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Down regulation of cerebellar memory related gene-1 following classical conditioning

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We have isolated and characterized the mRNA of a mouse gene named cerebellar memory related gene-1, previously found by microarray analysis to be differentially expressed following classical conditioning of the rabbit nictitating membrane response. Quantitative RT-PCR analysis showed a significant reduction in mRNA expression in cerebellar lobule HVI but not in the hippocampus of rabbits that received classical conditioning compared to control rabbits that received either unpaired stimulus presentations or were simply restrained. The mouse mRNA encodes a protein of 485 amino acids that includes different potential post-translational modification sites and five copies of the WD-repeat suggesting involvement in protein–protein interaction and regulatory function. *In-situ* hybridization experiments show highly localized expression of the transcript in mouse brain with the highest expression levels located in the cerebellum, hippocampus and cortex. Taken together, our results reveal a novel gene encoding a WD-repeat protein that is down-regulated in cerebellar lobule HVI as a result of learning and memory.

Keywords: Cerebellum, classical conditioning, gene expression, rabbit nictitating membrane response, WD-repeat

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Many experiments in the past have demonstrated the requirement of *de novo* gene expression during memory formation (Bracha *et al*. 1998; Chen & Steinmetz 2000; Davis & Squire 1984; Gomi *et al*. 1999; Stork & Welzl 1999). In contrast to the initial reductionist view that genes relevant to learning and memory would be easily found and would provide a simple key to understand this brain function, it is becoming apparent that the genetic contribution to memory is complex. In view of the broad variety of genes and the cross talk of genetic pathways involved in this regulation, gene expression profiles by cDNA-microarrays offer the new opportunity to study the complete behavior of these regulatory pathways (Cavallaro *et al*. 2002a; D'Agata & Cavallaro 2002). By performing gene expression analysis following classical conditioning of the rabbit nictitating membrane response (NMR) with high-density cDNA microarrays, we previously identified 79 genes that were differentially expressed in lobule HVI of the rabbit cerebellum (Cavallaro *et al*. 2001). Although some of these genes have significant similarity to known genes related to synaptic plasticity, memory or cognitive disorders, a large number of the identified genes are ESTs and have no name or known function. Consequently, gene expression profiling would seem to yield limited information about biological function if the role of the translated products (proteins) were to remain unknown. A lack of gene function information, however, need not be a deterrent to assaying gene expression using microarrays because follow-up analyses provide a viable means of adding to our knowledge of gene function. In particular, complete nucleotide sequence determination, conceptual translation, expression monitoring and biochemical analysis may provide a detailed functional understanding of the identified genes.

We have begun this type of analysis for one gene (EST GenBank Acc. No. AA000655), cerebellar memory related gene-1 (*CMRG1*), shown to be down-regulated by more than 2.5-fold in lobule HVI of rabbits given paired training relative to lobule HVI of rabbits given unpaired control stimulus presentations (Cavallaro *et al*. 2001). In the present study we confirmed the differential expression of *CMRG1*-mRNA in cerebellar lobule HVI of NMR conditioned rabbits and characterized the sequence and distribution of the mouse homolog in brain.

Materials and Methods

Classical conditioning

The subjects were 9 adult male albino rabbits (*Oryctolagus cuniculus*) each weighing 2.0–2.2 kg. Rabbits were individually

housed, given access to food and water, and maintained on a 12:12 light/dark cycle. Animals were allocated randomly to three groups and given paired training (paired, $n=3$), explicitly unpaired stimuli (unpaired, $n=3$) or restraint (sit, $n=3$). Paired and unpaired rabbits received one day of preparation and three sessions of stimulus presentation. Sit rabbits received one day of preparation and three sessions of restraint. On adaptation day, rabbits were prepared for periorbital electrical stimulation and nictitating membrane recording (right side) and acclimated to training chambers for the time of subsequent sessions (80 min). Paired subjects received 80 daily presentations of a 400-ms, 1000-Hz, 82-dB tone conditioned stimulus (CS) that coterminated with a 100-ms, 60-Hz, 2-mA electrical pulse unconditioned stimulus (US) delivered, on average, every 60 seconds. Unpaired subjects received the same 80 CS and 80 US but presented in an explicitly unpaired manner on average, every 30 seconds. Stimulus delivery and data collection were via a computer-controlled system (Gormezano *et al.* 1962; Schreurs & Alkon 1990). All animal procedures followed NIH guidelines and were approved by an Animal Care and Use Committee.

Quantitative RT-PCR

Transcript levels for EST AK017549 were assayed by real-time detection and evaluation of fluorometric PCR reactions to validate previous microarray data (Cavallaro *et al.* 2001). The sequences of the forward and reverse primers were GAACCGTGCAGTCTATGAG and GTTCTCACTCAGCGACGTGT respectively, and the length of PCR product generated was 103 bp. Twenty-four hours after three days of paired ($n=3$) or unpaired ($n=3$) stimulus presentations or restraint (sit, $n=3$), rabbits were killed and the right lobule HVI of each rabbit was removed and rapidly frozen on dry ice. Total RNA samples were reverse transcribed and then aliquots of cDNA (0.1 and 0.2 μg) and known amounts of external standard (purified PCR product, 10^2-10^8 copies) were amplified in parallel reactions using the forward and reverse primers. Each PCR reaction (final volume 20 μl) contained 0.5 μM of the primers, 2.5 mM Mg^{2+} and 1 X Fast start DNA master SYBR Green I mix (Roche Molecular Biochemicals, Mannheim, Germany). PCR amplifications were performed with a Light-Cycler (Roche Molecular Biochemicals) using four cycles: (i) denature cDNA (1 cycle: 95 °C for 10 min); (ii) amplification (45 cycles: 95 °C for 0 seconds, 57 °C for 5 seconds 72 °C for 10 seconds); (iii) melting curve analysis (1 cycle: 95 °C for 0 seconds, 67 °C for 10 seconds, 95 °C for 0 seconds); (iv) cooling (1 cycle: 40 °C for 3 min). Temperature transition rate was 20 °C/ second except for the third segment of the melting curve analysis where it was 0.2 °C/ second. Fluorometer gain value was 7. Real-time detection of fluorometric intensity of SYBR Green I, indicating amount of PCR product formed, was measured at the end of each elongation phase. Quantification was performed by comparing fluorescence of the PCR products of unknown concentration with the fluorescence of the external standards. Fluorescence values measured in the log-linear phase of amplification were considered using the second derivative maximum method of the Light Cycler Data Analysis software (Roche Molecular Biochemicals). Specificity of PCR products obtained was characterized by melting curve analysis followed by gel electrophoresis and DNA sequencing.

Cloning of CMRG1 from adult mouse brain

Single stranded cDNAs were synthesized incubating adult C57Bl6/J mouse brain mRNA (50 ng) with SuperScript II Rnase H-reverse transcriptase (200 U; Invitrogen Life Technologies, Carlsbad, CA, USA), oligo-$(dT)_{18}$ primer (100 nM), dNTPs (1 mM) and RNase-inhibitor (40 U) at 42 °C for 1 h in a final volume of 20 μl. Reaction was terminated by incubating at 70 °C for 10 min. Complementary DNAs were amplified by PCR using primers flanking the predicted start (5′-AACCAAAGAAGGAAGCCAG GGA−3′) and the stop codon (5′-GGCAGCACAGGGTGACAGAACC−3′) of EST AK017549. PCR reaction (final volume 20 μl) contained 0.4 μM of primers, 200 μM dNTPs, 1 μl of eLONGase enzyme mix (Invitrogen Life Technologies, Carlsbad, CA, USA) and 2 mM $MgCl_2$. PCR was performed on a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA, USA) using the following three cycle programs: (i) denaturation of cDNA (1 cycle: 95 °C for 1 min); (ii) amplification (25 cycles: 95 °C for 30 seconds, 60 °C for 30 seconds, 68 °C for 90 seconds); (iii) final extension (1 cycle: 68 °C for 7 min). Amplification products arising from RT-PCR were electrophoresed on a 10% acrylamide gel in 0.045 M Tris-borate/1 mM EDTA (TBE) buffer and visualized by ethidium bromide staining. For sequencing, PCR products were subcloned into ZERO Blunt TOPO PCR cloning vector (Invitrogen Life Technologies, Carlsbad, CA, USA).

In situ hybridization

Brains from adult male mice were rapidly frozen on dry ice, and brain sections (12 μM) were obtained with a cryostat set at −20 °C. *In situ* hybridization was performed with a specific mouse antisense [α-^{35}S] labeled-riboprobe produced using T7 RNA polymerase and an *Eco*RI linearized I.M.A.G.E. Consortium (LLNL) cDNA clone (ID/GenBank Acc. No. 424677/AA000655) as described previously (Cavallaro *et al.* 2001). Labeled mRNA signals were revealed with autoradiography. Figures were prepared from digitized images using Adobe PHOTOSHOP 5.0 and compared with a standard mouse brain atlas (Paxinos & Franklin 2001). In initial experiments characterizing the probe, coronal, horizontal and sagittal brain sections were used. Coronal sections presented in this report were derived from three individual adult mice (C57Bl6/J). Signal intensities in each of the three *in situ* hybridization section series were consistent with each other and were also verified using horizontal and sagittal sections (data not shown). Labeling intensity were denoted as very strong (++++) or just above background (+). Intermediate intensities were designated successively as strong (+++) and medium (++).

Table 1: Expression of *CMRG1* in hippocampus and cerebellar lobule HVI following classical conditioning of the rabbit nictitating membrane response

Area	Condition		
	Naive	Unpaired	Paired
Hippocampus	–	4288	4299
	2571 ± 219	2447 ± 124	2399 ± 201
Cerebellar HVI lobule	–	7366	4229
	4955 ± 323	4771 ± 381	1651 ± 210*

Transcript levels for *CMRG1* were assayed by real-time detection and evaluation of fluorometric PCR reactions. Results are the mean ± standard deviation of absolute RNA levels (transcipt copy number) from sit, unpaired and paired rabbits (data points were obtained from 3 individual animals; $*P < 0.01$). The sequence of the primers used and the length of PCR product generated are indicated in the *Materials and methods* section. Microarray values refer to the gene expression signal previously evaluated by microarray analysis (Cavallaro *et al.* 2001).

Results

Differential expression in cerebellar lobule HVI as a result of classical conditioning

CMRG1-mRNA was previously shown by microarray analysis to be down-regulated in lobule HVI of rabbits following classical conditioning of the rabbit NMR (Cavallaro *et al.* 2001). In the present study we confirmed this microarray data using quantitative RT-PCR. Table 1 shows a significant reduction in *CMRG1*-mRNA (transcript copy number) in cerebellar lobule HVI (per 100 pg RT-RNA) for three paired rabbits compared to three unpaired and three sit control rabbits ($P < 0.01$). The table also shows that there were no significant differences between paired, unpaired and sit groups in *CMRG1*-mRNA in the hippocampus.

Cloning of the mouse cDNA and molecular architecture of the deduced amino acid sequence

Complete sequence of I.M.A.G.E. Consortium clone ID: 424677 revealed a cDNA insert matching 1369 nucleotides on the 3′ end of a longer EST sequence present in GenBank (Acc. No. AK017549). The longest open reading frame (ORF) of this EST contains 1458 nucleotides proximal to its 5′ end. This ORF was verified by PCR using specific primers overlapping the ORF (GenBank Acc. No. AF513713). The predicted physico-chemical parameters, post-translational modifications and domain organization are illustrated in Fig. 1. The predicted protein sequence contains 485 amino acids, a molecular weight of 53 kDa and an isoelectric point of 9.33. The protein sequence includes several potential post-translational modification sites: one cAMP-cGMP dependent protein kinase phosphorylation site, five casein kinase II (protein kinase CK2) phosphorylation sites, eight protein kinase C phosphorylation sites and two N-glycosylation sites. Closer inspection of the amino acid sequence also reveals the existence of five copies of a domain, WD-repeat, each containing a central Trp-Asp motif.

Regional distribution in mouse brain

To determine the regional distribution of *CMRG1*-mRNA in the adult mouse brain we performed *in situ* hybridization. Figure 2 shows pseudocolor representative film autoradiograms of mouse brain sections. As summarized in Table 2, the brain regions examined showed various labeling intensities ranging from very low (+) (staining just above the background) to very strong (++++). Strong hybridization signal of *CMRG1*-mRNA was observed in the cortex, hippocampus and cerebellum. Within the hippocampus, strong intensities were evident in the dentate gyrus and the CA1, CA2 and CA3 neuronal fields. Labeling in the cerebellum appeared to be strongest in the granule cell layer and lighter in the proximal regions of the molecular layer.

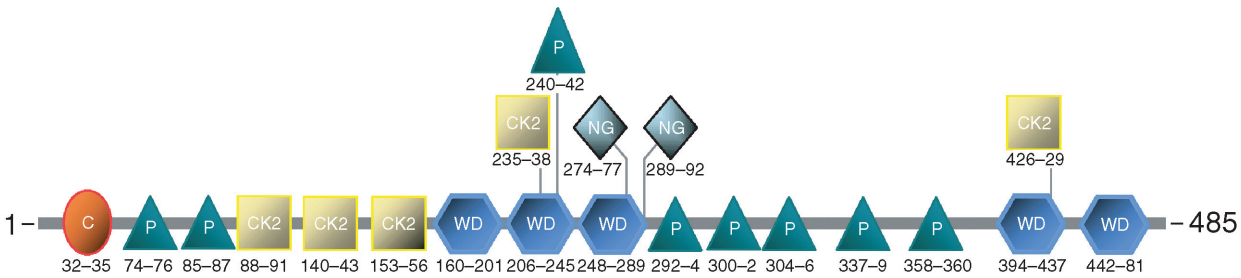

Figure 1: Predicted post-translational modification sites and domain organization of *CMRG1*. Abbreviations: C, cAMP-cGMP dependent protein kinase phosphorylation site; CK2, casein kinase II phosphorylation site; NG, N-glycosylation site; P, protein kinase C phosphorylation site; WD, WD repeat. WD-repeat consensus pattern: [LIVMSTAC]-[LIVMFYWSTAGC]-[LIMSTAG]-[LIVMSTAGC]-x(2)-[DN]-x(2)-[LIVMWSTAC]-x-[LIVMFSTAG]-W-[DEN]-[LIVMFSTAGCN].

Discussion

Many experiments in the past have demonstrated the requirement of *de novo* gene expression during memory formation (Bracha *et al*. 1998; Chen & Steinmetz 2000; Davis & Squire 1984; Gomi *et al*. 1999; Stork & Welzl 1999) and large-scale expression analysis is now starting to elucidate the genes involved (Cavallaro *et al*. 1997; Cavallaro *et al*. 2001; Cavallaro *et al*. 2002b; Gomi *et al*. 1999). Although further tests are required to determine exactly what role these genes play in memory, some of them have been related to mental retardation and cognition disorders. One of them for example, the cell division cycle 2-related protein kinase KKIAMRE, that has been shown to be induced following eyeblink conditioning (Gomi *et al*. 1999), was found decreased in a mouse model of fragile X mental retardation (D'Agata *et al*. 2002).

In the present study we have started the characterization of a novel gene, *CMRG1*, that was previously implicated by microarray analysis to be down-regulated in rabbit cerebellar lobule HVI following classical conditioning of the rabbit NMR (Cavallaro *et al*. 2001). By using quantitative RT-PCR analysis, we have now confirmed a significant reduction of *CMRG1*-mRNA in cerebellar lobule HVI but not the hippocampus of rabbits that received NMR classical conditioning when compared to controls.

The specific role of the down regulation of a gene following learning remains a matter of speculation. Down regulation

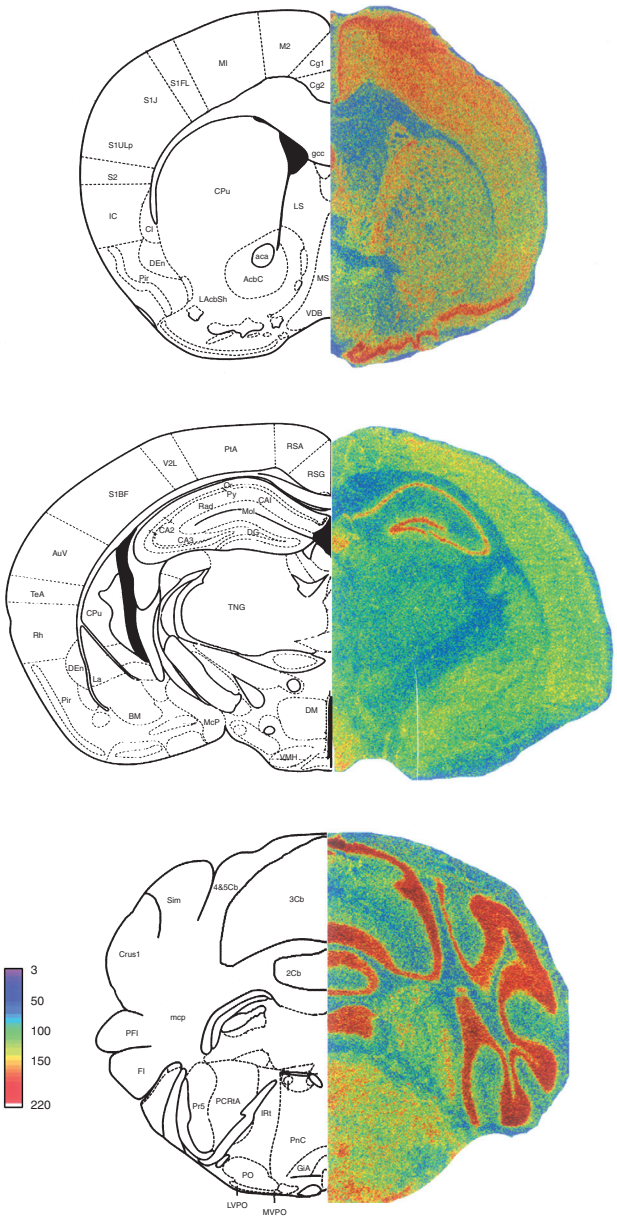

Figure 2: Regional expression of *CMRG1* in sections of adult mouse brain. Coronal sections of adult mouse brain. The color spectrum on the bottom left of the figure represents the pixel value of the gray levels. Abbreviations: Aca, anterior commissure; AcbC, accumbens nucleus core; AuV, secondary auditory cortex; BM, basomedial amygdaloid nucleus; Cb, cerebellar lobule; CA1, CA1 area of hippocampus; CA3, CA3 area of hippocampus Cl, claustrum; Cg, cingulate cortex area; CPu, caudate putamen (striatum); Crus1, crus 1 of ansiform lobule; DEn, dorsal endopiriform nucleus; DG, dentate gyrus; DM, dorsomedial nucleus; Fl, flocculus; ggc, genu of corpus callosum; GiA, gigantocellular reticular nucleus alpha; IC, insular cortex; Irt, intermediate reticular nucleus; La, lateral amygdaloid nucleus; LacbSh, lateral accumbens shell; LS, lateral septal nucleous; LVPO, lateroventral perioliviary nucleus; M1, primary motor cortex; M2, secondary motor cortex; mcp, middle cerebellar penducle; MeP; medial amygdaloid nucleus; Mol, molecular layer dentate gyrus; MS, medial septal nucleus; MVPO, medioventral perioliviary nucleus; Or, orien layer of hippocampus; PCRtA, parvicellular reticular nucleus alpha; PFl, paraflocculus; Pir, piriform cortex; PnC, pontine reticular nucleus; PO, periolivary region; Pr5, principal sensory 5 nucleus; PtA, parietal cortex; Py, pyramidal cell layer of hippocampus; Rad, stratum radiatum of hippocampus; Rh, rhinal cortex; RSA, retrosplenial agranular cortex; RSG, retrosplenial granular cortex; S1FL, S1 cortex forelimb region; S1J, S1 cortex jaw region; S1BF, S1 cortex barrel field; S1Ulp, S1 cortex upper lip region; S2, secondary somatosensory region; Sim, simple lobule (lobule HVI); TeA, temporal association cortex; TNG, thalamic nucleus group; V2L, secondary visual cortex; VMH, ventromedial hypothalamic nucleus.

Table 2: Regional distribution of *CMRG1* in adult mouse brain as determined by *in situ* hybridization

CNS region	Subfield	Abbreviation	mRNA expression
Cortex	Barrel field	S1BF	+ +
	Cingulate cortex, area 1	Cg1	+ + + +
	Cingulate cortex, area 2	Cg2	+ + + +
	Dorsal endopiriform nucleus	DEn	+ +
	Claustrum	Cl	+ +
	Insular cortex	IC	+
	Piriform cortex	Pir	+ + + +
	Primary motor cortex	M1	+ + + +
	Retrosplenial granular cortex	RSG	+
	S1 cortex, forelimb region	S1FL	+ + +
	S1 cortex, jaw region	S1J	+ + +
	S1 cortex, upper lip region	S1Ulp	+ +
	Secondary auditory cortex	AuV	+
	Secondary motor cortex	M2	+ + + +
	Secondary somatosensory region	S2	+ +
	Secondary visual cortex	V2L	+ + +
	Temporal association cortex	TeA	+ +
	Retrosplenial agranular cortex	RSA	+
	Anterior Commissure	aca	+ +
	Genu of corpus callosum	ggc	+ + +
Basal Ganglia	Accumbens Nucleus, core	AcbC	+
	Accumbens Nucleus, shell	AcbSh	+ +
	Caudate putamen (striatum)	Cpu	+ + +
	Lateral accumbens shell	LAcbSh	+ +
	Lateral septal nucleus	LS	+ + +
Amygdala	Basomedial amygdaloid nucleus	BM	+ +
	Lateral amygdaloid nucleus	La	+ +
	Medial amygdaloid nucleus	MeP	+ +
Hippocampus	CA1 field of hippocampus	CA1	+ + + +
	CA2 field of hippocampus	CA2	+ + + +
	CA3 field of hippocampus	CA3	+ + + +
	Dentate gyrus	DG	+ + + +
	Molecular layer dentate gyrus	Mol	–
	Orien layer of hippocampus	Or	+
	Pyramidal cell layer of hippocampus	Py	+ + + +
	Stratum radiatum of hippocampus	Rad	+
	Dorsomedial nucleus	DM	+ +
Thalamus	Thalamic nucleus group	TNG	+
Hypothalamus	Ventromedial hypothalamic nucleus	VMH	+ + +
Cerebellum	2nd cerebellar lobule	2Cb	+ + + +
	3rd cerebellar lobule	3Cb	+ + + +
	4th and 5th cerebellar lobules	4 & 5Cb	+ + + +
	Crus 1 of the ansiform lobule	Crus1	+ + + +
	Flocculus	Fl	+ + + +
	Middle cerebellar penducle	mcp	+ +
	Paraflocculus	PFl	+ + + +
	Simple lobule (HVI)	Sim	+ + + +
Brain Stem	Gigantocellular reticular nucleus, Alpha	GiA	+ + +
	Intermediate reticular nucleus	Irt	+ +
	Lateroventral periolivary nucleus	LVPO	+ + + +
	Mediventral periolivary nucleus	MVPO	+ + +
	Parvicellular reticular nucleus, Alpha	PCRtA	+ +
	Periolivary region	PO	+
	Pontine reticular nucleus	PnC	+
	Principal sensory trigeminal nucleus	Pr5	–

of a gene may be the end point in a dynamic gene expression process that begins with up regulation during acquisition of the learned response. Alternatively, memory storage may require a balance of up regulation of some genes and down regulation of genes that exert inhibitory constraints on memory formation (Alberini 1994). These latter genes might be termed memory suppressor genes (Abel & Kandel 1998; Abel & Lattal 2001; Abel *et al*. 1998; Cardin & Abel 1999).

To elucidate the possible functions of the *CMRG1* we have cloned its cDNA and characterized its distribution in the mouse brain by *in situ* hybridization. Our analysis revealed widespread expression of the *CMRG1* transcript with the highest levels located in specific regions of the cerebellum, hippocampus and cortex. Expression in the cerebellum was strongest and most discrete in the granule cell layer with each lobule being clearly defined by the labeled layer of granule cells. Labeling was also seen in lobule HVI (simple lobule) where the conditioning-specific differential expression had been detected in the rabbit (Cavallaro *et al*. 2001). There was a similarly strong and discrete expression of the EST transcript in the hippocampus with the dentate gyrus, CA1, CA2 and CA3 pyramidal cell body layer clearly defined. Expression in the cortex was strong but generally less discrete than in the cerebellum and hippocampus. The strongest labeling appeared in the cingulate cortex, motor cortex, piriform cortex and olfactory tubercle with slightly less labeling along the medial edge of the striatum (caudate/putamen).

CMRG1 comprises different potential post-translational modification sites and five WD-repeats. WD-repeat proteins belong to a large and expanding conserved protein family (Neer *et al*. 1994). Currently, the total number of hits in SWISS-PROT (release number: 40.7) is 1367 in 352 different proteins and the number of repeats within these proteins varies between 5 and 8. The WD-repeat proteins are important not only for their critical role in many crucial biological functions including signal transduction, transcription regulation and apoptosis, but also because they are associated with several human diseases that include cognitive impairment (Di Benedetto *et al*. 2001; Li & Roberts 2001; Smith *et al*. 1999). Perhaps the most studied WD-repeat containing proteins are the G-beta proteins, which act as intermediaries in the transduction of signals generated by transmembrane receptors (Smith *et al*. 1999). Among the major categories of Gβ effector targets are calcium channels and potassium channels (Clapham & Neer 1997). Given that increased membrane excitability modulated by calcium and potassium channels has been implicated in classical conditioning of the rabbit NMR (Schreurs & Alkon 1992) it is possible that down regulation of a Gβ protein may play a role in modulating calcium and potassium channels as a result of learning (Clapham & Neer 1997). The initial elucidation of *CMRG1* sequence and distribution described here should facilitate further research to test this hypothesis and fully elucidate its function and its role in learning and memory.

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