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Expression of a killer cell receptor-like gene in plastic regions of the central nervous system

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Abstract

A property common to the immune system and the nervous system is regulation by a highly complex and adaptable network of cellular interactions. Major histocompatibility complex (MHC) class I molecules, which are ligands of antigen-specific receptors on CD8 T cells and of inhibitory receptors on natural killer cells, have an important and surprising role in the control of activity-dependent neuronal plasticity in the central nervous system (CNS). While expression of MHC class I molecules in neurons has been reported, corresponding immune receptors have not been identified in the CNS. Here we show selective expression of a gene related to killer cell immunoglobulin-like receptor (KIR) genes in subregions of the mouse brain where synaptic plasticity and neurogenesis occur, including olfactory bulbs, rostral migratory stream and dentate gyrus of hippocampus. These results suggest new functions for KIR-like molecules in the CNS.
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Keywords: Dentate gyrus; MHC class I; NK cell receptor; Olfactory bulb; Plasticity

The immune and nervous system are interconnected (Tracey, 2002; Webster et al., 2002) and share intriguing common features at the molecular level (Blalock, 1984, 1994; Boulanger et al., 2001). Novel functions in the CNS have emerged for MHC class I molecules. MHC class I molecules, well known for their role in stimulating antigen-specific responses by CD8 T cells (Davis and Bjorkman, 1988), are also important for neuronal and sensory functions (Boulanger et al., 2001; Doherty, 2003). Mice deficient in both β_2-microglobulin and transporter for antigen presentation (TAP)-1, thereby lacking cell surface expression of MHC class I, have incomplete refinement of projections between the retina and the primary visual cortex during development, implying a role for MHC class I in visual system connectivity (Huh et al., 2000). In addition, the highly polymorphic MHC class I molecules control odorant receptor-mediated discrimination, and mating preference, among individual mice and rats (Doherty, 2003; Dulac and Torello, 2003; Schaefer et al., 2002). Expression of MHC class I is induced by interferon-γ, in conjunction with electrical silencing, in cultured rat hippocampal neurons (Neumann et al., 1995). Neuronal expression of MHC class I is also up-regulated by axotomy (Maehlen et al., 1988), and by acute and chronic inflammation (Foster et al., 2002). MHC class I expression is up-regulated by neuronal activity during remodeling of the cat visual system and in the dentate gyrus of rat hippocampus by kainic acid, which dramatically increases the activity of cortical and hippocampal neurons (Corriveau et al., 1998).

Major histocompatibility complex (MHC) class I molecules are expressed in many tissues and provide protection from natural cytotoxicity by binding to inhibitory receptors

Abbreviations: CNS, central nervous system; EST, expressed sequence tag; MHC, major histocompatibility complex; Ig, immunoglobulin; ITIM, immuno-receptor tyrosine-based inhibition motif; KIR, killer cell Ig-like receptor; Kirl, KIR-like; NK, natural killer.

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on natural killer (NK) cells (Long, 1999; Natarajan et al., 2002). NK cells in mice and rats recognize classical MHC class I through lectin-like receptors of the Ly-49 family (Natarajan et al., 2002). In contrast, the receptors used by higher primates to recognize classical MHC class I are the killer cell immunoglobulin (Ig)-like receptors (KIR) (Vilches and Parham, 2002). The human KIR gene family includes several genes, located on chromosome 19, that exhibit extensive polymorphism through a combination of variable gene content per haplotype and of allelic sequence polymorphism (Shilling et al., 2002; Trowsdale, 2001; Wagtmann et al., 1997). The extremely rapid evolution of KIR genes suggests that KIR may have other functions besides inhibition of NK cells (Khakoo et al., 2000). Orthologs of human KIR have been described in primate species, but not in rodents. However, mouse KIR-like (Kirl) cDNAs, derived from an X-linked gene, were isolated from RNA of thymus and NK cells (Hoelsbrekken et al., 2003; Welch et al., 2003). We have isolated full-length cDNAs for two mouse *Kirl* genes and performed RT-PCR with mouse cells and tissues, and in situ hybridizations on mouse brain sections. We found that one of two *Kirl* genes is expressed selectively in regions of neuronal plasticity and neurogenesis.

1. Experimental procedures

Mouse NK cells and mast cells were isolated and cultured as previously described (Rojo et al., 2000). The mouse NK cell line LNK (Tsutsui et al., 1996) was provided by Dr. Howard Young (NCI, Frederick, MD). The T-cell lines EL4, 3D0 and YAC-1, the macrophage cell line J774.1A, and the fibroblast cell line NIH-3T3 were from the American Type Culture Collection (Manassas, MD). The B-cell lines A20, CH27 and WEHI-231 were provided by Dr. Susan Pierce (NIAID, Rockville, MD).

Full-length cDNA was amplified by RACE PCR from C57BL/6 mouse IL-2-activated NK cells with a forward primer (5′-TTTGTTCTGGCTCTGCTCCTTGC-3′) designed on the basis of an EST sequence (GenBank accession BB644731), and a reverse primer to a 3′ adaptor sequence introduced with the oligo(dT) primer during first strand cDNA synthesis according to the manufactures instructions (SMART RACE cDNA Amplification Kit, Clontech, Palo Alto, CA). PCR amplification was performed with Taq polymerase (Invitrogen). PCR products were cloned into the pCRII vector by TOPO TA cloning (Invitrogen). Three independent clones were selected and sequenced on both strands. To investigate expression of *Kirl* in mouse cells and to amplify *Kirl* from different mouse strains, a reverse primer (5′-GCCAGTGAGGACCATAGTCTTT-3′) was used in conjunction with the forward primer in RT-PCR reactions. The templates for these reactions were made by oligo(dT)-primed reverse transcription of poly(A)$^+$ RNA. Primers to GP3DH (forward 5-GGCATGGACTGTGGTCATGAG-3′ and reverse 5′-TGCACCACCAACTGCTTAGC-3′) and CD3ϵ (forward 5-TGGAGCAAGAATAGAAAGGCCAAG-3′ and reverse 5′-CTCTGATTCAGGCCAGAATACAGG-3′) were also used.

In situ hybridizations of ^{35}S-labeled RNA probes, transcribed from the sense and anti-sense strands of the 1553 bp full-length mouse *Kirl1* cDNA, to sections of mouse brain were performed as described (Foster et al., 2002). To determine the cellular localization of hybridized probes, sections were coated with nuclear track emulsion (NTB-2, Eastman Kodak or LMN1, Amersham), exposed for 3 weeks, developed (D19, Kodak) and counterstained. For double-label histochemistry, sections were processed for in situ hybridization with *Kirl1* cRNA probe as described above, followed by immunohistochemistry with anti-neuronal nuclei (NeuN) monoclonal antibody (mAb) (Chemicon International, Temecula, CA) at a dilution of 1:1000 (Foster et al., 2002).

cDNA encoding full-length mouse KIRL was cloned in frame with an N-terminal HA-tag and inserted into the pIRES2-EGFP vector (Clontech). Sequence of the constructs can be obtained upon request. 293T cells were transfected with Lipofectamine (Invitrogen). Cell surface expression was detected with anti-HA mAb HA.11 (Covance Research Products, Cumberland, VA) and secondary PE-conjugated goat anti-mouse F(ab′)$_2$ (Jackson Immunoresearch, West Grove, PA). Whole cell lysates were prepared as previously described (Watzl et al., 2000). Membranes were Western-blotted with rabbit polyclonal anti-HA Ab (Santa Cruz Biotechnology, Santa Cruz, CA).

2. Results

Nucleotide sequences of the mouse genome, and of an EST (GenBank accession BB314335 and BB644731) isolated from a *corpora quadrigemina* cDNA library, indicated the existence of two mouse KIR-like genes. PCR primers were designed to amplify *Kirl* cDNA from NK cells of C57BL/6 mice. Two bands of about 1550 bp and 1250 bp were observed (Fig. 1). RT-PCR with RNA from the NK cell line LNK also produced these two bands (Fig. 1). No cDNA was amplified from RNA of other immune cells, such as T, B and macrophage cell lines, and mast cells (Fig. 1). As controls, primers for CD3ϵ and for GP3DH were used with the same cDNA samples. Transcripts of CD3ϵ were detected in T-cell lines and in NK cells, as expected (Biassoni et al., 1988; Lanier et al., 1992), but not in the NK cell line LNK. Nucleotide sequencing of the KIRL PCR products revealed a 1553 bp full-length cDNA (GenBank accession AY530782), which encodes a receptor with high similarity to the three Ig domain-containing human KIR molecules (68% nucleotide identity in the Ig domain-coding regions). However, the mouse KIRL lacks the cytoplasmic immuno-receptor tyrosine-based inhibition motifs (ITIM) typical of inhibitory KIR

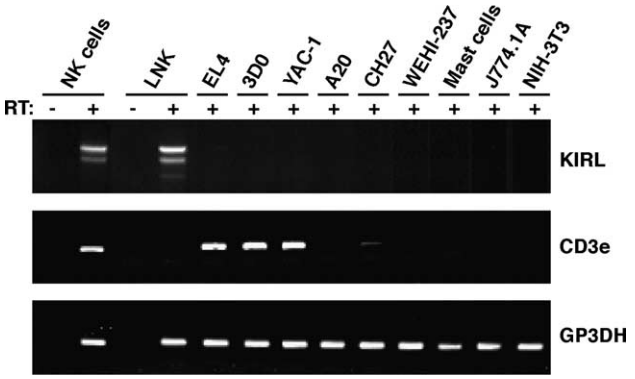

Fig. 1. Transcription of *Kirl* in NK cells. RT-PCR was performed with primers that amplify full-length cDNA from both *Kirl* genes. RNA tested was from C57BL/6 NK cells, the NK like cell line LNK, the T-cell lines EL4, 3D0 and YAC-1, the B-cell lines A20, CH27 and WEHI-231, in vitro cultured mast cells from C57BL/6; the macrophage cell line J774.1A and the fibroblast cell line NIH-3T3, as indicated. Some of the reactions were performed without reverse transcriptase (RT), as indicated. CD3ε primers were used as positive controls for NK and T cells. Primers for GP3DH served as positive control.

(Burshtyn et al., 1996). The shorter KIRL PCR product of 1256 bp corresponded to an isoform lacking the third, membrane-proximal Ig domain. Similar mouse *Kirl* cDNAs have been isolated recently from RNA of C57BL/6 thymus and NK cells (Hoelsbrekken et al., 2003; Welch et al., 2003). Our sequence is derived from the *Kirl1* gene and its translation corresponds to that reported by Welch et al. (2003), including the frame-shift mutation that introduces an early stop codon, upstream of the conserved ITIM-encoding sequences.

As the only reported KIR-related mouse EST had come from a library of a brain tissue sample, we decided to perform in situ hybridizations with *Kirl* ribonucleotide probes on C57BL/6 mouse brain sections. Expression was detected with the anti-sense probe in the olfactory bulb, dentate gyrus and cerebellum (Fig. 2A). No signal was observed with the corresponding sense probe (Fig. 2A). RT-PCR was performed with poly(A)$^+$ RNA isolated from olfactory bulbs, hippocampus, cerebellum and cortex of C57BL/6 mouse brains, and with poly(A)$^+$ RNA from mouse NK cells. Two prominent PCR products appeared in the hippocampus, olfactory bulb, cerebellum and NK cell samples, but not in cortex (Fig. 2B), consistent with the in situ hybridization data. The signal obtained in the lane from the brain RNA was approximately one-tenth the signal from NK cell RNA, suggesting lower expression levels or an expression limited to a subset of cells in brain tissue.

To further characterize *Kirl* expression in the CNS, in situ hybridizations were performed on coronal and sagittal sections of mouse C57BL/6 brains. Expression was clearly visible in the dentate gyrus, rostral migratory stream, olfactory bulb and cerebellum in sagittal section (Fig. 3A). The coronal section revealed expression in the dentate gyrus, the habenula, the CA1 field of Ammon's horn and lower expression in the amygdala and hypothalamus (Fig. 3B). To determine the cellular localization of hybridized probes, sections were coated with nuclear track emulsion and counter-stained with cresyl violet. Silver grains appeared concentrated over neurons, as identified by Nissl stain criteria (pale and moderate-to-large diameter), of the dentate gyrus (Fig. 3C) and amygdala (Fig. 3D). Labeled cells in the rostral migratory stream (RMS in Fig. 3E) were oval-shaped, darker stained cells (Fig. 3F) characteristic of newly formed neurons of the rostral migratory stream (Altman, 1969). Identification of neuronal cells was also confirmed with a mAb specific for neuronal nuclei (anti-NeuN) (not shown).

As in situ hybridization could not discriminate between the two *Kirl* genes, which have 96% sequence identity in the coding region, nucleotide sequencing was performed with RT-PCR products. Several full-length clones were sequenced after independent RT-PCR reactions with hippo-

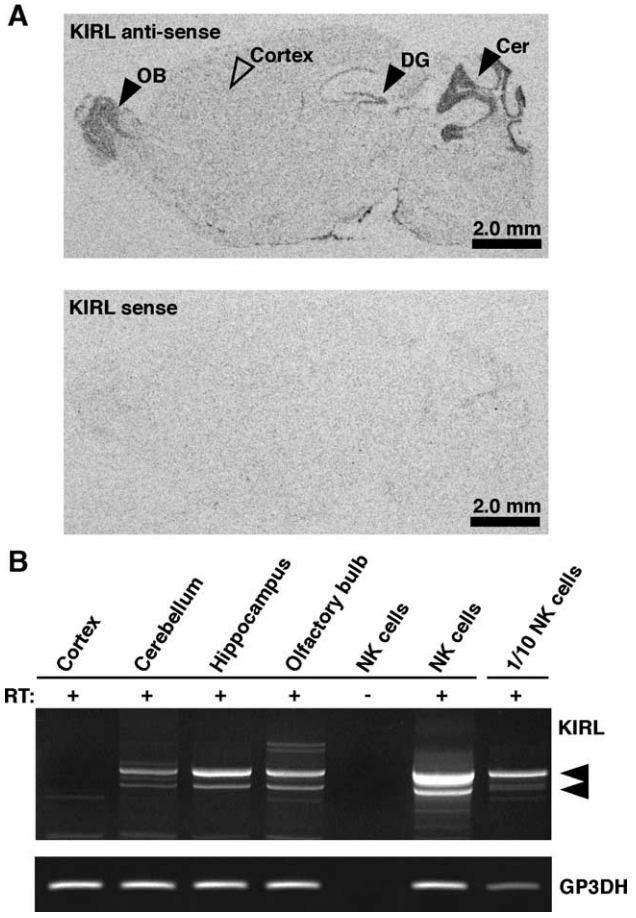

Fig. 2. (A) A ^{35}S-labeled *Kirl1* anti-sense probe of 1553 bp was hybridized to sagittal sections of mouse brain. Radioactivity is visible in the olfactory bulb (OB), in dentate gyrus (DG) of the hippocampus and in the cerebellum (Cer). The corresponding sense probe (lower panel) gave much weaker signals. (B) *Kirl* transcripts were amplified from equivalent amounts of poly(A)$^+$ RNA of NK cells and the indicated brain tissues. Two splice variants are indicated with arrowheads. A lane with 1/10 the amount of poly(A)$^+$ RNA from NK cells is included. One sample was amplified in the absence of reverse transcriptase (RT), as indicated.

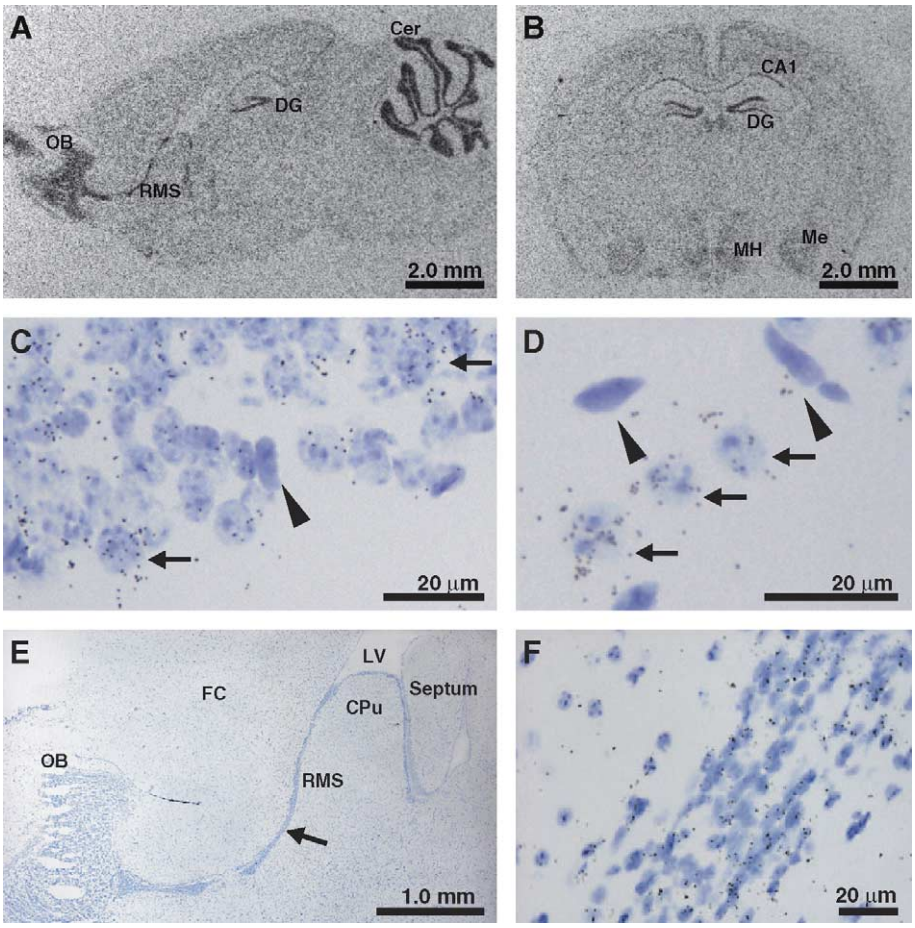

Fig. 3. Transcription of *Kirl* in brain cells. In situ hybridization with ^{35}S-labeled mouse *Kirl1* cDNA of 1553 bp was performed on mouse brain tissue sections. (A) A sagittal section revealed expression in the olfactory bulbs (OB), rostral migratory stream (RMS), dentate gyrus (DG) and in granule cell layer of cerebellum (Cer). (B) A coronal section revealed expression in the DG, Ammon's horn field CA1 of the hippocampus, the medial nucleus of the amygdala (Me), and in the ventromedial and dorsomedial nuclei of the hypothalamus (MH). (C–F) Nuclear track emulsion and counter-staining with cresyl violet shows expression of *Kirl* transcripts in neurons of the dentate gyrus (C), amygdala (D) and rostral migratory stream (E, F). Some of the neurons are indicated by arrows and non-neuronal cells by arrowheads (C, D). In the low magnification in E, septum, lateral ventricle (LV), caudate putamen (Cpu) and frontal cortex (FC) are discernable. The arrow in E points to the area magnified in F.

campus RNA; each one corresponded to *Kirl2* (GenBank accession AY530784). A shorter splice variant, lacking the first Ig domain, was also derived from *Kirl2*. Independent clones from olfactory bulbs, hippocampus and cerebellum were identified as *Kirl2* by restriction enzyme digestions and sequencing. The EST isolated from a brain sample had also been derived from a *Kirl2* transcript (Hoelsbrekken et al., 2003; Welch et al., 2003). In contrast, the nucleotide sequence of several clones that were obtained from independent RT-PCR reactions with NK cell RNA of both C57BL/6 and CBA/J mice corresponded to *Kirl1*, as did the published sequences obtained from thymus and NK cells (Hoelsbrekken et al., 2003; Welch et al., 2003). Therefore, it appears that *Kirl1* is expressed predominantly in NK cells, whereas *Kirl2* is expressed selectively in specific regions of the brain.

To determine if the mouse *Kirl2* gene encodes an expressible receptor, a KIRL2 cDNA was engineered to include an N-terminal HA tag and was transiently expressed in 293T cells (Fig. 4). A vector that includes an intra-ribosomal entry site for co-expression of EGFP was used in order to identify transfected cells by EGFP fluorescence on flow cytometry. KIRL2 was expressed at the cell surface (Fig. 4A). To monitor protein expression in the transfected cells, lysates were tested by Western blotting with anti-HA polyclonal Ab after SDS-PAGE under reducing and non-reducing conditions. KIRL2 was clearly expressed at the protein level (Fig. 4B). The predicted molecular mass, including N-linked glycosylation, of KIRL2 is 52 kDa. KIRL2 migrated with an apparent molecular mass of 48 kDa (Fig. 4B). Higher bands of approximately 95 kDa that were visible under non-reducing conditions, but not in the reduced samples, suggest the formation of disulfide-linked dimers (Fig. 4B,C). KIRL2 has three cysteines in the stem region, which connects the membrane-proximal Ig domain to the transmembrane region. These cysteines could mediate disulfide-linked associations of either KIRL2 homo-dimers or hetero-dimers with other molecules of similar size. We

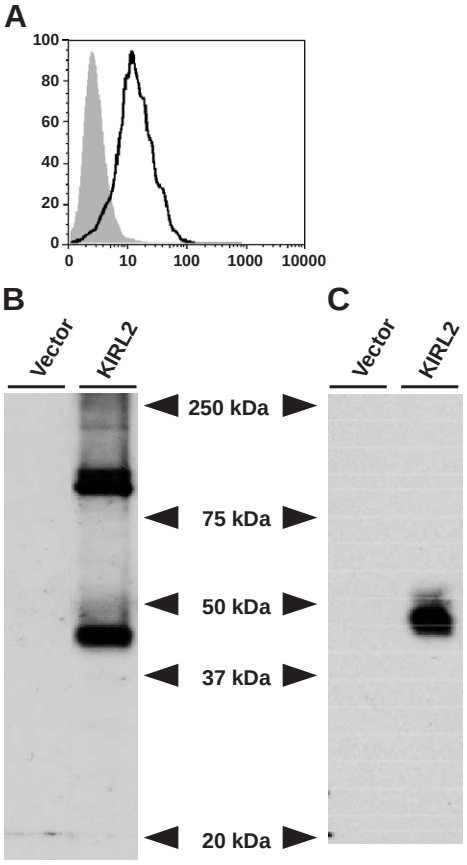

Fig. 4. Cell surface expression of KIRL2 in transfected cells. (A) 293T cells transfected with an HA-tagged *Kirl2* cDNA and EGFP were stained with an anti-HA mAb and gated on $EGFP^+$ cells. A mock transfected control is shown as shaded area. (B, C) Whole cell lysates of 293T cells transfected with the indicated plasmids were separated on a non-reducing (B) or reducing (C) SDS-PAGE gel and Western-blotted with an anti-HA polyclonal Ab.

conclude that the *Kirl2* gene encodes a receptor that can be expressed at the cell surface.

3. Discussion

The present study reports novel observations of expression of the *Kirl2* gene in specific regions of the mouse CNS. While several groups have reported the presence of MHC class I in CNS neurons in vivo (Corriveau et al., 1998; Foster et al., 2002; Huh et al., 2000; Kimura and Griffin, 2000; Lidman et al., 1999; Linda et al., 1999; Maehlen et al., 1988, 1989; McGavern et al., 2002; Pereira and Simmons, 1999; Pereira et al., 1994; Schultzberg et al., 1989), a receptor for MHC class I in the CNS has not been identified yet. MHC class I molecules are constitutively present in several brain regions (Corriveau et al., 1998; Huh et al., 2000; Lidman et al., 1999; Linda et al., 1999) and can be induced in specific neuronal populations (Corriveau et al., 1998; Foster et al., 2002; Maehlen et al., 1989; Pereira and Simmons, 1999; Pereira et al., 1994). The *Kirl2* expression pattern reported here overlaps with that previously reported for MHC class I. Specifically, we observed *Kirl2* expression in olfactory bulb, rostral migratory stream, hippocampus, habenula, hypothalamus, amygdala and cerebellum.

The current description of *Kirl2* expression in mouse brain is intriguing because it suggests yet another role for immune molecules in normal neuronal function. MHC class I molecules are important for innate and adaptive immune responses through their role in antigen presentation. Similarly, peptide presentation by MHC class I plays an important role in the brain's immune response. Furthermore, there is accumulating evidence suggesting that immune molecules may serve additional functions in neurons. In fact, it has been suggested that ligands and receptors shared by the immune and nervous systems might contribute to the regulation of synaptic plasticity in the CNS (Boulanger et al., 2001). Direct evidence that MHC class I and the ζ chain of T-cell receptor (a key transmembrane signaling molecule of the antigen-specific receptor complex of T lymphocytes) are important to neuronal plasticity was provided by Huh et al. (2000) and yet the molecular basis underlying these effects is unknown (Syken and Shatz, 2003). A functional role of KIRL2 in neuronal plasticity has not been shown. The structural similarity of KIRL2 with human KIR (50% sequence identity in the Ig domains), and the overlapping expression profile of KIRL2 with MHC class I in mouse CNS suggest that KIRL2 function may be regulated by binding to MHC class I in the CNS. Unfortunately, direct binding assays are still lacking because it has not been possible to express soluble forms of KIRL2 (data not shown). The search for a KIRL2 ligand will continue using other approaches.

The striking expression of *Kirl2* in the rostral migratory stream, olfactory bulb and dentate gyrus is interesting because these regions correspond to sites of neurogenesis (Alvarez-Buylla et al., 2002; Gage, 2000). Our data with transfected 293T cells shows that KIRL2 can be expressed as a cell surface molecule. An interesting possibility is that KIRL2 may contribute specificity in the formation of neuronal synapses by binding to MHC class I. Expression of MHC class I in the CNS is important for the refinement of the developing visual system (Huh et al., 2000). KIRL2 may serve other functions, such as control of neuron chemotaxis and apoptosis, and may do so by binding to ligands other than MHC class I. Fine mapping of KIRL expression in the CNS during development and in adult mice, and receptor–ligand binding studies should provide further insight into the potential role of KIRL2 in neuronal function, including neurogenesis and neuroplasticity.

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