

Regional distribution of the leucine-rich glioma inactivated (*LGI*) gene family transcripts in the adult mouse brain.

Authors: Herranz-Pérez, Vicente^{1,3}, Olucha-Bordonau, Francisco Eliseo², Morante-Redolat, José Manuel^{1,3*}, Pérez-Tur, Jordi^{1, 3†}.

¹ Unitat de Genètica Molecular. Institut de Biomedicina de València – CSIC. València (Spain).

² Departament d'Anatomia i Embriologia Humana, Facultat de Medicina i Odontologia. Universitat de València (Spain).

³ Ciberneted.

* Current address: Departament de Biologia Cel·lular i Parasitologia, Facultat de Ciències Biològiques. Universitat de València (Spain).

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† Corresponding author:

Dr. Jordi Pérez-Tur

Unitat de Genètica Molecular – Ciberneted

Departament de Genòmica i Proteòmica

Institut de Biomedicina de València – CSIC

Jaume Roig, 11. E 46010 València (Spain)

Phone: +34 96 339 1755

Fax: +34 96 339 3774

E-mail: jpereztur@ibv.csic.es

ABSTRACT

The leucine-rich glioma inactivated (*LG*) gene subfamily contains four highly conserved members (*LG1*, 2, 3 and 4), which have been described in human, mouse and other mammals. Although their main roles remain unknown, *LG1* gene mutations have been found in human partial temporal lobe epilepsy. Moreover, previous studies showed that the products of these genes exert their function in the nervous system. The anatomical distribution of these gene transcripts in the brain might give some insight to elucidate their possible function. In this study, the pattern of expression of the four *LG* genes was assessed in the brain of C57BL/6J adult mice by in situ hybridization. We found that the *LG1* transcript is mainly expressed in the dentate gyrus and CA3 field of the hippocampus. *LG2* and *LG4* genes, which showed a similar pattern of distribution with minor differences, were mostly expressed in the medial septal area, thalamic reticular nucleus and substantia nigra pars compacta. *LG3*-expressing cells were distributed widespread, but were more consistently observed in the hippocampal formation, thalamic and hypothalamic nuclei, substantia nigra and reticular formation.

In summary, *LG1* gene expression is very restricted to intrahippocampal circuitry, which might be related to its involvement in temporal lobe epilepsy. The patterns of expression of *LG2* and *LG4* genes are very similar and their distribution in the vertical limb of the diagonal band and in putative hippocampal interneurons, suggest that the function of these genes might be related to the generation of hippocampal theta rhythm. Finally, *LG3* gene widespread expression in the brain suggests that its transcripts might be involved in a common cellular process present in different neuronal types.

Section: Cellular and Molecular Biology of Nervous Systems.

Keywords: *LG1*; *LG2*; *LG3*; *LG4*; C57BL/6J mice; In situ hybridization.

Abbreviations: ac, anterior commissure; Acb, accumbens nucleus; aci, anterior commissure, intrabulbar part; ACo, anterior cortical amygdaloid nucleus; AD, anterodorsal thalamic nucleus; AHA, anterior hypothalamic area; AOB, accessory olfactory bulb; AOD, anterior olfactory bulb, dorsal part; AOE, anterior olfactory bulb, external part; AOL, anterior olfactory bulb, lateral part; BA, basal amygdala; BLA, basolateral anterior amygdala; BMA, basomedial amygdaloid nucleus, anterior part; BST, bed nucleus of the stria terminalis; CA1, Ammon's horn 1; CA2, Ammon's horn 2; CA3, Ammon's horn 3; Ce, cerebellum; CeA, central amygdala; CIC, central nucleus of the inferior colliculus; CM, centromedial thalamic nucleus; CPu, caudate putamen; DC, dorsal cochlear nucleus; DCIC, dorsal cortex of the inferior colliculus; DD, deep dorsal nucleus; DeCe, dentate nucleus of the cerebellum; DeN, dentate nucleus; DEnd, dorsal endopiriform; DG, dentate gyrus; DGSC, deep gray layer of superior colliculus; Dk, Darkschewitsch nucleus; DpMe, deep

mesencephalic nucleus; DR, dorsal raphe nucleus; DSub, dorsal subiculum; ec, external capsula; Ent, entorhinal cortex; EPL, external plexiform layer of the olfactory bulb; f, fornix; FC, fasciola cinereum; Gl, glomerular layer of the olfactory bulb; GrL, cerebellar granular layer; GrO, granular cell layer of the olfactory bulb; Hb, habenula; ic, inner capsula; icp, inferior cerebellar peduncle; IF, interfascicular nucleus; IGSC, inner gray layer of the superior colliculus; IO, inferior olive; IP, interpeduncular nucleus; IPI, internal plexiform layer of the olfactory bulb; La, lateral amygdala; LD, laterodorsal thalamic nucleus; LG, lateral geniculate nuclei; LH, lateral hypothalamic area; IHb, lateral habenula; LL, lateral lemniscus; LM, lateral mammillary nucleus; LP, lateral posterior thalamic nucleus; LPO, lateral preoptic area; LSd, lateral septal nucleus, dorsal part; LSi, lateral septal nucleus, intermediate part; LVe, lateral vestibular nucleus, dorsal part; MdR, medullary reticular nucleus; MeA, medial amygdala; mfb, medial forebrain bundle; MG, medial geniculate body; MGd, dorsal division of the medial geniculate body; MGv, ventral division of the medial geniculate body; mHb, medial habenula; Mi, mitral cell layer of the olfactory bulb; ML, lateral division of the medial mammillary nucleus; MM, medial division of the medial mammillary; MMn, median division of the medial mammillary nucleus; MnR, median raphe nucleus; MOB, main olfactory bulb; MoV, motor nucleus of the trigeminal nerve; MPO, medial preoptic area; MS, medial septal nucleus; MS/LS, transition area between medial and lateral septum; mta, mamillothalamic tract; mtg, mamillotegmental tract; NTS, nucleus of the solitary tract; Pa, pallidal nucleus; PAG, periaqueductal gray; PaV, paraventricular hypothalamic nucleus; pcvLGN, parvocellular division of the lateral geniculate nucleus; PIN, posterior intralaminar cortex; Pir, piriform cortex; Pn, pontine nuclei; PrV, principal nucleus of the trigeminal nerve; PTA, pretectal area; Pu, putamen; PVP, paraventricular thalamic nucleus, posterior division; pyx, pyramidal decussation; Re, reuniens thalamic nucleus; RF, reticular formation; RLi, rostral linear nucleus of the raphe; RN, red nucleus; RPC, red nucleus, parvocellular part; RPO, reticularis pontis oralis; Rt, reticular thalamic nucleus; SC, superior colliculus; SI, substantia innominata; sm, stria medullaris of the thalamus; SNC, substantia nigra, pars compacta; SNr, substantia nigra, pars reticulata; SpV, spinal trigeminal tract; st, stria terminalis; SuM, supramammillary nucleus; TT, taenia tecta; TS, triangular septal nucleus; VCA, ventral cochlear nucleus, anterior part; VCP, ventral cochlear nucleus, posterior part; vDB, nucleus of the vertical limb of the diagonal band; VL, ventrolateral thalamic nucleus; VP, ventral pallidum; VPL, ventral posterolateral thalamic nucleus; VPo, ventral posterior thalamic nucleus; VTA, ventral tegmental area; ZI, zona incerta.

1. INTRODUCTION

The leucine-rich glioma inactivated (*LGI/Epitempin*) gene subfamily belongs to the large leucine-rich repeat protein family. Searching for homology sequences in public databases, based on the previously known LGI1 protein sequence, revealed the existence of a highly homologous group of proteins that contain the so-called EPTP repeats (Pfam **PF03736**): LGI1, LGI2, LGI3, LGI4, VLGR1 and TNEP1 {Staub, 2002 #4522}. The EPTP repeats were first identified in the LGI1 sequence and are thought to generate a beta-propeller structure, a potential protein-protein interaction domain {Kobe, 2001 #4388}. Moreover, the four LGI proteins also contain cysteine-flanked leucine-rich repeats, which confer them a highly similar domain structure {Staub, 2002 #4522}. Another common feature of this group of proteins is that overexpression in several cell lines of either *LGI* shows that the products of these genes are glycosylated secreted proteins {Senechal, 2005 #5017}. In addition, defective *LGI1* gene gives rise to a mutant protein that is neither secreted nor unstable {Senechal, 2005 #5017}.

Information about the *LGI1* gene and protein is more abundant than for the rest of the family members. *LGI1* gene was discovered in the T98G glioblastoma cell line, where it was rearranged as a result of a t(10;19)(q24;q13) balanced translocation {Chernova, 1998 #3080}. Thus, it was proposed to be a tumor suppressor gene based on its lower expression in human glial tumor and glioblastoma cell lines when compared to normal brain samples {Chernova, 1998 #3080}. Further investigation led to the discovery that *LGI1* gene mutations caused autosomal dominant lateral temporal epilepsy (ADLTE) {Kalachikov, 2002 #4318; Morante-Redolat, 2002 #4526; Gu, 2002 #4527}. Analysis of *LGI1* gene expression revealed that it is strongly transcribed in neurons, but not in glial cells {Kalachikov, 2002 #4318; Senechal, 2005 #5017}; nevertheless, *LGI1* gene is also expressed in other tissues {Head, 2007 #5037}. It has been hypothesized that LGI1 might be involved in cell motility and invasiveness {Kunapuli, 2003 #4625; Kunapuli, 2004 #4936}. Furthermore, {Schulte, 2006 #5039} have reported that the LGI1 protein binds to the presynaptic voltage-gated K⁺ channel (Kv) where, as part of the Kv complex, prevents the N-type inactivation mediated by the Kvβ1 subunit. Mutant LGI1 proteins may fail in inactivating Kvβ1, which would result in channels closing faster. Thus, slowly repolarized terminals of LGI1 defective neurons may induce focal seizures {Schulte, 2006 #5039}. Additional information on putative LGI1 function arises from the observation that LGI1 oligomers interact with post-synaptic ADAM22 receptors {Fukata, 2006 #5040}, which suggests that secreted LGI1 protein enhances AMPA receptor-mediated excitatory transmission {Fukata, 2006 #5040}. [But the most relevant proof about the function of LGI1 lie on the discovery by Zhou and co-workers, that this protein mediates postnatal structural pruning and functional maturation of glutamatergic synapses in hippocampus, while mutant LGI1 prevents this process in transgenic mice. Moreover, they suggest that LGI1 acts at](#)

the presynaptic terminus to reduce Kv β 1-mediated inhibition over the Kv1.1 channel {Zhou, 2009 #5614}.

Conversely, there is scarce data on the function of *LGI3* and *LGI4* and no data about *LGI2*. Several studies have shown that the beta amyloid peptide (Abeta) upregulates and colocalizes with LGI3 protein in the cell membrane and with internalized Abeta in astrocytes {Kimura, 2007 #5533; Okabayashi, 2008 #5531; Okabayashi, 2007 #5532}. Western blot analyses demonstrated that both glial fibrillary acidic protein (GFAP) and apolipoprotein E (ApoE) significantly increased with simultaneous Abeta-induced upregulation of LGI3, suggesting that activated LGI3 protein may be involved in the astroglial response against Abeta {Kimura, 2007 #5533}. Furthermore, it has been reported that LGI3 colocalizes with Abeta in cultured rat astrocytes, whereas RNA interference experiments demonstrate that *LGI3* downregulation clearly inhibits Abeta uptake by cultured astrocytes {Okabayashi, 2008 #5531}. These results strongly suggest that the LGI3 protein is involved in Abeta uptake by astrocytes {Okabayashi, 2008 #5531}. With respect to neurons, in brains from aged monkeys, LGI3 protein accumulates on or near neuronal plasma membranes, where it colocalizes with endocytosis-associated proteins and lipid raft markers {Okabayashi, 2007 #5532}. On the other hand, syntaxin-1, a SNARE component in exocytosis, has been identified as an LGI3-associated protein by pull-down, coimmunoprecipitation and colocalization techniques {Park, 2008 #5446}. The results obtained from these studies suggest that LGI3 protein may play a regulatory role in neuronal endocytosis {Okabayashi, 2007 #5532} and exocytosis via interaction with syntaxin 1 {Park, 2008 #5446}.

With regard to *LGI4*, its ablation is associated with a defective Schwann cell signalling pathway, resulting in abnormal peripheral nerve development that causes the *claw paw* mutation in mice {Bermingham, 2006 #5032}. Results from immunoprecipitation and mass spectrometric analysis from mice brain proteins also link LGI4 and LGI1 to ADAM22, revealing that either LGI1 or LGI4 can bind to ADAM22, ADAM11 and ADAM23 with different affinity. In fact, it appears that ADAM23 works as an LGI1 receptor in the ADAM22-negative cells and therefore, these systems could be regulated not only by affinity, but also by the cell-type-specific expression of each protein {Sagane, 2008 #5510}.

Data regarding on the distribution pattern of neurons expressing the different types of LGI proteins are scarce. The expression of these genes in human brain has been assessed by semi-quantitative PCR {Gu, 2002 #4347}, but little is known about their expression in other tissues. In mouse brain, {Kalachikov, 2002 #4318} described the regional distribution of *LGI1* expressing neurons in the hippocampus and cortex. In addition, {Senechal, 2005 #5017} reported the comparative distribution of *LGI1-4* expressing neurons; however, the reported maps only contained one sagittal section for each gene. Finally, {Lee, 2006 #5600} showed that *LGI3*

transcripts are widespread expressed in the mouse brain, but the authors did not analyze the expression pattern of this gene in detail. Thus, the aim of the present study is to describe the detailed neuroanatomic distribution of the transcripts of the four *LGI* genes (*LGI1-4*) in the mouse brain using in situ hybridization (ISH). An accurate map of the regional distribution of these transcripts in the brain might provide some insight about the putative function/s of each *LGI* gene. Thus, a stronger expression of one of these genes in a distinct brain region most likely will point out towards a particular function of the encoded protein in that specific neuronal group.

2. RESULTS

In the present study, we used in situ hybridization to determine the anatomical distribution of cells expressing transcripts of the *LG1* gene family, *LG1*, 2, 3 and 4, in the mouse brain. Two sense and two antisense digoxigenin-labeled probes were designed and synthesized for each of the genes mentioned above. The pattern of expression of each *LG1* gene is summarized in Figure 1 and Table 2. To demonstrate the specificity of the binding of the designed probes to the different *LG1* transcripts, brain sections were processed using sense probes instead of antisense. We verified that the complementary sense probe for each antisense probe did not show signal (this is illustrated in Figure 2A for *LG11* transcripts in the hippocampus). Each pair of antisense probes showed an equal labeling pattern for their corresponding gene transcript. No regional or intensity differences were observed in the labeling within each pair of probes.

***LG11* gene expression**

The majority of *LG11* expressing cells were located in anatomically defined areas. The entire brain, including the olfactory bulb, and the cervical spinal cord were analyzed, although we only found *LG11*-labeled cells in the telencephalon (Figure 1, A1-S1).

The strongest *LG11* expression signal was observed in the dentate gyrus and in the CA3 and CA2 fields of the hippocampal formation (Figure 2B). Labeling in the CA1 field was weak but evident. There was also some strong signal in the subicular area (Figure 2C). *LG11* expression in the hippocampal formation was restricted to the pyramidal and granular layers, whereas scattered labeling was observed outside these areas (Figure 2C).

Labeling in the cerebral cortex was weak but evident. No regional area showed a higher labeling than others. In contrast, within each area inner layers (V-VI) showed a stronger labeling than outer (I-IV) ones, which showed almost no signal. There were clusters of labeled perikarya in both medial and lateral entorhinal cortices, which we have identified as layers II and III (Figure 2D). Some labeling was also present in the piriform cortex. *LG11*-labeled cells were also scarce and dispersed in the amygdala, mainly in the basolateral complex. The central and medial amygdala, along with the cortical amygdala, contained few or no labeled cells (Figure 2E). The lateral septum contained labeled perikarya specifically in the caudal dorsal nucleus. Some scattered labeled cells were also observed in the caudate putamen area (Figure 2F). *LG11*-labeled cells were absent in the olfactory bulb, diencephalon, brainstem and spinal cord.

***LG12* gene expression**

A consistent pattern of *LG12* transcription was observed throughout the entire brain (Figure 1 B2-S2). The *LG12* gene was expressed in scattered cells of all layers of the main

olfactory bulb (Figure 3A). There were also labeled cells in the accessory olfactory bulb, and a strong signal was observed in the external division of the anterior olfactory nuclei (Figure 3B).

Within the cerebral cortex, labeling was present in all cortical areas with no apparent regional differences. In contrast, for each area cells were intensely labeled (although in a diffuse way) in the inner layers (V-VI) whereas no labeling was found in the outer layers (I-IV) (Figure 3C). Some labeled cells were observed in layers II and III of the piriform cortex (Figure 3D).

In the hippocampal formation, *LG/2* expressing cells were dispersed in all layers of both the dentate gyrus and Ammon's horn. Interestingly, both pyramidal and granular layers were unlabeled (Figure 3E). This observation, together with the scattered distribution of the labeled cells and the absence of cellular labeling in olfactory bulbs or spinal cord among other areas, strongly suggests that the *LG/2* expressing cells in the hippocampus are interneurons. In addition, dense labeling was observed in the deep layers of the subiculum.

Expression of *LG/2* in the amygdalar complex was diffuse (Figure 3F), whereas strong labeling was found specifically in the supracapsular part of the bed nucleus of the stria terminalis.

Within the septal area, strong labeling was present in the vertical limb of the diagonal band-medial septum, specifically in the most medial area. Conversely, the horizontal limb and lateral areas of the medial septum-diagonal band complex were unlabeled (Figure 3G). Only some clusters of *LG/2*-labeled cells were observed lateral to the medial septum in the medial septal/lateral septal transition zone.

The expression pattern of *LG/2* in the basal ganglia showed a specific distribution: labeled cells were dispersed in the caudate putamen area, whereas strong and concentrated labeling was found in the pallidum.

A consistent pattern of labeling was obtained in the thalamus. No labeling was observed in the most dorsal thalamic nuclei, with the exception of the anterodorsal thalamic nucleus. The strongest labeling was observed at all levels of the reticular nucleus (Figure 3E). Clusters of labeled cells were found in the magnocellular ventral division of the lateral geniculate nucleus. Dense labeling also occurred in the deep dorsal nucleus of the posterior intralaminar complex, between the dorsal and ventral divisions of the medial geniculate body. Labeled cells also appeared in the medial division of the lateral habenular nucleus. In addition, some labeling was also present in both divisions of the zona incerta (Figure 4A).

Some weak but consistent labeling was noted in both the medial preoptic nucleus and in the lateral preoptic area. The medial division of the medial mammillary nucleus showed a robust *LG/2* transcription signal (Figure 4B). Faint or no expression was observed in the rest of the hypothalamic nuclei.

At the mesencephalic level, strong labeling was observed in both the pars reticulata and the pars compacta of the substantia nigra, and also in the ventral tegmental area. In addition, *LG/2* expression was also observed in the interpeduncular nuclei. Scattered labeled cells were observed

in several areas of the brainstem such as the deep mesencephalic reticular formation, red nucleus, interstitial nucleus of Cajal, and deep gray layer of the superior colliculus. Some labeling occurred in the pericentral nuclei of the inferior colliculus and also in the intercollicular nucleus.

At the pontine and medullary levels, some *LG/2* expression was present in the median and dorsal raphe nuclei. Scattered labeled cells were found in the reticular formation. Some *LG/2* signal was present in the granular layer of the dorsal cochlear nucleus; whereas some dispersed labeled cells were located in the ventral anterior and ventral posterior cochlear nuclei. This same pattern of diffuse labeling was observed in the vestibular nuclei and in the principal and spinal trigeminal nuclei. In contrast, strong *LG/2* labeling was observed in the inferior olive (Figure 4C).

In the cerebellum, high expression *LG/2* levels were observed in a fraction of cells of the granular layer of the cerebellar cortex. Some weak but consistent labeling was also observed in the deep cerebellar nuclei (Figure 4D). *LG/2* labeling was not found in any division of the spinal cord.

***LG/3* gene expression**

In contrast to the rest of the *LG/* genes, *LG/3* was expressed more diffusely in the diencephalon, and presented a restricted labeling pattern in the rest of the brain (Figure 1 A3-S3). Labeled cells were scarce in the granular layer of the main and the accessory olfactory bulb, and some labeling was found in the mitral cell layer of the main olfactory bulb.

The neocortex showed diffuse *LG/3* labeling with no apparent regional differences. Strong labeling was found in both the granular layer of the dentate gyrus and the pyramidal layer of the Ammon's horn (Figure 5A). In addition, some sparse *LG/3* labeling was present in different layers of the hippocampal formation. The lateral and central nuclei of the amygdala showed strong *LG/3* labeling (Figure 5B), as well as the pallidum and some cells in the caudate-putamen area. Many dispersed *LG/3* labeled cells were found mainly in the dorsal thalamus, although all the thalamic area was labeled (Figure 5C). Although the *LG/3* signal was present in nearly all nuclei of the thalamus including the medial habenular nucleus (Figure 5D), the intensity of the signal was weak. In the subthalamus, some faint mRNA expression was detected in the zona incerta and the medial and lateral nuclei. Weak *LG/3* signal was observed in the supraoptic, paraventricular, ventromedial and mammillary nuclei of the hypothalamus. No *LG/3* labeling was observed in both the superior and inferior colliculi, contrasting with the strong labeling found in the periaqueductal gray. The oculomotor nuclei showed lower levels of labeling. Some *LG/3* labeling was observed in the substantia nigra pars compacta, which was higher than in the pars reticulata (Figure 5E). No *LG/3* labeling was detected in the interpeduncular and interfascicular nuclei, but some labeling was present in the ventral tegmental area.

Along the reticular formation, strong *LG/3* labeling was observed associated with large cells located in the mesencephalic reticular formation, the reticularis pontis oralis and caudalis, and the

medial magnocellular division of the medullary reticular formation. In the cerebellum some labeled cells were observed in the deep cerebellar nuclei and in the Purkinje cell layer. No labeling was observed in the spinal cord.

***LG14* gene expression**

The pattern of expression of the *LG14* gene was very similar to that observed for the *LG12* gene expression, and corresponded to well defined anatomical areas (Figure 1 A4-S4).

LG14 labeling in the olfactory bulb was similar to that described for *LG12* transcripts. Some labeling was found in the granular layer of the main olfactory bulb and in the accessory olfactory bulb.

The *LG14* expression in the cerebral cortex was very similar to *LG11* and *LG12* distribution, where scattered cells showed strong *LG14* labeling in the deep layers (V-VI), whereas the superficial layers (I-IV) remained unlabeled (Figure 6A).

Some labeling occurred in the layer II of the piriform cortex and in the endopiriform nucleus. *LG14* labeling in the hippocampal formation was slightly different to that observed for *LG12*. Weak *LG14* labeling was observed in the pyramidal and granular layers of the hippocampal formation, whereas scattered putative interneurons showed strong labeling (Figure 6B). Weak *LG14* labeling was also observed in the amygdala, although a group of labeled perikarya was seen in the medial lateral division of the central nucleus. A stronger *LG14* signal was observed in the supracapsular bed nucleus of the stria terminalis.

LG14 labeling in the medial septum was identical to that observed for *LG12* transcripts, being restricted to the vertical limb of the diagonal band and its most medial subdivision. This labeling was the strongest *LG14* signal observed in the brain (Figure 6C). In addition, some *LG14* labeled cells were concentrated in the lateral septum/medial septum transition zone.

Faint *LG14* labeling was observed in the caudate-putamen whereas, like in the *LG12* pattern, strong labeling was found in the lateral globus pallidus (Figures 6D and E).

As with *LG12* transcripts, the strongest *LG14* signal in the thalamus was observed in the nucleus reticularis (Figure 6E). Some labeled cells were also found in the zona incerta and in the lateral nucleus of the habenula. In addition, some *LG14* labeling was specifically observed in the deep dorsal nucleus of the posterior intralaminar complex (Figure 6F).

In the mesencephalon, some *LG14* labeled cells were observed in the deep mesencephalic reticular formation, anterior pretectal nuclei, nucleus of Darkschewitch and nucleus of the posterior commissure. Strong *LG14* labeling was also found in both the pars reticulata and the pars compacta of the substantia nigra (Figure 7A), the ventral tegmental area, and the rostral dorsal interpeduncular nucleus (Figure 7B and C). Some labeling was detected in the pericentral nuclei of the inferior colliculus, and an intense *LG14* signal was observed in some dispersed cells of the

intercollicular area. No *LG/4* labeling was observed in the superior colliculus and in the periaqueductal gray.

In the pons, medulla and cerebellum *LG/4* labeling was nearly identical to that observed for *LG/2*. Some diffuse signal was observed in the median and dorsal raphe nuclei and the reticular formation. *LG/4* expression was also present in the cochlear vestibular and trigeminal nuclei, whereas strong labeling was observed in the inferior olive. In the cerebellum, strong *LG/4* signal was present in some cells of the granular layer of the cerebellar cortex, whereas some weak labeling was seen in the deep cerebellar nuclei. *LG/4* labeling was not detected in the spinal cord.

3. DISCUSSION

With this procedure, we have generated a detailed map of the pattern of expression of each *LG/* gene within defined anatomical divisions in the brain. Based on the regional distribution of the transcripts for each gene, we have speculated about their possible involvement in a particular function.

We have used two antisense digoxigenin-labeled probes for each gene. Their complementary sense probes were also assayed to avoid unspecific labeling. We observed an equal pattern of expression for the two antisense probes of each *LG/* gene we have used. In all cases, incubation with the sense probe did not produce any labeling in the tissue, demonstrating that the signal obtained for each probe is specific of each gene transcript, which was our main goal. The use of digoxigenin-labeled probes instead of the more sensitive radioactive probes, could lead us to an underestimation of the neurons expressing these genes, but in general this is a good approach for descriptive anatomy, which is the scope of the present study. In addition, digoxigenin-labeled probes provide a higher resolution in the observation of labeled areas, allowing us to discern within concrete brain areas.

There are very few antibodies for the proteins encoded by these genes and when available, these antibodies did not work reliably in our hands, resulting in the absence of exhaustive immunohistochemical studies. Therefore, the importance of the mRNA expression of these genes becomes even more relevant. Besides, in situ hybridization minimizes usual problems that appear as a result of the use of antibodies, like weak signal or the lack of knockout mice for these genes to test specificity.

To date the only comparative data on the distribution of *LG/1-4* expressing cells was reported by Senechal et al. {Senechal, 2005 #5017}, in a study that focused on demonstrating that the *LG/* genes give rise to secreted protein products. In that study, the patterns of distribution for each *LG/* gene transcript were presented in a single sagittal section for each gene, using digoxigenin-labeled probes. Their results for *LG/1* and *LG/2* expression were similar to ours, but here we show several differences that were seen for *LG/3* and *LG/4* transcripts. In addition, we observed some gene expression in additional areas, which were not considered in Senechal et al. study.

The pattern of *LG/1* expression in the hippocampus and cerebral cortex matches with the distribution reported in other studies using digoxigenin based ISH {Kalachikov, 2002 #4318;Senechal, 2005 #5017}. In agreement with our findings, in these studies the highest *LG/1* signal was observed in the granular and pyramidal layers of the dentate gyrus and the CA3 field. Similarly to our results, some *LG/1* expression was reported in the piriform cortex, amygdala and deep layers of the cerebral cortex.

In addition, we observed clusters of *LG/1* labeled perikarya in the superficial layers II and III of both medial and lateral entorhinal cortices. This observation is in agreement with the results

reported in a study using *LG11* immunocytochemistry, in which *LG11* labeling for the protein was found in the molecular layer of both dentate gyrus and Ammon's horn {Schulte, 2006 #5039}. This molecular layer corresponds to the terminal field of the classical perforant pathway that arises from the superficial layers II and III of the entorhinal cortex {Witter, 1991 #5041}, suggesting that the *LG11* gene product is transported from the perikarya to the terminals. These data are consistent with the notion proposed by Schulte et al. that the *LG11* protein may be a subunit of the presynaptic voltage gated K^+ channels {Schulte, 2006 #5039}.

The caudal part of the dorsal division of the lateral septum (LSd) receives bilateral projections from the CA3 field and projects to the lateral supramammillary and the medial septum/diagonal band complex {Risold, 1997 #5042}. Then, the medial septum and lateral supramammillary nucleus project back to the hippocampal formation being, in part, responsible of theta synchronization {Kocsis, 1994 #5611}. In addition, electrical or chemical stimulation of the lateral supramammillary nucleus enhances granule cell synaptic excitability {Mizumori, 1989 #5048;Carre, 1991 #5050;Nakanishi, 2001 #5049}. The majority of components of this loop express *LG11* transcripts and, in addition, a mutation of *LG11* protein produces an increase in cellular excitability through its association with presynaptic $Kv1$ channels when compared to wild-type *LG11* {Schulte, 2006 #5039}. Taking together, these observations suggest that the connectivity among *LG11*-expressing cell groups may be involved in the increased excitability that leads to the epileptic phenotype. In such conditions, theta-burst activity or other high-frequency excitations may trigger focal epileptic activity.

Our results agree with those using ISH for *LG11* showing a restricted expression of *LG11* in the telencephalon {Kalachikov, 2002 #4318;Senechal, 2005 #5017}. However, *LG11* expression studies by Northern blot in mouse {Chernova, 1998 #3080} and by Western blot in human {Furlan, 2006 #5615}, also showed expression of this gene in thalamus, cerebellum and spinal cord. These discrepancies could be due to the different sensitivity of the methods used in detecting *LG11* expression. Besides, the expression of *LG11* in hippocampus and restricted cortical areas is in agreement with the development of lateral temporal epilepsies as it has been reported previously {Berkovic, 1991 #5616;Senechal, 2005 #5017} and also agrees with the fact that *LG11* protein mediates in the postnatal maturation of glutamatergic excitatory synapses in the hippocampus, which is arrested in transgenic *LG11*-mutated mice {Zhou, 2009 #5614}.

Senechal et al {Senechal, 2005 #5017} reported a specific *LG12* expression in the nucleus reticularis thalami and a diffuse pattern throughout the brain. These results are in agreement with the strong *LG12* signal we observed in the nucleus reticularis thalami. In addition, we were able to find other important locations where *LG12* labeling was robust and specific. Thus, we observed *LG12* labeled neurons in the hippocampus, pallidum, vertical limb of the diagonal band/medial septum, medial division of the lateral habenula, zona incerta and substantia nigra pars reticulata. The distribution of *LG12* genes in non principal neurons of the hippocampus, out of the pyramidal

and granular layers and in the vertical limb of the diagonal band allow us to speculate a possible role of *LG12* in theta rhythm synchronization. Most, if not all, non principal hippocampal neurons are GABA-ergic and contributes to hippocampal population oscillations (theta, gamma and 200 Hz ripples) (Freund and Buzsaki 1996). Also the medial septum contributes to synchronization of the hippocampal neurons at theta frequency, in fact, the medial septum is considered to be the hippocampal theta pacemaker (Vertes and Kocsis 1999). The medial septum is composed of a heterogeneous population of glutamatergic, GABAergic and cholinergic neurons (Colom 1999, Sotty 2003) each one contributing in a different way to hippocampal synchronization (Sotty 2003). Besides, parvalbumin GABAergic neurons of the medial septum project to the hippocampal GABAergic non principal neurons {Freund, 1988 #5055; Freund, 1989 #5601}. The exact role of *LG12* in this system remains to be investigated.

Our data show widespread expression of *LG13* in the diencephalon, whereas it is much lower in the rest of the brain. The fact that *LG13* is expressed in granular and pyramidal neurons of the hippocampal formation might indicate a possible relation of the expression of this gene with excitatory aminoacid neurons. Although the widespread distribution of the *LG13* transcripts does not allow postulating a particular function, the fact that the strongest activity was found in hippocampal cells suggests that this might be considered as a good system for studying *LG13*-Abeta interactions in glia and pyramidal cells.

Our data show that the regional distribution of *LG14* transcripts is similar to that observed for *LG12*. Indeed, we found *LG14* labeled cells in both the nucleus reticularis thalami and medial septum, similar to the distribution of *LG12* transcripts. Nevertheless, some differences were observed in the patterns of expression of both genes, suggesting possible different roles for *LG12* and *LG14* products. In addition to the labeling observed in putative interneurons, *LG14* signal was also weakly, but consistently, expressed in the granular and pyramidal layers of the hippocampus, whereas *LG12* signal was absent. *LG12* was highly expressed in the lateral globus pallidus whereas *LG14* expression was weak. *LG12* expression was present in the medial division of the medial mammillary nucleus, which was devoid of *LG14* signal. In contrast, *LG14* labeling was stronger than *LG12* labeling in the ventral tegmental area. Our pattern of *LG14* expression differs somewhat from that reported by Senechal et al. {Senechal, 2005 #5017}, who showed some *LG14* signal in the rostral migratory stream leading to the olfactory bulb. In our study, we used one probe located in the 3'-end of the cDNA and a second probe in the coding region of the *LG14* gene, and both probes provided the same pattern of labeling.

The *LG14* gene product has been proposed to play a role in peripheral nerve development. Bermingham et al. demonstrated *LG14* expression in Schwann cells, and found that defective forms of the *LG14* gene result in the *claw paw* mutation {Bermingham, 2006 #5032}, characterized by axonal shorting and hypomyelination throughout the peripheral nervous system in mice. In addition, the authors also discovered that silencing the *LG14* gene in Schwann cell cultures inhibits

myelination, which is restored by applying exogenous LGI4 protein product {Birmingham, 2006 #5032}.

In summary, we have found that *LGI* genes can be classified into three main groups of *LGI* genes: *LGI1*, *LGI2/4* and *LGI3*. *LGI1* gene products might be involved in the presynaptic regulation of voltage-gated K⁺ channels through several pathways. One is the classical trisynaptic pathway that originates in the entorhinal cortex and ends in the CA1 field; the second is a longer pathway, which emerges from the CA3 field towards the caudal dorsal lateral septum, and from there, towards the lateral supramammillary nucleus. This projects backwards to the dentate gyrus and the CA3 field in a projection that did not contain *LGI1* regulation. The role of LGI1 along the pathway remains to be determined. Defective forms of the LGI1 protein might maintain depolarization throughout these pathways leading to ADLTE. The pattern of expression of *LGI2* and *LGI4* genes coincides in the medial septum and nucleus reticularis thalami, (and seems to be associated with GABAergic neurons) although regional differences were also found. Finally, the widespread expression of *LGI3* gene in numerous brain areas suggests that the product of this gene could be involved in a common cellular process in different neuronal types. The non-restricted pattern of expression of this gene precludes proposing a particular function for its products.

4. EXPERIMENTAL PROCEDURE

Animals:

Adult C57BL/6J male mice (n=20; weight=25-35 gr) from Harlan, were maintained at a 12:12 hours light/dark cycle at 22 °C, with ad libitum access to food and water. All experiments were carried out conformed to the guidelines for animal experimentation on the ethical use of animals of the CSIC and the School of Medicine of the University of Valencia. All efforts were made to minimize the number of animals used and their suffering.

Design and synthesis of probes:

We designed two probes for each *LG1* gene mRNA (*LG11-4*). Homology searches using NCBI-BLAST were performed to ensure that each probe had no homology with any other sequence in the public databases. Special caution was taken in the case of *LG14*, given that its 3'-UTR overlaps with the 3'-UTR region of the *Fxyd3* gene, encoded in the reverse strand. Such region was avoided in order to guarantee the specificity of our probes.

The selected probes were the complementary sequences to the following mouse mRNA genome regions: Nucleotides 379 to 909 corresponding to exons 3 to 7 (probe 1) and nucleotides 1659 to 2174 within exon 8 and the 3' untranslated region (3'-UTR; probe 2) for *LG11* (GenBank accession no. [NM_020278](#)); nucleotides 1700 to 2343 from the 3'-UTR (probe 1) and nucleotides 482 to 1019 within exons 3 and 6 (probe 2) for *LG12* (GenBank accession no. [NM_144945](#)); nucleotides 280 to 823 within exons 1 and 6 (probe 1) and nucleotides 1790 to 2344 in the 3'-UTR (probe 2) for *LG13* (GenBank accession number: [NM_145219](#)); nucleotides 538 to 1091 corresponding to exons 5 and 8 (probe 1) and nucleotides 1707 to 2252 in the 3'-UTR (probe 2) for *LG14* (GenBank accession no. [NM_144556](#)). All the sequences mentioned above were PCR-amplified from a mouse cDNA library (Invitrogen). Primers used at this stage, along with the size of the corresponding probe, are shown in Table 1. The amplification conditions were as follows: 5 minutes of initial denaturalization at 94 °C, 30 cycles of 30 seconds at 94 °C, 30 seconds at 58 °C and 1 minute at 72 °C, followed by a final extension of 10 minutes at 72 °C. *Pfu turbo* polymerase from Stratagene was used for these amplifications following the manufacturer's indications. The PCR product was resolved in 1% agarose gel in tris-borate-EDTA buffer (TBE, 89 mM tris, 89 mM boric acid, 20 mM EDTA, pH 8). The band corresponding to the expected fragment size was isolated and purified using a Minelute gel extraction kit (Qiagen). Subsequently, the fragment was digested with *Sal*I and *Xho*I for the *LG11* probes, and with *Eco*R I and *Xho*I for the remaining probes (all restriction enzymes were purchased from Fermentas. The resulting sequences were cloned into pBluescript KS II (-) (Stratagene) with a Rapid ligation kit (Roche) and transformed into DH5- α electrocompetent bacteria. These constructs were subsequently sequenced to verify their integrity.

Once cloned, the constructs were amplified in bacteria. Following phenol purification, an in vitro synthesis of the RNA probes with digoxigenin-labeled nucleotides was performed using an RNA oligonucleotide synthesis kit (Roche), following the manufacturer's instructions. The integrity of the probes was verified by resolving them in 1% agarose gel in TBE buffer, and the products were stored with 50% formamide to prevent the formation of secondary structures.

In situ hybridization:

To avoid the activity of ribonucleases, all material was washed with ethanol and heated for at least two hours at 200 °C prior to in situ hybridization. In addition, all solutions were treated with 1:10⁴ diethyl-pyrocabonate (DEPC, Sigma-Aldrich) and autoclaved. All steps were performed at 4 °C if not otherwise specified.

Animals were killed by subcervical dislocation and the brains were quickly removed, washed in 0.1 M phosphate buffered saline (PBS; 137 mM NaCl; 2.68 mM KCl; 6.48 mM Na₂HPO₄; 647 mM KH₂PO₄) and fixed overnight in 4% paraformaldehyde in PBS. Brains were then washed for 30 minutes in PBS and embedded in gelatin. Then, 50 µm sections were obtained with a Leica vibratome and stored in 4% paraformaldehyde until further use. For each brain four parallel subseries were obtained and reacted with the correlative set of *LG1-4* probes (two sense and antisense probes were used for each gene). To remove the residual paraformaldehyde, free-floating sections were washed twice with PBST (0.1% Tween 20 in PBS) followed by gradual dehydration with 25%, 50%, 75%, and 100% methanol in PBST and vice versa. Next, sections were washed with PBST and incubated with 1% H₂O₂ in PBST for 10 minutes. Afterwards, sections were washed again with PBST, treated with 5 µg/ml of proteinase K for 7 minutes at room temperature and postfixed with 4% paraformaldehyde for 20 minutes. Sections were then washed again before a prehybridization step of at least 2 hours with hybridization solution (50% deionized formamide; 5X SSC; 2% blocking powder (Roche), 0,1% Triton X-100; 1 mg/ml yeast tRNA; 5 mM EDTA; 50 µg/ml heparin) at 57 °C. Next, fresh solution was added, followed by the addition of the digoxigenin labeled RNA probes at a final concentration of 1 µg/ml. The probes were left to hybridize overnight at 57 °C. The next day, 5 high stringency washes were carried out for 30 minutes each with 0.1% CHAPS (Sigma, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate), 2X SSC in DEPC treated H₂O at 57 °C. After blocking with 20% fetal calf serum in KTBT (50 mM Tris-HCl pH 7.5; 150 mM NaCl; 10 mM KCl; 0.3% Triton X-100) for 3 hours, sections were incubated with 1:1000 sheep anti-digoxigenin alkaline phosphatase-conjugated antibody (anti-DIG-AP) (Roche) in blocking solution overnight. To prevent unspecific reactivity of the antibody, we pre-absorbed the anti-DIG-AP antibody with brain powder. Brain powder was obtained by homogenizing several mouse brains in a minimal volume of PBS. Following centrifugation of the brain homogenate at 3000 g for 30 minutes, the pellet was washed with ice-cold acetone, dried, and crushed to obtain a fine powder. The next day we carried out

5 one-hour washes of the sections with KTBT at room temperature. An alkaline phosphatase-mediated color reaction was obtained by incubating the sections in 333 mg/ml of 4-nitroblue tetrazolium chloride (Roche) and 165 mg/ml of 5-bromo-4-chloro-3-indolyl phosphate (Roche) in NTMT buffer (100 mM Tris-HCl pH 9.5; 50 mM MgCl₂; 100 mM NaCl; 0.1% Tween-20) to obtain a blue-purple precipitate. When the desired level of color reaction was observed (after 2-3 hours incubation), sections were gently washed in KTBT overnight, followed by a final wash with PBS prior to mounting the sections on superfrost/plus slides (Microm). Slides were dried overnight and were coverslipped next day with Eukitt (Zeiss).

Parallel control experiments were carried out for all the probes using the sense sequence of the probe, always at the same concentration as the respective antisense probe, to assess possible non-specific hybridization.

Analysis of the anatomical distribution of *LGI* transcripts:

Each section series reacted with a *LGI* probe was analyzed with a Nikon Eclipse E600 microscope at 4x, 10x and 20x objectives in bright field illumination. Each section was compared with the corresponding level of the Paxinos and Franklin mouse brain atlas {Paxinos, 2001 #5612}, from which we obtained a set of diagrams for the representation of the expression of the genes. Our set of diagrams (Figure 1) starts at Bregma level +3.08 mm (A1-A4) and finishes at level -7.64 mm (S1-S4). Consecutive diagrams are separated 0.5 mm. Dots represent labeling in the represented area under our subjective criteria for quantification. Additionally, expression levels were also registered for the most representative areas. Quantification was estimated under our subjective criteria (Table 2).

Some representative pictures showing the distribution of the labeled transcripts for the four *LGI* genes were obtained in brightfield, using a Nikon DMX-1200 camera connected to a computer. The images were captured using software ACT1 (Nikon). The images were only adjusted for brightness and contrast using Adobe Photoshop.

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TABLE AND FIGURE LEGENDS

Table 1.- Primers and restriction sites used for cloning the different probes designed for in situ hybridization.

Table 2.- Relative levels of expression for each *LG1* gene estimated by visual comparison in representative areas of the mouse central nervous system.

Figure 1.- Diagrams showing the distribution and relative strength of the ISH signal for each *LG1* gene (*LG1-4*) across the mouse brain. Diagrams were obtained by modifying templates from the Paxinos and Franklin's mouse atlas (2001). The diagrams start at Bregma level +3.08 mm (A1-A4) and finish at level -7.64 mm (S1-S4). Consecutive diagrams are separated 0.5 mm. The strong similarity between *LG2* (right side, first column) and *LG4* (right side, second column) expression is apparent. All scale bars are 1 mm.

Figure 2.- Photomicrographs showing the expression of *LG1* gene in several brain regions. A) Absence of *LG1* labeling after incubation with the sense probe. B) *LG1* signal obtained with the antisense probe 1, which is strong in the dentate gyrus (DG) and CA3, and low in fasciola cinerea (FC) and in CA1. C) *LG1* labeling in the dentate gyrus and in some scattered cells in the dorsal subiculum (DSub) of the posterior hippocampus is shown at higher magnification. Similarly, a detail of the entorhinal cortex (Ent), illustrating the weak but consistent labeling in the superficial layer, is shown in D). *LG1* signal in the anterior basolateral (BLA) and central (CeA) nuclei of the amygdala can be appreciated in E). In F) low *LG1* signal was also observed in the dorsal lateral septal nucleus (LSd) and in putamen (Pu). Scale bars in A and B = 500 μ m; rest of images = 200 μ m.

Figure 3.- Photomicrographs showing the expression of *LG2* gene A) Dispersed labeled cells were observed in all layers of the olfactory bulb (arrows). B) Intense *LG2* labeling of the majority of neurons of the external division of the anterior olfactory nuclei (AOE) along the olfactory peduncle. C) Illustrates the labeling in the parietal cortex, mainly in the deep layers, as it occurs in the rest of the cerebral cortex. D) *LG2* labeling in the piriform cortex (Pir) was strong in layer II and diffuse in layer III. E) Shows dispersed labeled cells in both the Ammon's horn and dentate gyrus, whereas pyramidal and granular layers remain unlabeled. *LG2* labeling is also shown in the lateral habenular nucleus (IHb) whereas, in contrast, is absent in the medial habenular nucleus (mHb). F) *LG2* labeling in the thalamus and basal ganglia; strong signal was observed in the reticular thalamic nucleus (Rt), with some labeling also apparent in the lateral pallidal nucleus (Pa). Scattered labeled cells were also seen in the putamen (Pu) and in several amygdalar nuclei

such as the lateral (LA), central (CeA) or medial amygdala (MeA). G) *LG/2* signal in the medial septum and diagonal band nuclei; strong labeling was observed in the vertical limb of the diagonal band, although some scattered labeled cells were also detected in the transition between medial and lateral septum (MS/LS). *LG/2* labeling was absent in the horizontal limb (hDB). Scale bars in E and F = 500 μm ; rest of images = 200 μm .

Figure 4.- Photomicrographs showing some details of *LG/2* expression. A) *LG/2* labeling was found in the zona incerta (ZI), ventral to the mammillothalamic tract (mta). B) In the mammillary bodies, *LG/2* was specifically and strongly expressed in the median division of the medial mammillary nucleus. C) In the caudal medulla, strong *LG/2* labeling was observed in the large neurons of the inferior olivary complex. Some labeled neurons were also apparent in the medullary reticular formation (RF). D) In the cerebellum, a strong *LG/2* signal was observed in some regularly dispersed neurons of the granular layer of the cerebellar laminae. In addition, *LG/2* expression was also found in neurons of the deep cerebellar nuclei, such as the dentate nucleus. Scale bars in C and D = 100 μm ; rest of images = 200 μm .

Figure 5.- Photomicrographs showing the expression of *LG/3* gene. A) There was strong *LG/3* expression in the granular and pyramidal neurons of the dentate gyrus and Ammons's horn. In addition, some disperse neurons, most likely interneurons, were observed in the rest of layers. B) The *LG/3* labeling in the amygdalar complex was strong in the lateral nucleus and in the medial division of the central nucleus. C) Widespread and weak *LG/3* expression in the laterodorsal thalamic nucleus. D) *LG/3* labeling in the medial habenula (mHb) contrasts with the lack of signal in the lateral habenula (lHb). In addition, some weak *LG/3* labeling was seen in the posterior paraventricular thalamic nucleus (PVP). E) Some labeling was also apparent in the substantia nigra (SN), ventral tegmental area (VTA) and interpeduncular nucleus (IP). F) *LG/3* signal was also present in the median raphe (MnR). Scale bar in C = 100 μm ; the rest of images = 200 μm .

Figure 6.- Photomicrographs showing the expression of *LG/4* gene in some brain regions. Note that the pattern of labeling is similar to that of *LG/2* transcripts. A) In the parietal cortex, *LG/4* labeling was mainly observed in the deep layers. B) *LG/4* signal in the hippocampus was found in dispersed cells, but was also present in the granular and pyramidal layers, and in the reticular nucleus. C) *LG/4* labeling was found in the vertical limb of the diagonal band of the medial septum (MS). D) Strong labeling was present in the supracapsular division of the bed nucleus of the stria terminalis (BST). In contrast, scattered labeled cells were observed in the ventral pallidum (VP). *LG/4* expression was almost absent in nearly all hypothalamic nuclei, including the medial preoptic nucleus (MPO). E) Some *LG/4* labeling was evident in the putamen and in the lateral pallidum. F) A group of *LG/4* labeled cells was specifically located in the deep dorsal nucleus (DD) of the

posterior intralaminar complex (PIN), between the dorsal and ventral divisions of the medial geniculate body (MGd and MGv, respectively). Scale bars in B and D = 500 μm ; rest of images = 200 μm .

Figure 7.- Photomicrographs showing some details of *LG14* expression in the mesencephalon.

A) A strong *LG14* labeling is clearly seen in the pars compacta of the substantia nigra (SNc) and the ventral tegmental area (VTA). Scattered labeled cells were present in the deep mesencephalic reticular formation (DeMe) and the pars reticulata of the substantia nigra (SNr). *LG14* labeling was absent in the supramammillary (SuM) nucleus and in all mammillary nuclei (mMM, IMM, LM).

B) High magnification of the *LG14* labeling observed in the substantia nigra in photomicrograph B.

C) In the caudal mesencephalon, the *LG14* labeling was observed in the interpeduncular nucleus (IP) and in the interfascicular nucleus (IF). Scale bar in A = 500 μm ; rest of images = 200 μm .