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MHC class I protein is expressed by neurons and neural progenitors in mid-gestation mouse brain

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ABSTRACT

Proteins of the major histocompatibility complex class I (MHCI) are known for their role in the vertebrate adaptive immune response, and are required for normal postnatal brain development and plasticity. However, it remains unknown if MHCI proteins are present in the mammalian brain before birth. Here, we show that MHCI proteins are widely expressed in the developing mouse central nervous system at mid-gestation (E9.5–10.5). MHCI is strongly expressed in several regions of the prenatal brain, including the neuroepithelium and olfactory placode. MHCI is expressed by neural progenitors at these ages, as identified by co-expression in cells positive for neuron-specific class III β-tubulin (Tuj1) or for Pax6, a marker of neural progenitors in the dorsal neuroepithelium. MHCI is also co-expressed with nestin, a marker of neural stem/progenitor cells, in olfactory placode, but the co-localization is less extensive in other regions. MHCI is detected in the small population of post-mitotic neurons that are present at this early stage of brain development, as identified by co-expression in cells positive for neuronal microtubule-associated protein-2 (MAP2). Thus MHCI protein is expressed during the earliest stages of neuronal differentiation in the mammalian brain. MHCI expression in neurons and neural progenitors at mid-gestation, prior to the maturation of the adaptive immune system, is consistent with MHCI performing non-immune functions in prenatal brain development. These results raise the possibility that disruption of the levels and/or patterns of MHCI expression in the prenatal brain could contribute to the pathogenesis of neurodevelopmental disorders.

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Introduction

Proteins of the major histocompatibility complex class I (MHCI) present antigenic peptides for cellular immune surveillance, a key element of the vertebrate adaptive immune response (Neefjes and Momburg, 1993). The central nervous system (CNS) is functionally "immune privileged", meaning that adaptive immune responses in the brain are often blunted or delayed relative to other sites. This was long thought to be due to a lack of expression of MHCI proteins by healthy neurons under basal conditions (Neumann et al., 1995; Rall et al., 1995). However, numerous studies show that MHCI mRNA and protein are expressed by healthy neurons in the postnatal central and peripheral nervous system (e.g., Corriveau et al., 1998; Datwani et al., 2009; Huh et al., 2000; Ishii and Mombaerts, 2008; Ishii et al., 2003; Lidman et al., 1999; Linda et al., 1998; Loconto et al., 2003; McConnell et al., 2009; Miralves et al., 2007; Needleman et al., 2010; Ribic et al., 2011; Thams et al., 2008).

In addition, recent studies show that MHCI has unexpected, non-immune functions in the developing and adult brain. MHCI protein is enriched in synaptic fractions of adult rodent brain (Huh et al., 2000) and co-localizes with the post-synaptic density protein PSD-95 in dendrites of acutely-dissociated postnatal hippocampal neurons (Goddard et al., 2007), consistent with a role for MHCI in regulating synapse function (Boulanger, 2009). Indeed, in the adult mouse hippocampus, genetic reduction of cell surface MHCI is associated with increased NMDAR-mediated synaptic transmission (Fourgeaud et al., 2010) as well as an increase in the magnitude of NMDAR-dependent long-term potentiation (LTP) and loss of NMDAR-dependent long-term depression (LTD) (Huh et al., 2000). In the adult cerebellum, loss of the genes encoding the classical MHCIs H2-K and H2-D is associated with a lower threshold for the induction of LTD at parallel fiber-Purkinje cell synapses (McConnell et al., 2009). Thus endogenous MHCI is essential for normal synaptic transmission and plasticity in multiple regions of the adult mammalian brain.

MHCI is also expressed at earlier postnatal ages. MHCI mRNA is expressed in rodent and cat lateral geniculate nucleus (LGN) during the first two postnatal weeks (Corriveau et al., 1998; Huh et al., 2000), and reduced cell surface expression of MHCI protein in rodent genetic models disrupts postnatal development of projections

Abbreviations: β2m, beta-2 microglobulin; CNS, central nervous system; HES cell, human embryonic stem cell; LGN, lateral geniculate nucleus; LTD, long-term depression; LTP, long-term potentiation; MHCI, major histocompatibility complex class I; NSC, neural stem cell; PNS, peripheral nervous system; VNO, vomeronasal organ.

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from retina to LGN during this time (Datwani et al., 2009; Huh et al., 2000). MHCI mRNA expression has also been detected in prenatal cat LGN (E43 and E52; gestation is 65 days), where it is downregulated by chronic blockade of sodium-based action potentials (Corriveau et al., 1998). However, it is unknown if MHCI plays a role in prenatal brain development *in vivo*. A key first step is to determine if MHCI protein is normally present in the mammalian brain before birth.

In this study, we show that MHCI protein is widely expressed in the developing mouse CNS at mid-gestation. MHCI is expressed by cells positive for markers of neuronal precursors (Pax6, nestin, Tuj1) as well as newly-differentiated neurons (MAP2). Thus MHCI is expressed at the earliest stages of neuronal differentiation in the developing mammalian brain.

Results

To determine if MHCI proteins are expressed prenatally, mouse embryos were stained using a mouse monoclonal antibody (OX-18) that recognizes a monomorphic epitope of rat MHCI (RT-1A; Fukumoto et al., 1982) and reacts with mouse MHCI in Western blots and immunohistochemistry (Corriveau et al., 1998; Datwani et al., 2009; Huh et al., 2000; Needleman et al., 2010; Rolleke et al., 2006). Multiple lines of evidence support the specificity of this antibody in labeling MHCI in neurons (Table 1). First, cell surface immunofluorescence is greatly attenuated in both $\beta 2m^{-/-}$ and $\beta 2m^{-/-}TAP^{-/-}$ cells (Needleman et al., 2010; Goddard et al., 2007), in which reduced levels of MHCI reach the cell surface (Dorfman et al., 1997; Zijlstra et al., 1989). Second, OX-18 recognizes proteins of the expected molecular weight in western blots of adult mouse brain (Corriveau et al., 1998; Huh et al., 2000; Needleman et al., 2010), and similar banding patterns are seen with a rabbit polyclonal anti-MHCI antibody that recognizes a distinct epitope (Needleman et al., 2010). Third, light- and electron- microscopic localization of OX-18 signals in cortex parallels patterns seen with another anti-MHCI monoclonal antibody raised against a distinct epitope (Needleman et al., 2010). Fourth, OX-18 labeling in marmoset brain is reduced by treatment with an immunosuppressive drug (FK506; Rolleke et al., 2006). We see OX-18 staining in the adult mouse that is identical to published results in all brain regions examined, including cortex, cerebellum, and hippocampus, as well as in acutely-dissociated perinatal mouse hippocampal neurons *in vitro* (Corriveau et al., 1998; Datwani et al., 2009; Glynn and McAllister, 2006; McConnell et al., 2009; Needleman et al., 2010). Our adult mouse brain staining with OX-18 is abolished if the antibody is replaced with an isotype control or applied to MHCI-deficient tissue (not shown). In fetal mouse, we also find that omitting the primary antibody or incubating with an isotype-control antibody both abolish staining (see Fig. 1B–C). Taken together, these studies provide strong support for the specificity of OX-18 in recognizing MHCI in the brain in our hands.

Whole mouse embryos were stained using the anti-MHCI antibody OX-18 at mid-gestation (E9.5–E10.5), based on date of mating and observation of vaginal plug, and confirmed by comparison to anatomical markers of Theiler stages (stages 14–18; http://www.emouseatlas.org/emap/ema/theiler_stages/house_mouse/book.html). These ages correspond roughly to days 22–28 of human embryonic development, or E11-12 in rat, based on Carnegie stage comparisons (http://php.med.unsw.edu.au/embryology/index.php?title=Carnegie_Stage_Comparison).

Table 1
Previous experimental approaches and results obtained supporting the specificity of the OX-18 antibody in detecting MHCI proteins in the brain.

Neuronal technique	Observation	Reference
Immunohistochemistry (OX18)	• Stains neurons in most layers of rat cortex • Stains hippocampal neurons in vitro • Cell surface immunofluorescence attenuated in MHCI-deficient ($\beta 2m^{-/-}$ and $\beta 2m^{-/-}TAP^{-/-}$) cells • Light-level labeling parallels patterns seen with another anti-MHCI monoclonal antibody raised against a distinct epitope • Labels neurons in marmoset brain • Labeling reduced following treatment with immunosuppressive drug (FK506)	Needleman et al. (2010), Rolleke et al. (2006), Goddard et al. (2007)
Western blot (OX18)	• Bands of expected size detected in rat primary somatosensory cortex membranes • Bands of expected size detected in rat synaptosomal fractions • Bands of expected size detected in rat visual cortex • Similar banding patterns seen with a rabbit polyclonal anti-MHCI antibody that recognizes a distinct epitope	Corriveau et al. (1998), Huh et al., (2000), Needleman et al. (2010)
Electron microscopy (OX18)	• MHCI found in synapses (pre- and post-synaptic), axon terminals and dendrites of layer V, adult rat visual cortex • EM-level labeling parallels results seen with another anti-MHCI monoclonal antibody raised against a distinct epitope	Needleman et al. (2010)

MHC class I is expressed in prenatal brain

Immunofluorescence microscopy of coronal and parasagittal sections revealed specific staining for MHCI throughout the embryo, including the developing somites (not shown), organs, nasal process, and CNS (Fig. 1G). Similar patterns were seen in four rounds of immunostaining, and representative images are shown. Hoechst staining of nuclei (e.g., Fig. 1D–F and J) provides anatomical landmarks. In prenatal brain, MHCI is expressed throughout the developing neuroepithelium, including the mesencephalon, diencephalon and telencephalon (Fig. 1A). Incubation with a mouse isotype control IgG (Fig. 1B) or in the absence of primary antibody (Fig. 1C) did not yield appreciable staining, supporting the specificity of MHCI immunolabeling. In a more lateral section, MHCI expression is detected in the nasal process (Fig. 1G–I). At higher magnification (Fig. 1I), MHCI staining fills the cell soma, consistent with cytosolic and/or membrane localization in these permeabilized sections. Similar somatic labeling is also observed in dorsal neuroepithelial cells in the same section (Fig. 1K and L). Overall, MHCI staining intensity in the embryonic brain was comparable to, or stronger than, staining with the same antibody in adult brain (not shown).

Co-expression of MHCI and early neuronal marker MAP2

To determine if MHCI is expressed by newly-differentiated neurons, double-label immunostaining was performed using an antibody that recognizes microtubule-associated protein 2 (MAP2). MAP2 is a major component of the neuronal cytoskeleton that is critical for neuronal morphology and differentiation (Matus, 1988), and MAP2 is widely used as an early neuronal marker (e.g., Cheyne et al., 2011; Chun and Shatz, 1989; Ferri and Levitt, 1993). In parasagittal sections, MAP2 is expressed mainly by peripheral neuroepithelial cells in both mesencephalon and diencephalon (Fig. 2B). At higher magnification, it is evident that MAP2 is expressed only by cells at the periphery of the neuroepithelium (Fig. 2F), while MHCI is detected more widely throughout the neuroepithelium at this age

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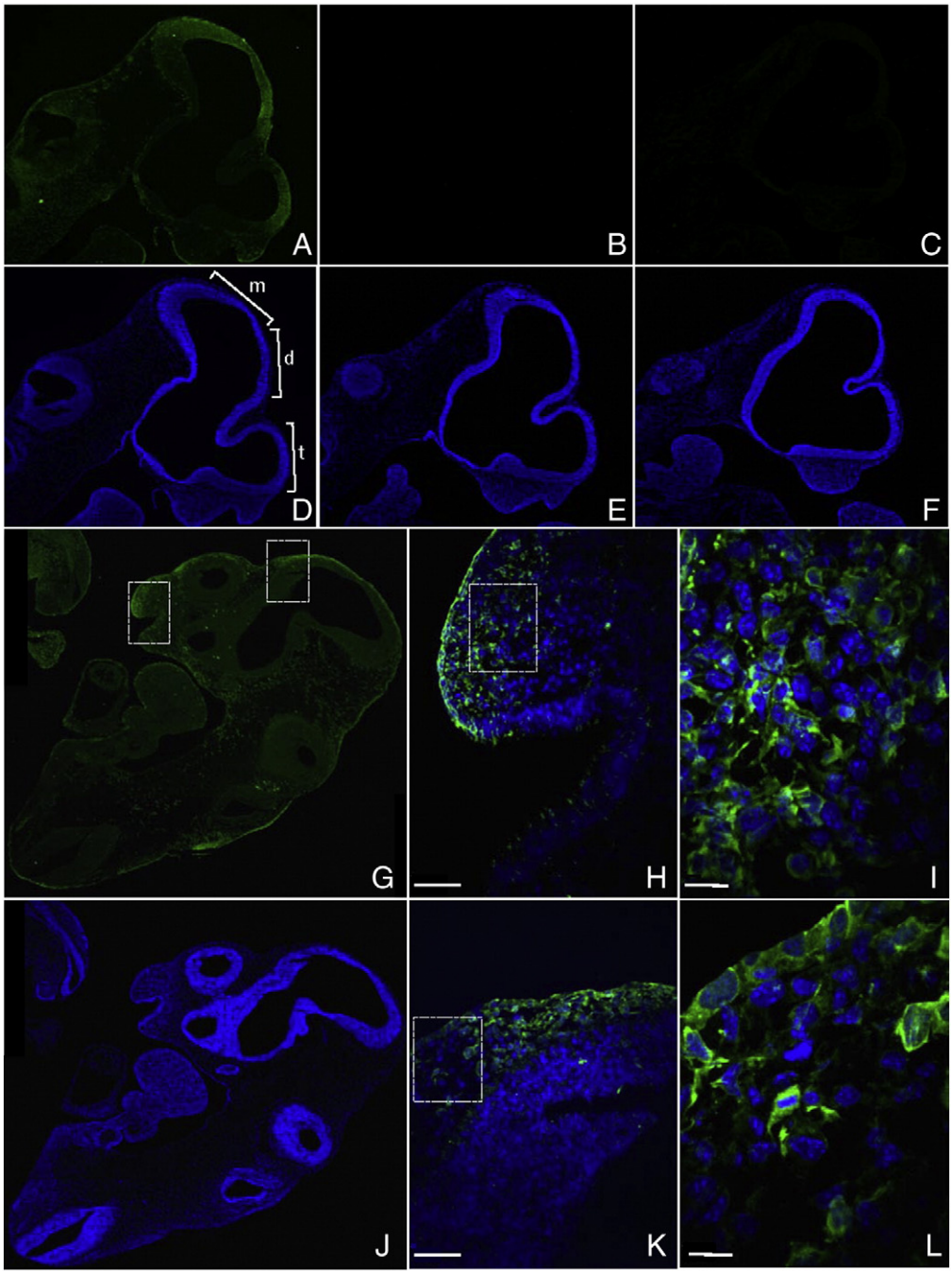


Fig. 1. MHCI is expressed in prenatal mouse embryos at mid-gestation. (A). MHCI immunostaining in parasagital sections through telencephalon, diencephalon and mesencephalon of E9.5–E10.5 mouse embryo. Note the lack of staining in mouse IgG isotype (B) and no-primary (C) controls. Hoechst staining (D–F) shows morphology of the sections directly above. In a more lateral parasaggital section (G, MHCI; J, Hoechst), MHCI expression is enriched in the nasal process and neuroepithelium (G, left and right square, respectively). Magnification of the nasal process (H,I) and neuroepithelium (K,L) shows that MHCI staining (green) surrounds the nucleus (blue). A–F, G and J, 4× magnification. Scale bars: H and K, 100 μm; I and L, 25 μm.

(Fig. 2A). Since MAP2 labels cytoskeletal elements in developing neurites, and OX-18 labeling at this age is primarily cytosolic/cell surface, these two labels are not expected to colocalize perfectly if they are expressed in the same cell. Closer examination suggests that MHCI is expressed by most if not all MAP2-positive cells (Fig. 2E–H). Thus MHCI is expressed in the small population of post-mitotic neurons that are found in the E9.5 mouse brain. MHCI is also expressed in a number of cells in the brain primordium that do not express MAP2 at this age, and therefore are not post-mitotic neurons.

Co-expression of MHCI and neuronal lineage marker Tuj1

To determine if MHCI is expressed by neuronal precursors, double-label immunostaining was performed using an antibody that recognizes neuron-specific class III beta-tubulin (Tuj1; Lee et al., 1990). Class III beta-tubulin is expressed by neuronal precursors in the central and peripheral nervous systems (Memberg and Hall, 1995). Tuj1 has been used widely as a marker of neuronal precursors (e.g., Bolteus and Bordey, 2004; Doetsch et al., 1997; Luskin, 1998), and does not label the form of β-tubulin found in glial cells (Burgoyne et al., 1988).

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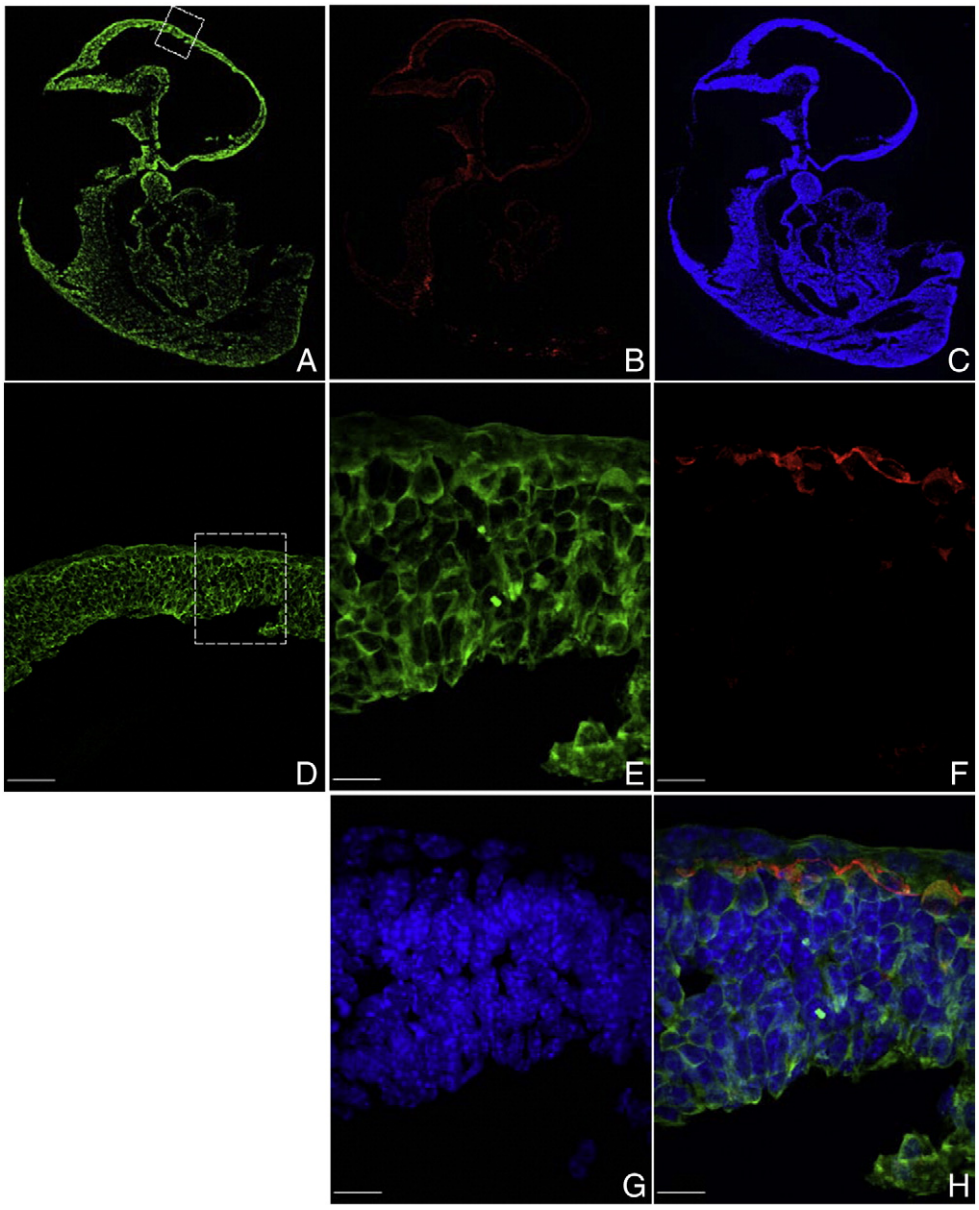


Fig. 2. MHCI is expressed by MAP2-positive cells in E9.5 neuroepithelium. (A–C), low magnification micrographs of a parasagittal section of a mouse embryo, and (D–H) higher-magnification views of the same section at the mesencephalic level, stained for MHCI (green; A, D, E, H), MAP2 (red; B, F, H) or Hoechst (blue; C, G, H). D, boxed region in A; E–H, boxed region in D. Scale bars: A–C, 4× magnification; D, 100 μm; E–H, 25 μm.

In parasagittal sections, MHCI is detected throughout the neuroepithelium (Fig. 3A), and qualitatively similar staining of the neuroepithelium is observed for Tuj1 (Fig. 3B). At higher magnifications, it is apparent that MHCI is extensively co-expressed by Tuj1-positive cells in neuroepithelium (Fig. 3D–H). Thus MHCI is expressed by neuronal precursors in the mid-gestation embryo.

Co-expression of MHCI and neuronal progenitor/dorsal neuroepithelium marker Pax6

To determine if MHCI is expressed at earlier stages of neuronal differentiation, sections were co-immunolabeled with antibodies against MHCI and Pax6 (paired box protein, also known as aniridia type II protein (AN2) or oculorhombin), a paired-box transcriptional regulator that is expressed in progenitors of the dorsal neuroepithelium after neural tube closure in mouse (Manuel and Price, 2005; Schmahl et al., 1993; Walther and Gruss, 1991). During differentiation, Pax6 represses pluripotent genes and activates neural genes. During the pro-neurogenic to neurogenic transition in proliferating progenitor cells, postmitotic neurons either elevate or eliminate Pax6 expression in a cell type-specific manner (Hsieh and Yang, 2009). Pax6 is ubiquitously expressed in the human neural plate, and is necessary and sufficient for human neuroectoderm specification (Zhang et al., 2010).

As shown above, MHCI expression is broadly distributed in cell somas throughout dorsal and ventral telencephalon (Fig. 4A). Consistent with Pax6's role as a transcriptional regulator, Pax6 labeling is primarily nuclear, and therefore co-expression with MHCI would appear as purple (Pax-6-(red) and Hoechst-(blue) positive) nuclei embedded in green, MHCI-positive cell bodies. Strongly Pax6-positive cells are found throughout dorsal telencephalon, as well as in specific

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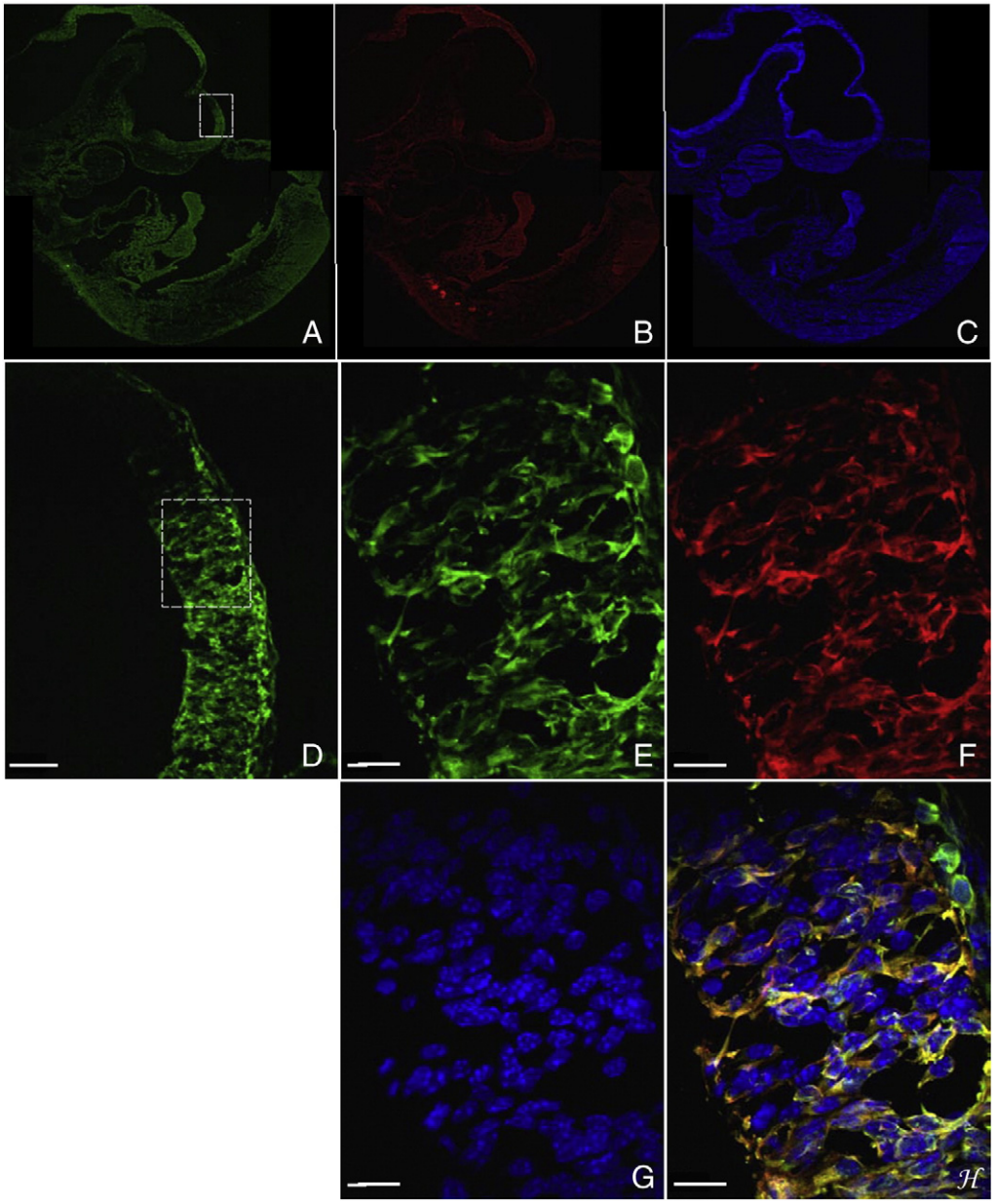


Fig. 3. MHCI is expressed by Tuj1-positive neuronal precursors in prenatal brain. (A–C), low magnification micrographs of a parasagittal section of a mouse embryo, and (D–H) higher-magnification views of the same section at the telencephalic level, stained with MHCI (green; A, D, E, H), Tuj1 (red; B, F, H) or Hoechst (blue; C, G, H). D, boxed region in A; E–H, boxed region in D. A–C, 4× magnification. Scale bars: D, 100 μm; E–H, 25 μm.

domains of ventral telencephalon, including neuroepithelium and olfactory placode (Fig. 4B). Higher-magnification analysis shows that MHCI is expressed in the somas of most cells with Pax6-positive nuclei in dorsal telencephalon (Fig. 4E–H). MHCI is also expressed in populations of Pax6-positive cells in ventral telencephalon. Colocalization here is also extensive, and very few MHCI-positive, Pax6-negative cells can be found in ventral mesenchyma, indicating that most of the MHCI-expressing cells in these regions are progenitors of the neural lineage. In diencephalon, MHCI is found throughout the neuroepithelium, although stronger staining is observed dorsally (Fig. 5A). Pax6 is more restricted to dorsal regions, as expected (Fig. 5B). At higher magnification, MHCI is found in the somas of most cells with Pax6-positive nuclei in dorsal diencephalon (Fig. 5E–H), as in telencephalon (Fig. 4). In mesencephalic sections, MHCI shows broad, strong expression (Fig. 6A). In contrast, only a few Pax6-positive cells are found, mainly in the periphery of the mesencephalon (Fig. 6B), and this staining was not nuclear (see Discussion), colocalizing in only a few cells with MHCI (Fig. 6E–H). Thus MHCI is widely expressed in Pax6-negative cell types in mesencephalon, but is expressed by most if not all Pax6-positive cells in other regions of the brain primordium.

Limited co-expression of MHCI and neural progenitor marker nestin

To determine if MHCI is expressed in neuronal precursors prior to differentiation, antibodies against nestin were used in immunohistochemical analysis. Nestin is a type VI intermediate filament protein expressed by dividing cells during the early stages of development in many tissues, including the CNS and peripheral nervous system

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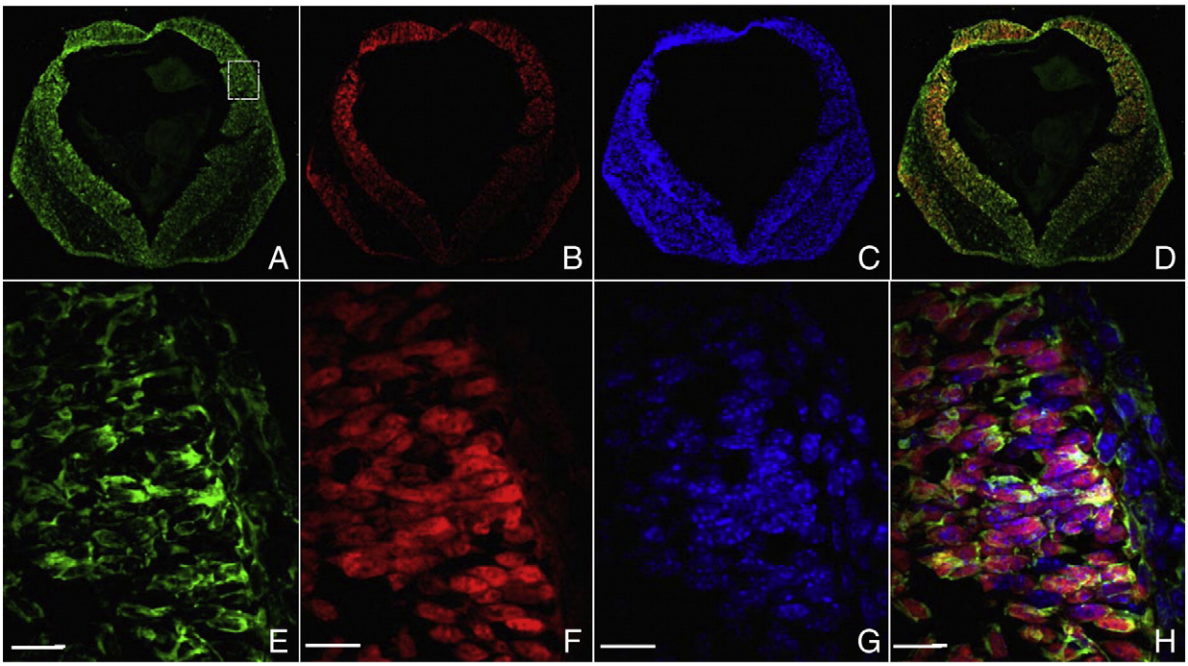


Fig. 4. MHCI is expressed by Pax6-positive cells in telencephalon. (A–D), coronal sections of telencephalon stained for MHCI (A), Pax6 (B) and Hoechst (C); merge of (A) and (B) shown in D. (E–H), higher magnification of dorsal telencephalon (boxed region in A) stained for MHCI (E), Pax6 (F) and Hoechst (G) (merge, H). A–D, 4× magnification. Scale bars: E–H, 25 μm.

(PNS), and has been used as a marker of neural stem/progenitor cells (Lendahl et al., 1990). Upon differentiation, nestin is downregulated and is replaced by tissue-specific intermediate filament proteins (e.g., neurofilament in neurons, and glial fibrillary acidic protein (GFAP) in glia).

In coronal sections of telencephalon, MHCI is broadly expressed in the neuroepithelium, including dorsal and ventral regions of the telencephalon and the olfactory placode (Fig. 7A). In contrast, nestin is enriched in dorsal neuroepithelial cells, although some cells in ventral regions, including olfactory placode, are also stained (Fig. 7B). Higher-magnification views show that MHCI and nestin are extensively co-expressed in cells in the olfactory placode (Fig. 7I–L). In other regions, red nestin-positive intermediate filaments are seen in processes that are MHCI-negative (Fig. 7A–H), suggesting localization

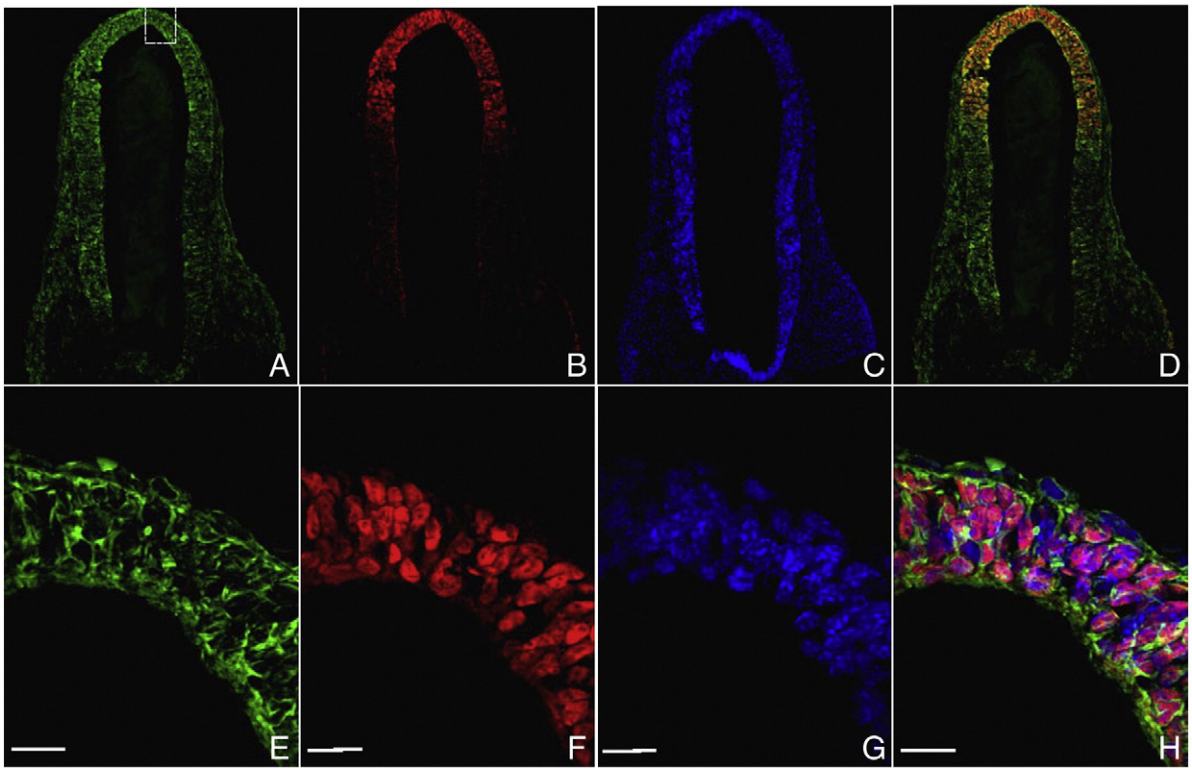


Fig. 5. MHCI is by Pax6-positive cells in diencephalon. (A–D), coronal sections of diencephalon stained for MHCI (A), Pax6 (B) and Hoechst (C). Merge of (A) and (B) shown in D. (E–H), higher magnification of dorsal diencephalon (boxed region in A) stained for MHCI (E), Pax6 (F) and Hoechst (G) (merge, H). A–D, 4× magnification. Scale bars: E–H, 25 μm.

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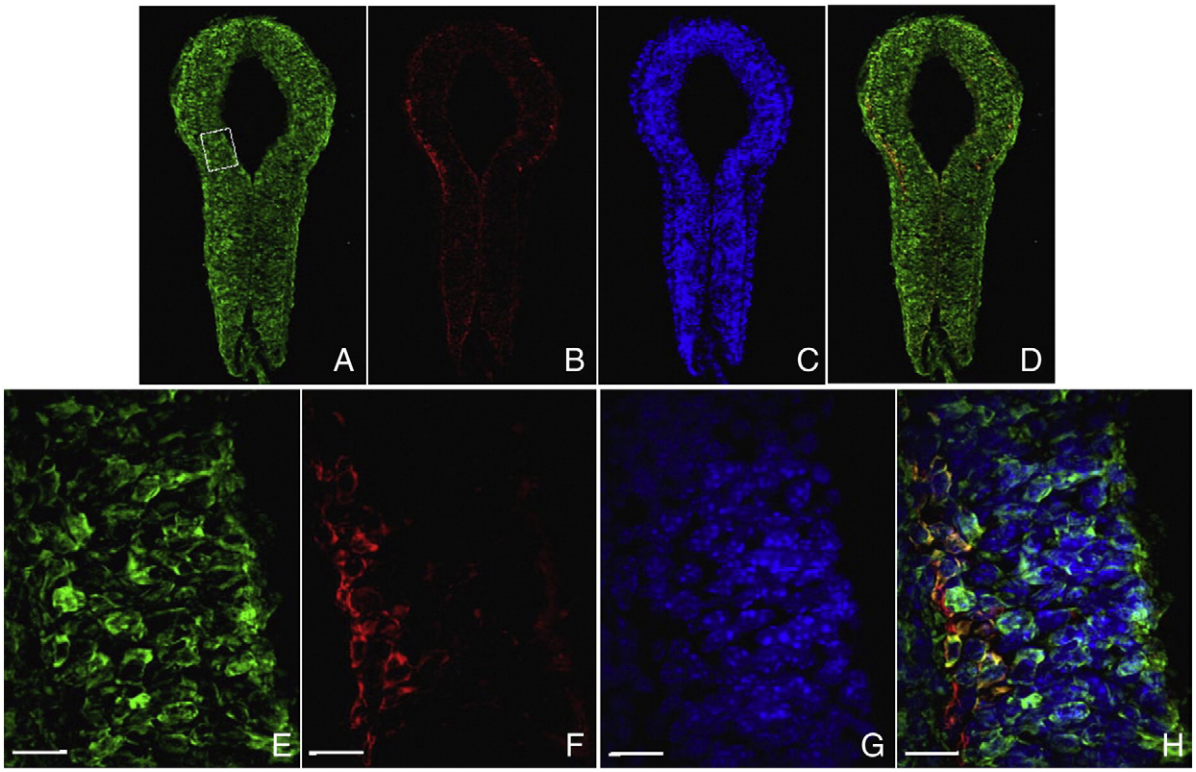


Fig. 6. MHCI is expressed by Pax6-positive cells in mesencephalon. (A–D) coronal sections of mesencephalon stained for MHCI (A), Pax6 (B) and Hoechst (C). Merge of (A) and (B) shown in D. (E–H), higher magnification of dorsal mesencephalon (boxed region in A) stained for MHCI (E), Pax6 (F) and Hoechst (G) (merge, H). A–D, 4× magnification. Scale bars: E–H, 25 μm.

to distinct cellular or subcellular compartments. In all regions, as expected, the subcellular appearance of MHCI and nestin staining differs qualitatively: nestin, a cytoskeletal component, appears filamentous, while MHCI, a transmembrane protein, appears to fill the soma and/or cover the cell surface. In diencephalic sections, MHCI is broadly expressed in the neuroepithelium, with slightly stronger labeling in dorsal areas (Fig. 8A). Here, as in the telencephalon, nestin is enriched in the dorsal region (Fig. 8B). MHCI is expressed in both nestin-positive and nestin-negative populations in diencephalon (Fig. 8E–H), as in telencephalon. In mesencephalic sections, MHCI is broadly expressed (Fig. 9A). In contrast, nestin is expressed in the periphery of the mesencephalon, ringing the dorsal and ventral regions (Fig. 9B). At higher magnification, filamentous nestin (Fig. 9G) does not overlap with cell surface/cytosolic MHCI (Fig. 9E). The same staining pattern is found in rhomboencephalic sections (not shown). Thus MHCI is expressed by Tuj1-positive neuronal progenitors and in a subset of nestin-positive and/or Pax6-positive neuronal precursors at E9.5–10.5. Furthermore, MHCI is also detected in the emerging population of differentiated neurons (labeled by MAP2) at this early stage of brain development.

Discussion

MHCI proteins are known for their roles in the adaptive immune system. However, increasing evidence shows that MHCI proteins have non-immune functions in the postnatal CNS (recently reviewed in Boulanger, 2009; Elmer and McAllister, 2012; Shatz, 2009). Here we show that MHCI proteins are broadly expressed by neuroepithelial cells in the prenatal mouse brain at mid-gestation. MHCI is detected in cells that express a neuronal lineage marker (TuJ1), as well as in subsets of neuronal precursors (nestin-positive, or Pax6-positive) and post-mitotic neurons (MAP2-positive). Together, these results raise the possibility that MHCI has as-yet unidentified roles during prenatal brain development.

At mid-gestation, MHCI protein is detected in the cell bodies of newly-differentiated neurons, as determined by co-expression of the neuronal marker MAP2. Since whole-mount embryos must be permeabilized for immunolabeling, it is difficult to determine if MHCI is expressed at the cell surface or intracellularly. mRNA encoding β2 microglobulin (β2m), the MHCI light chain, is also detected in the prenatal mouse brain, at E14.5 (EMAGE gene expression database Richardson et al., 2010, http://www.emouseatlas.org/emage/). Since β2m is required for stable cell-surface expression of most MHCI proteins (Dorfman et al., 1997; Zijlstra et al., 1989), the presence of β2m suggests that MHCI proteins may be able to reach the neuronal cell surface prenatally. MHCI is also present in the developing neuropil, consistent with the possibility that MHCI may be expressed at prenatal synapses, as it is postnatally (Datwani et al., 2009; Goddard et al., 2007; Needleman et al., 2010). Since MHCI protein has been detected in postsynaptic densities in postnatal hippocampal neurons (Goddard et al., 2007), but both pre- and post-synaptically in postnatal cortical neurons (Needleman et al., 2010), it will be important to determine if MHCI is expressed pre- or post-synaptically by neurons at these earlier ages.

In telencephalon and diencephalon, MHCI is expressed in most neuronal progenitors with Pax6-positive nuclei. In mesencephalon, MHCI colocalizes with non-nuclear Pax6 staining, an unusual expression pattern for a transcription factor. In mouse, quail and *Caenorhabditis elegans*, an isoform of Pax6 lacking the paired domain is found in both nucleus and cytoplasm of non-neuronal cells (Carriere et al., 1995; Mishra et al., 2002; Zhang et al., 1998), although the presence and subcellular localization of this isoform in neurons remain unknown. Although both nestin-positive/MHCI-negative and nestin-negative/MHCI-positive cells are detected in prenatal brain, a substantial subset of nestin-positive cells express MHCI. It is likely that, as with MAP2, we are underestimating the extent of the colocalization of MHCI with nestin, given that the subcellular localizations of these two proteins (somatic vs filaments within processes) are so different, and colocalization may be difficult to determine.

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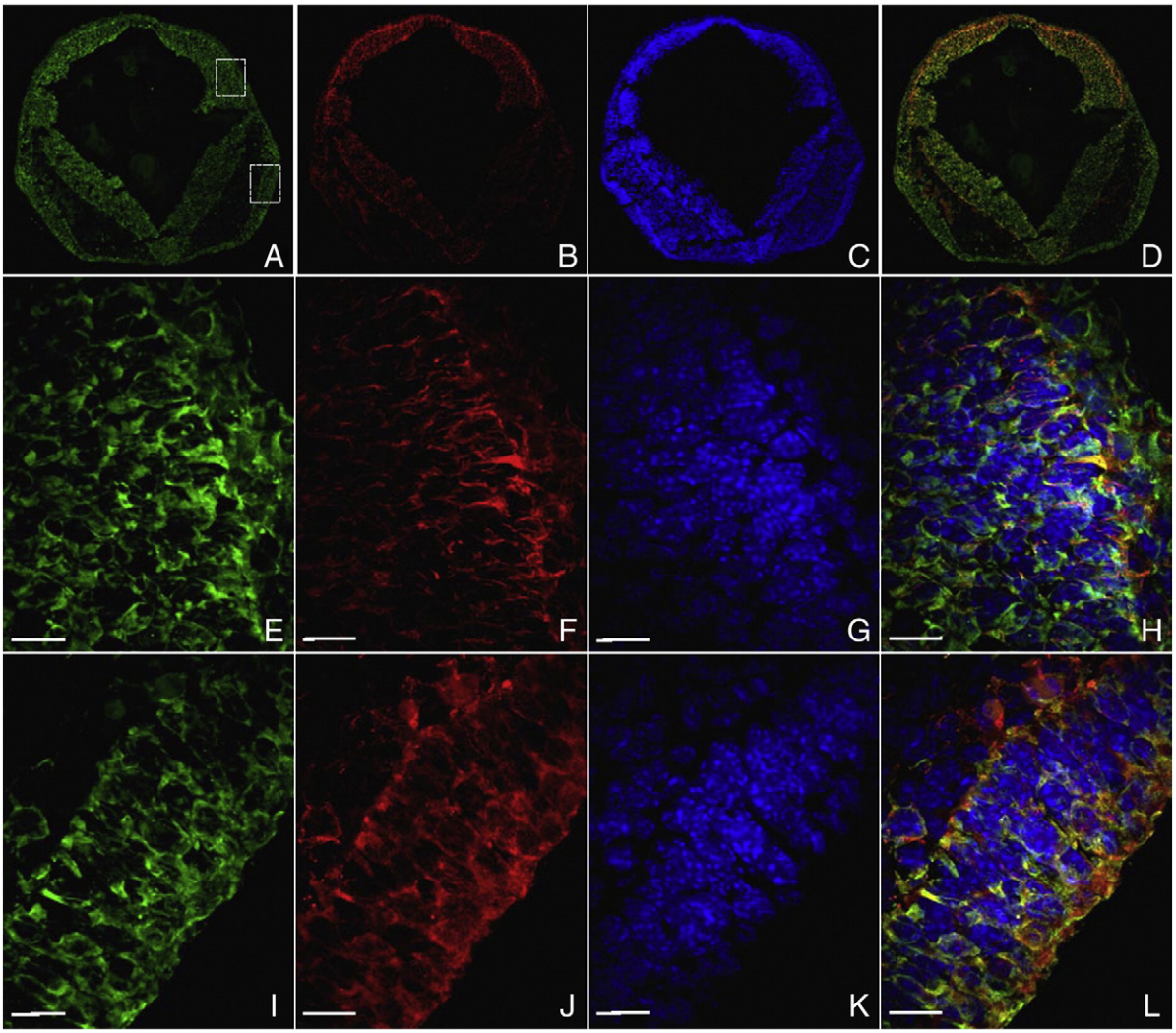


Fig. 7. MHCI is expressed in nestin-positive cells in telencephalic neuroepithelial cells and olfactory placode. (A–D), low-power views of coronal sections of telencephalon immunostained for MHCI (green; A), nestin (red; B), and Hoechst (blue; C). Merge of (A) and (B) shown in D. Below, higher magnification of boxed regions in A. (E–H), dorsal telencephalon and (I–L), olfactory placode, stained for MHCI (E, I), nestin (F, J) and Hoechst (G, K) (merge, H, L). A–D, 4× magnification. Scale bars: E–L, 25 μm.

MHCI protein is strongly expressed in the embryonic olfactory placode (Figs. 4A and 7A), which gives rise to the main olfactory epithelium (MOE) and vomeronasal organ (VNO) in mouse (Brunjes and Frazier, 1986). Two families of non-classical MHCI proteins, M1 and M10, are expressed in the adult mouse VNO (Loconto et al., 2003; Ishii et al., 2003; Ishii and Mombaerts, 2008), and MHCI peptides may act as chemosensory signals in the VNO (Leinders-Zufall et al., 2004), supporting a potential role for MHCI proteins in pheromone detection. Our results raise the possibility that in addition to a role in VNO-mediated sensory processing in the adult, MHCI contributes to the prenatal development of the main and/or accessory olfactory systems.

Expression of specific nonclassical MHCI genes has been detected prenatally in non-neuronal cell types as early as the first few hours post-fertilization. The preimplantation embryo development (*Ped*) gene product Qa-2 is a mouse non-classical (or class Ib) MHCI protein that is expressed on the surface of preimplantation mouse embryos (Warner et al., 1987). Changes in Qa-2 levels are sufficient to bidirectionally regulate the rate of cleavage of preimplantation embryos in C57Bl/6 mice (Tian et al., 1992; Wu et al., 1999; Xu et al., 1994). MHCI is also detected in the placenta and fetal trophoblast, where it may play roles in implantation, uterine vascularization, fetal growth, MHCI-based pregnancy block, and preventing rejection of the fetus by the maternal immune system (Madeja et al., 2011). Based in part on this very early prenatal expression, a role for MHCI in embryogenesis has been proposed (Ohno, 1977). MHCI expressed in thymus could also be involved in thymic selection prenatally (Sasada et al., 2003), since in mice, the thymus is evident by E12–13. However, T cells do not leave the thymus before birth (Fehling and von Boehmer, 1997), suggesting that MHCI expressed in other tissues, including brain, is unlikely to contribute to thymic selection or mediate T cell-mediated immune recognition prenatally. It is tempting to speculate that expression of MHCI during prenatal development, prior to the maturation of the adaptive immune response, reflects an evolutionarily basal role for MHCI in non-immune processes.

The current results demonstrate that MHCI is expressed in the right time, at the right place, to influence prenatal neuronal development *in vivo*. MHCI protein has been detected in perinatal neurons grown *in vitro* for one or more days, including E16 mouse cortical (Zohar et al., 2008) and hippocampal (Bilousova et al., 2012) neurons, and E14 mouse dorsal root ganglion neurons (Wu et al., 2011). Furthermore, manipulations of MHCI levels and/or function in these *in vitro*

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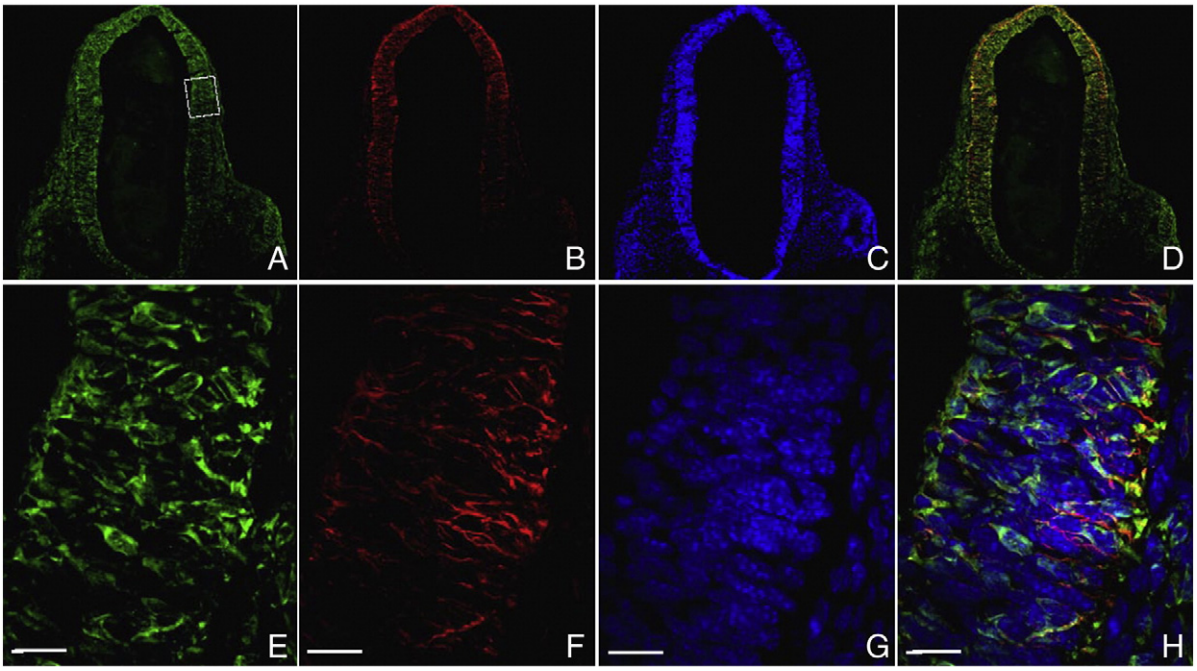


Fig. 8. MHCI is expressed by neuroepithelial cells in mouse diencephalon, but is not co-expressed with nestin in these regions. (A–D), coronal sections of diencephalon stained for MHCI (A), nestin (B) and Hoechst (C). Merge of (A) and (B) shown in D. (E–H), higher magnification of dorsal diencephalon (boxed region in A) stained for MHCI (E), nestin (F) and Hoechst (G) (merge, H). A–D, 4× magnification. Scale bars: E–H, 25 μm.

systems can have a significant impact on neuronal morphology. MHCI molecules inhibit neurite outgrowth in *in vitro* preparations of perinatal retinal (Escande-Beillard et al., 2010; Washburn et al., 2011), hippocampal (Bilousova et al., 2012), cortical (Zohar et al., 2008), and dorsal root ganglion (DRG) (Wu et al., 2011) neurons. In retinal and DRG neurons, this inhibition is at least partially rescued by the addition of anti-MHCI antibodies (Washburn et al., 2011; Wu et al., 2011). These findings, together with a growing literature in older neurons, show that MHCI has

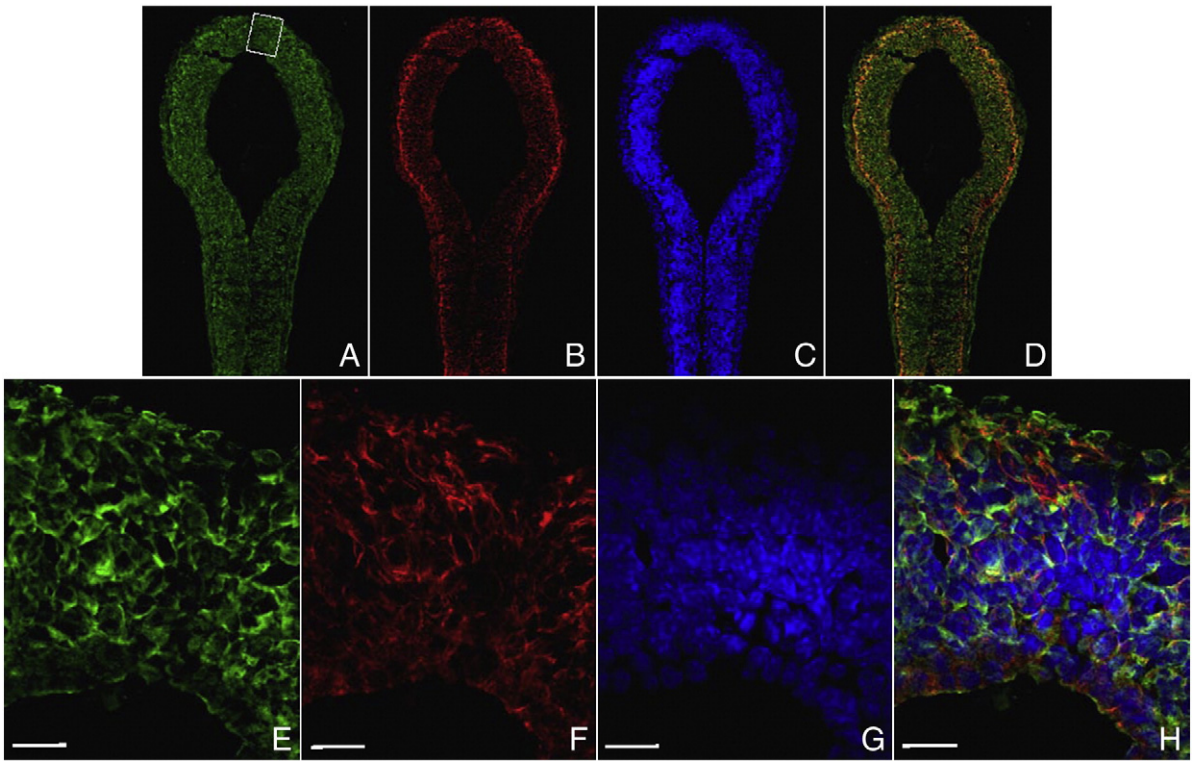


Fig. 9. A subset of MHCI-expressing mesencephalic cells are nestin-positive. (A–D), coronal sections of mesencephalon stained for MHCI (A), nestin (B), and Hoechst (C). Merge of (A) and (B) shown in D. (E–H), higher magnification of dorsal mesencephalon (boxed region in A) stained for MHCI (E), nestin (F) and Hoechst (G) (merge, H). A–D, 4× magnification. Scale bars: E–H, 25 μm.

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normal, apparently non-immune functions in perinatal and postnatal neurons *in vitro*. The current results suggest that MHCI may have additional functions in prenatal brain development *in vivo*.

In cats, MHCI mRNA is found in the ventricular zone (Corriveau et al., 1998), an area that contains progenitor cells undergoing mitosis (Luskin and Shatz, 1985), at E43, E52, and P0. In adult mice, the MHCI-like protein RAE-1 is expressed in the subventricular zone and promotes the proliferation of neurospheres (Popa et al., 2011). These results are consistent with our current finding that MHCI protein is expressed in neural progenitors in prenatal mouse brain, and support a potential role for MHCI in early neuronal differentiation throughout life.

Undifferentiated human embryonic stem (HES) cells express low levels of MHCI on the cell surface (Drukker et al., 2002), protecting them from immune surveillance (Li et al., 2004). However, MHCI levels increase moderately as HES cells differentiate. Neural stem cells (NSCs) derived from embryonic rat forebrain also express MHCI molecules at low levels (Yin et al., 2008), and MHCI mismatch inhibits differentiation and retention of new neurons (Chen et al., 2011). The current results support a model in which neural progenitors and newly-differentiated neurons can both express MHCI proteins. Characterizing the normal role of MHCI in embryonic development may help maximize the efficacy of stem-cell therapies while reducing the risk of tissue rejection.

The normal presence of MHCI in the prenatal brain *in vivo* raises the possibility that changes in MHCI expression before birth could impact prenatal brain development. Activation of the maternal immune response, which may increase the risk of autism in humans (Atladottir et al., 2010), is sufficient to disrupt brain development in the offspring in animal models (Brown, 2006; Patterson, 2011). Maternal immune activation is associated with changes in the levels of cytokines, including interferon-γ (IFN-γ) (Boksa, 2010; Deverman and Patterson, 2009; Garay et al., in press), which regulate expression of MHCI in neurons (Neumann et al., 1995; Wong et al., 1984) and neural stem cells (Cheeran et al., 2008; Yin et al., 2008). Prenatal immune activation can inhibit postnatal neurogenesis in mouse cerebral cortex (Soumiya et al., 2011), and although MHCI does not affect neurogenesis in adult mice (Laguna Goya et al., 2010), it remains to be determined if MHCI can modify neurogenesis prenatally. It will also be important to determine if changes in cytokines in the fetal brain in the wake of maternal immune challenge (Garay and McAllister, 2010; Needleman and McAllister, 2012) influence prenatal MHCI expression in the brain. Genetic, as well as epigenetic, changes in MHCI could contribute to neurodevelopmental disorders. MHCI mRNA and protein expression are elevated in specific regions of the brain in murine trisomy 16 (a mouse model of Down's syndrome) (Kornguth et al., 1991), and genetic studies have demonstrated an association between the MHCI region and the risk of developing either schizophrenia or autism (Purcell et al., 2009; Stefansson et al., 2009; Torres et al., 2006). Future studies are needed to test whether or not altered MHCI expression can affect prenatal brain development *in vivo*, and if such changes could contribute to the pathogenesis or progression of neurodevelopmental disorders. The current results demonstrate that MHCI proteins are widely expressed in neuronal cell lineages in the normal vertebrate brain at mid-gestation, suggesting that MHCI may play important non-immune roles in the developing brain well before birth.

Experimental methods

Mouse embryo dissection and fixation

Timed-pregnant C57BL/6 mice were obtained from JAX (The Jackson Laboratories, Maine, USA) at day 8 of gestation. At E9.5, pregnant mice were deeply anesthetized with isoflurane and the embryos quickly dissected and immersed in cold 4% PFA/PBS for 30 min with gentle agitation. Embryos were immersed in 10% sucrose overnight at 4 °C and then sunk in 30% sucrose. Fixed, cryo-protected embryos were placed on a thin layer of dry ice-chilled OCT (Sakura Finetek USA, Torrance, CA, USA) on the bottom of a plastic biopsy Cryomold (Sakura Finetek USA, Torrance, CA, USA) and were oriented for coronal or parasaggital sectioning under a dissecting scope. The mold was filled with OCT and quickly put on dry ice until the embryo-containing block froze. Embedded embryos were stored at −80 °C for later cryosectioning.

Immunohistochemistry

12–14 μm thick sections of whole-mount embryos were cut on a cryostat (Leica CM3050 S, Buffalo Grove, IL, USA) and mounted on cold Superfrost slides (Fisher Scientific, Pittsburgh, PA, USA). Slides were allowed to air-dry for >30 min on a slide warmer and stored at −80 °C until immunostaining. Upon removal from the −80 °C freezer, slides were allowed to dry for 10 min at room temperature (RT). OCT was washed off in PBS (2×5 min washes). Tissue was blocked and permeabilized in 3% BSA, 5% TX-100 in PBS for 1 h at RT. Slides were incubated with primary antibodies (rabbit anti-Pax6, 1:1000, Millipore; chicken anti-nestin, 1:1000, Novus Biologicals; mouse anti-β III tubulin (Tuj1; clone D-10), 1:1000, Santa Cruz Biotechnology, Inc.; rabbit anti-microtubule-associated protein 2, 1:1,000, Millipore; mouse OX-18 1:200-1:500, AbD Serotec), diluted in the same blocking/permeabilization solution overnight at 4 °C with gentle agitation in a humidified chamber. The following day, slides were washed in PBS (2×5 min) and incubated with the appropriate species' secondary antibodies (1:1,000, labeled with Alexa 488 or Alexa 568, Life Technologies, Grand Island, NY, USA) for 1 h at RT. Slices were washed (3×5 min) in PBS. Hoechst dye (1 μg/ml) was included in the last wash. Coverslips were mounted using Fluorescent Mounting Media (EMD Millipore, Billerica, MA, USA) and slides were stored at 4 °C until imaging via epifluorescence (Zeiss AxioObserver Z1) or spinning-disk confocal microscopy (3I Marianas SDC equipped with appropriate filter cubes and laser lines).

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