suggests that changes in galanin expression in these regions was inconsistent with species survival during evolution. However, it is clear from previous studies that 20kb of the promoter region of the galanin gene could not produce marker gene expression in the brain of transgenic mice (Bacon *et al*, 2007). Therefore, prior to the current study, a critical functional component of the galanin locus remained unidentified. By virtue of its extreme conservation the current study succeeded in predicting and identifying a regulatory region that lay 42kb 5' of the human GAL gene and was able to support the expression of marker genes in cells of the amygdala, PVN and arcuate nucleus that also express galanin mRNA. The importance of galanin gene regulation in the mode of action of galanin is highlighted by the extreme conservation of GAL5.1 which greatly exceeds the conservation of the galanin coding region itself. This degree of conservation demonstrates that, whilst there has been a significant degree of sequence change in the the galanin coding region since bird-mammal divergence, evolution has maintained strong selection against changes in the GAL5.1 sequences to ensure the correct expression of galanin in these regions of the brain.

It has been shown that cis-regulatory sequences with importance to the expression of specific genes are not only highly conserved but are retained in cis with these genes often for hundreds of millions of years (Mackenzie *et al*, 2004a; Mongin *et al*, 2009; Navratilova and Becker, 2009a; Navratilova *et al*, 2009b). This appears to be the case with the GAL5.1 enhancer and the galanin gene which have remained in-cis for at least 310 million years in all of the higher vertebrate species examined to date. Considering the propensity of genomes to rearrange through evolution this strong “in-cis” relationship suggests that separation of the GAL gene and GAL5.1 is inconsistent with vertebrate survival and serves as the first piece of evidence functionally linking the GAL5.1 sequence to the galanin gene.

A second piece of evidence is the remarkable similarity of the patterns of expression of GAL5.1-h $\beta$ g-LacZ transgene derived  $\beta$ -galactosidase and the expression of galanin mRNA in the PVN, the arcuate nucleus and the amygdala of both transgenic lines produced in the current study. The fact that both lines generated were able to produce patterns of expression of the transgene that matched the expression of the endogenous galanin gene argues against the possibility that these patterns were caused by insertional effects i.e. random integrations of the transgene into parts of the genome which are transcriptionally active in these specific cells. The correspondence in patterns strongly suggests that GAL5.1 is not only involved in galanin expression but that it may be the only cis-regulatory

element required for the tissue specific expression of the galanin gene in the hypothalamus and amygdala.

Thirdly, we also show using high resolution confocal imaging that the transgene and the endogenous peptide are co-expressed at the cellular level in individual neurones of the amygdala and the PVN.

Together, these three lines of evidence argue strongly in favour of a role for the GAL5.1 enhancer in the regulation of the GAL in the hypothalamus and the amygdala. Based on its strong conservation and its activity in galanin expressing neurones we predict that genomic deletion of the GAL5.1 element by embryonic stem cell targeting in mice would produce a similar phenotype to that previously observed following deletion of the galanin gene coding region (Wynick and Bacon, 2002).

Although our evidence suggests that GAL5.1 is involved in the regulation of GAL in these tissues we cannot rule out the possibility that specific enhancer-promoter interactions may be required to allow appropriate responses to specific stimuli or signal transduction systems. Thus, it would be interesting to see how GAL5.1 interacted with the Galanin promoter regions previously identified (Bacon *et al*, 2007). Moreover, an interesting contrast can be drawn between GAL5.1 and the ECR2 enhancer shown to control the expression of the TAC1 gene in sensory neurones (Shanley *et al*, 2010; Shanley *et al*, 2011). ECR2 could only drive tissue specific expression in the presence of the endogenous TAC1 promoter whereas GAL5.1 can support expression in the presence of

an exogenous weak promoter such as the human beta-globin promoter or HSV-TK promoter. These observations suggest that different enhancers possess varying levels of autonomy or interdependence and that the GAL5.1 is functionally self contained relative to some other previously characterised enhancers (Shanley *et al*, 2010; Shanley *et al*, 2011).

In addition to determining that GAL5.1 is able to drive marker gene expression in cells of the hypothalamus and amygdala that also express the galanin peptide we wanted to determine quantitatively whether GAL5.1 could enhance the activity of an already well characterised promoter. We used a human neuroblastoma cell line, called SHSY-5Y, that displays neuronal characteristics (Scott *et al*, 1986). Using these cells we were unable to show that GAL5.1 could act as an enhancer of promoter activity. However, we have previously shown that many regulatory sequences are only able to act as enhancers in response to activation of an appropriate signal transduction cascade (Shanley *et al*, 2010; Shanley *et al*, 2011). Thus, we were able to show that GAL5.1 acted as an enhancer of promoter activity following activation of the PKC pathway, that is known to modulate gene expression in the hypothalamus and amygdala, but not PKA or MAPkinase pathways. These results suggest that GAL5.1 is an enhancer responsible for transmitting the activation of PKC pathways to the core transcriptional apparatus. Intriguingly, it was observed that one of the allelic variants of the GAL5.1 enhancer reduced the predicted ability of the ETS-1 transcription factor to bind. This prediction is interesting as ETS-1 expression overlaps that of the galanin

gene and GAL5.1 in the PVN (Figure 2A insert). In addition, a number of studies have shown that the regulatory activity of ETS-1 is modulated by the PKA signal transduction pathway (Naito *et al*, 2002; Vetter *et al*, 2005).

In addition to establishing the cells in which the GAL5.1 enhancer is active and that GAL5.1 is activated by PKC activation we identified two polymorphisms within the GAL5.1 sequence. Examination of the population frequency of these polymorphisms suggests little evidence of the existence of other allele combinations other than GG or CA. In addition, the numbers of individuals homozygous for CA is much lower than expected supporting the hypothesis that the CA allele has reduced fitness in the population in the past. In keeping with this hypothesis we observed that the equivalent loci within the GAL5.1 enhancer of other species are always GG suggesting that divergence from the GG allele has not been compatible with species fitness during evolution. If we consider our observation that the CA allele is significantly less active in primary hypothalamic neurones than the GG allele, together with the observation that GAL5.1 is active in the PVN and that galanin expression in PVN controls appetite for fatty foods and alcohol, then it is possible that the CA allele may reduce appetite for these nutrients. Thus, the CA allele may have been selected against through mammalian evolution because maintenance of a preference for high energy fatty foods and alcohol during evolution may have been essential for survival. Moreover, there is little genetic evidence that polymorphisms around the GAL locus are

associated with obesity (Schauble *et al*, 2005). Instead, it may be possible that polymorphisms around the GAL locus, including the CA allele of GAL5.1, might reduce preferences for high fat foods and alcohol. Indeed, one study that examined the effects of different gene polymorphisms on weight loss through reduced carbohydrate intake demonstrated that polymorphisms in the GAL gene were significantly associated with accelerated weight loss (Ruano *et al*, 2006). Thus, it would be interesting to determine the GAL5.1 allele frequencies in populations of individuals attempting to lose weight to determine if the CA allele can be associated with accelerated weight loss.

With respect to depression and anxiety there have been a number of association studies linking polymorphisms around the GAL locus with mood disorders. For example, one polymorphism; rs948854, was associated with depression and anxiety disorder in female patients together with worse treatment response to antidepressants (Unschuld *et al*, 2008; Unschuld *et al*, 2010). In addition, a larger GWAS study suggested that a polymorphism; rs2156464, that lies 25kb from the GAL locus may be involved in major depressive disorder (Wray *et al*, 2010). However, we were unable to identify significant LD between this SNP and those within our study. Although we largely concentrated our efforts on understanding the effects of the CA allele on the activity of GAL5.1 in the hypothalamus it would be intriguing to analyse the effects of this allele on expression within primary amygdala neurones once we have optimised culture conditions and transfection protocols for these cells.

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**Disclosure/conflict of interest.**

The authors declare no conflict of interest.

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## Figure legends

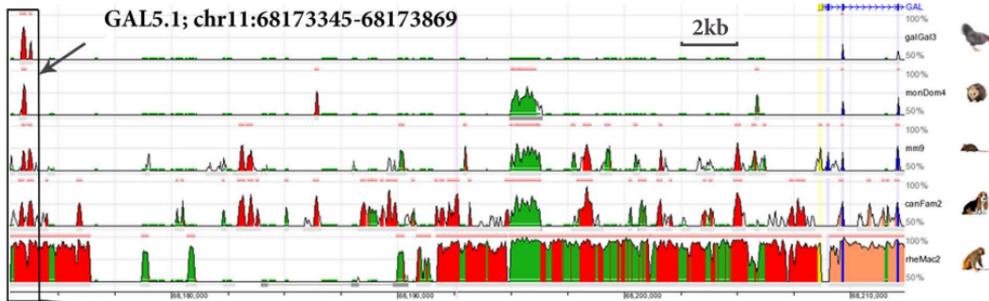
**Figure 1. A** VISTA plot from the ECR Browser comparing 45kb of DNA to the 5' of the GAL locus with the genomes of (from top to bottom) chicken, marsupial, mouse, dog and rhesus monkey. The x-axis represents linear distance with reference to the human genome sequence. The y-axis represents levels of sequence conservation between 50% and 100%. Blue lines with chevrons represent the genomic extent of each gene. Red, green, blue, pink and yellow peaks represent areas of sequence conservation (> 75% over 100 base pairs) in intergenic non-coding, repetitive sequence, exonic, intronic and untranslated regions respectively. **B.** a vertebrate Multiz-alignment and phast-con 28 species conservation plot showing where the rs2513280 and rs2513281 SNPs fall in relation to the most conserved areas of the enhancer sequence. **C** and **D** multiple alignment plots of human, chimp, rhesus monkey, mouse rat and chicken DNA within the GAL5.1 enhancer and surrounding the (**C**) rs2513280 and (**D**) rs2513281 loci demonstrating levels of conservation (red boxes) and aligned binding matrices of a number of different transcription factor proteins that are known to be expressed within the hypothalamus. The tables above both alignments (C and D) display predicted probabilities of transcription factor binding based on the the known binding matrix of each transcription factor where 1=perfect match and 0 =no match as predicted by the regSNP algorithm. Thus, column 1 represents binding probabilities to the dominant G allele for both loci and column two represent those of the (C) C and (D) A alleles.

**Figure 2.** A, B, E and F photomicrographs of the expression of the  $\beta$ -galactosidase reporter gene, as detected using X-gal staining, within the (A and B) the hypothalamic region and (E and F) the amygdala and arcuate nucleus region of transgenic lines (A) 21 and (B) 18 of mice containing the GAL5.1h $\beta$ g-LacZ reporter construct. C and G, expression of galanin mRNA within the (C) hypothalamus and (G) amygdala region of the mouse brain by in-situ hybridisation. D and H; 1 micron optical confocal sections through the (D) paraventricular nucleus and (H) amygdala region of a mouse transgenic for the GAL5.1h $\beta$ g-lacZ transgene (line 21) following fluorescent immunohistochemistry using antisera against the galanin peptide (false colour green) and  $\beta$ -galactosidase (false colour red). Co-localisation within cells is indicated by false colour yellow and highlighted with white arrows. Nucleii are stained with TO-PRO3 (blue). PVN; paraventricular nucleus; CeA; central amygdala, MeA; medial amygdala, VMH; ventromedial hypothalamic nucleus, Arc; arcuate nucleus, TU tuberal nucleus. Scale bar in D and H= 20 microns.

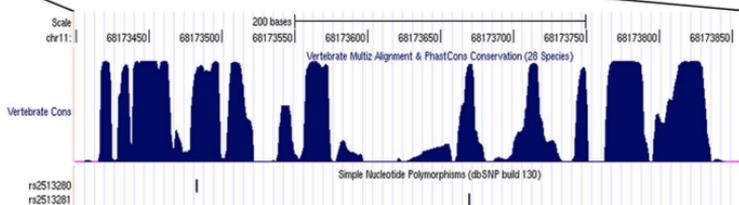
**Figure 3A.** diagrammatical representation of the different constructs used in the current study (not to scale) and showing the different alleles reproduced in each construct using site directed mutagenesis. LacZ,  $\beta$ -galactosidase reporter; h $\beta$ gprom, human beta globin promoter; TKprom, herpes simplex virus thymidine kinase promoter. **B,** Graph comparing levels of induction of the pLuc plasmid against that of the pGAL5.1(GG)-Luc plasmid (Figure 3A) following their transfection into SHSY-5Y cells and treatment with forskolin, angiotensinII or PMA. Relative levels of induction

are normalised against renilla luciferase and vehicle control (DMSO or H<sub>2</sub>O). **C**, graph showing the relative expression levels of the luciferase reporter constructs shown in figure 3A and normalised to co-transformed renilla expressing plasmid and the pLuc plasmid. \*, p<0.05; \*\*, p<0.01; *ns*, not significant.

A



B

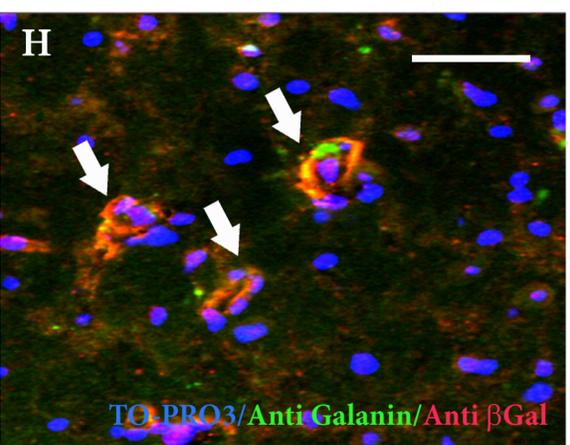
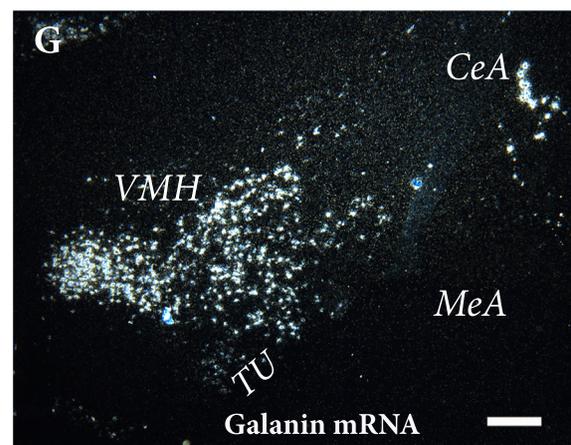
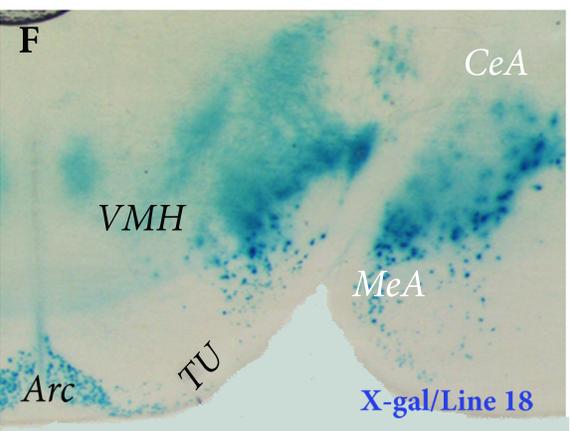
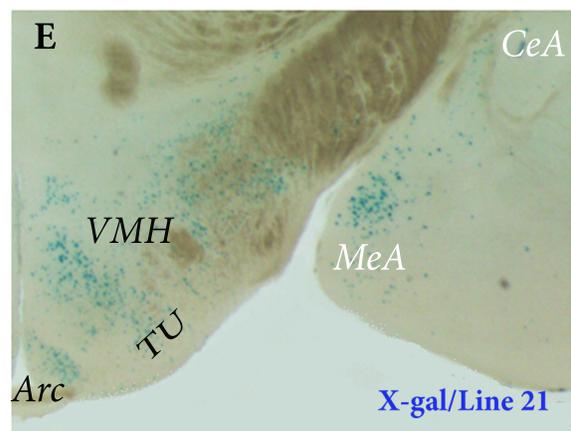
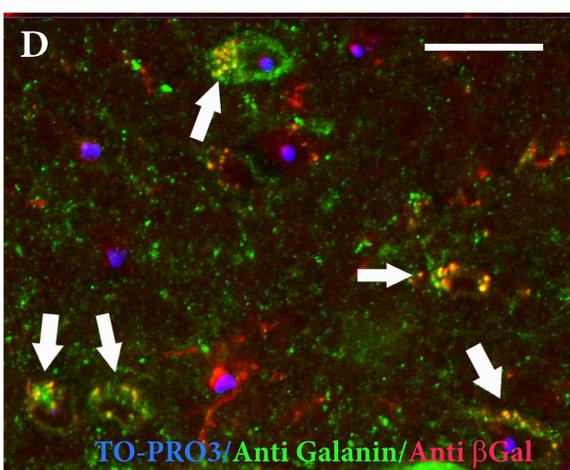
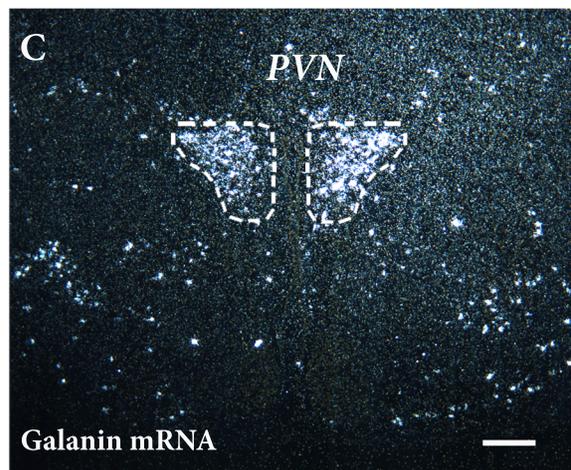
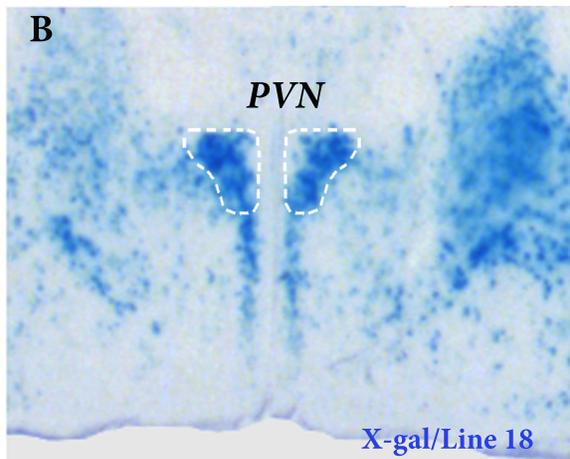
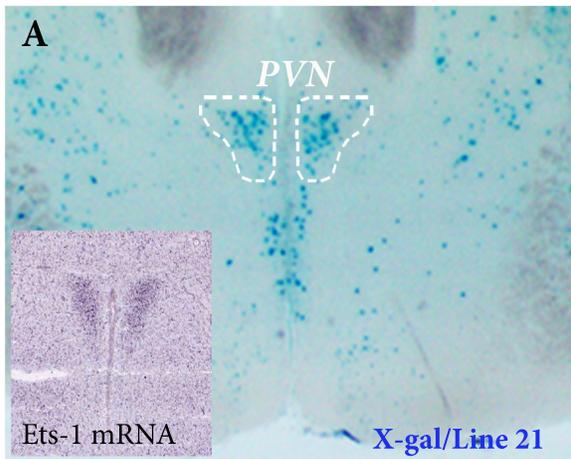


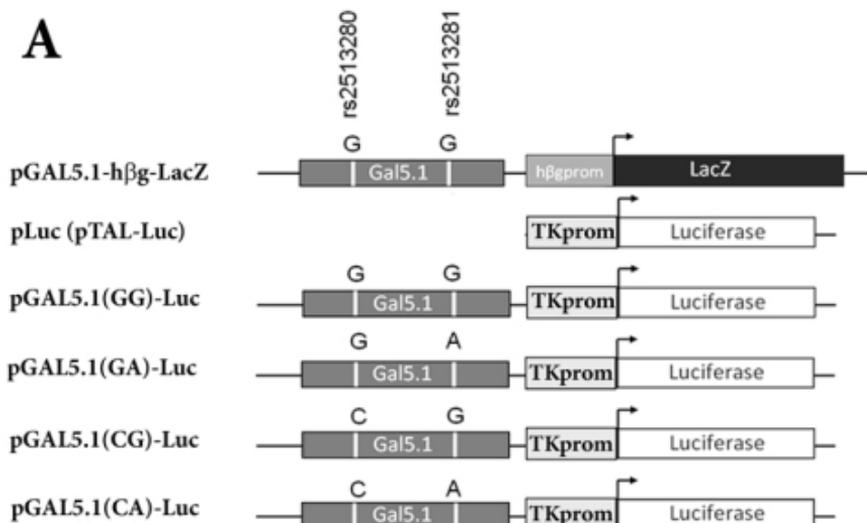
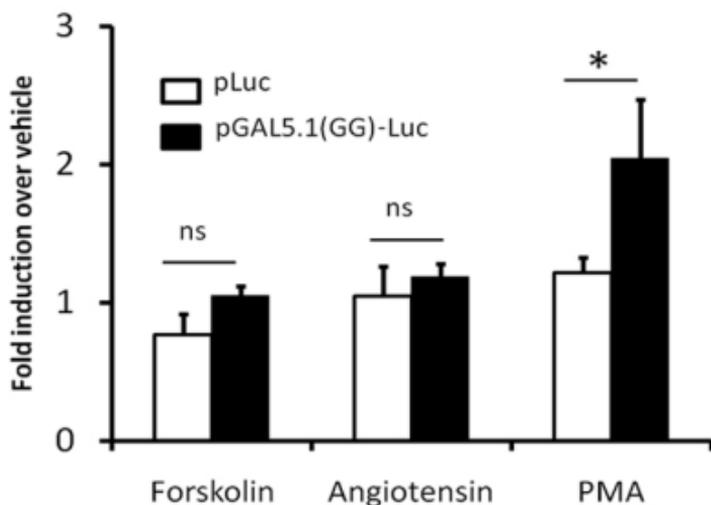
C. rs2513280



D. rs2513281





**A****B****C**

Relative transcriptional activity of different alleles of GAL5.1 in primary hypothalamic neurones

