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STAT5A/B activity is required in the developing forebrain and spinal cord

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Formation of the CNS requires the coordination and integration of processes such as cell proliferation, neuronal differentiation, neuronal migration, axon tract formation and synaptogenesis, all of which must occur at precise times and places during development. Although growth factors are known to play a role in regulating many of these processes, very little is known of the signaling events immediately downstream of ligand–receptor interactions in the developing CNS. Here we present evidence that STAT5, an important mediator of cytokine signaling, is required for some aspects of CNS development. We show that phosphorylated and hence activated forms of STAT5 (pSTAT5) are expressed in a temporally restricted manner in a subset of early-born telencephalic neurons and axons. Accordingly, *Stat5* mutants have reduced numbers of interneurons in the cortical marginal zone, suggestive of migration defects. Moreover, corticofugal axons develop aberrantly in *Stat5* mutants, indicative of a role for pSTAT5 in axon guidance. Notably, pSTAT5 is also expressed in commissural axons in the embryonic spinal cord, where it is also required for their guidance. Taken together, we provide the first evidence that STAT5 is a key effector molecule in the developing mammalian CNS.

Keywords: *Stat5a/b*; Nervous system; Telencephalon; Spinal cord; Development; Axon tracts; Neurogenesis

Introduction

Formation of a functional central nervous system (CNS) is the successful culmination of coordinated rounds of cellular proliferation, differentiation, survival, migration and the formation of synaptic connections. Many of these cellular events are orchestrated by growth factors and/or cytokines, acting through their respective downstream signaling pathways. One prominent signal transduction pathway involves the *Ja*nus *k*inases (JAK), which are recruited to the membrane and phosphorylated following growth factor or cytokine stimulation, leading to the selective activation and phosphorylation of a *s*ignal *t*ransducer and *a*ctivator of *t*ranscription (STAT) transcription factor. Once activated (i.e., phosphorylated), the STATs dimerize and translocate to the nucleus where they directly bind their target consensus sequence. To date, four mammalian JAKs (JAK1, JAK2, JAK3 or TYK2) and seven STATs have been identified [STAT1, 2, 3, 4, 5a, 5b (referred to as STAT5) and 6]. Each cytokine and growth factor receptor recruits different combinations of JAKs and STATs, thereby controlling at least in part the specificity of downstream transcriptional events.

Members of the JAK and STAT families are expressed during embryonic development in a number of species, including mice (Duncan et al., 1997), *Xenopus* (Pascal et al., 2001; Turpen et al., 2001), zebrafish (Lewis and Ward, 2004), *Dictyostelium* (Kawata et al., 1997), mosquito (Lin et al., 2004), *C. elegans* (Wang and Levy, 2006) and *Drosophila* (Li et al., 2003). Evolutionary studies suggest that mammalian *Stat3*, *Stat5a* and *Stat5b* arose from the duplication of a common primordial gene corresponding to the single *Drosophila Stat92E* (Miyoshi et al., 2001). Indeed, *Stat3*, *Stat5a* and *Stat5b* lie adjacent to each other on mouse chromosome 11 and based on transcriptional assays, the three genes are functionally similar (Miyoshi et al., 2001). It is interesting then that during *Drosophila* development mutations in *Stat92E* or *hopscotch*, encoding a JAK, lead to deficits in formation of the CNS and peripheral nervous system (Li et al., 2003). Moreover, during development of the murine CNS, STAT3 has been implicated in the maintenance of neural progenitor cells (Yoshimatsu et al., 2006). Although a role for STAT5 has not yet been reported, a number of receptors implicated in the selective activation of STAT5 such as the erythropoietin receptor, ErbB4, the EphA receptors and the chemokine receptor CXCR4 have been implicated in various aspects of brain development, including axon guidance, interneuron migration and cortical layer formation (Shingo et al., 2001; Dufour et al., 2003; Stumm et al., 2003; Flames and Marin, 2005). In addition, cytokine stimulation of an immortalized rat striatum-derived cell line (ST14A) expressing the interleukin-3 receptor led to phosphorylation of JAK2 with subsequent STAT5 phosphorylation, suggesting a role for this pathway in neuroblast signaling (Cattaneo et al., 1996; De-Fraja et al., 1998). Cumulatively this evidence suggests that STAT5 may play a role in mediating some aspects of embryonic CNS development.

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In this study we show that the active or phosphorylated form of STAT5 (pSTAT5) is present in the developing forebrain during the active period of neurogenesis, where it specifically localizes to early-born post-mitotic neuronal populations and later is expressed in developing axon tracts. Examination of *Stat5a/5b* double mutants (hereafter referred to as *Stat5*$^{-/-}$ or *Stat5* mutants) revealed a reduction in the number of cortical interneurons in the marginal zone. The formation of corticofugal axon projections was also abnormal and developmentally delayed. In addition, activated STAT5 was detected in the developing commissural axons of the spinal cord and, accordingly, crossing defects were evident post-midline, suggestive of axon guidance defects in *Stat5* mutants. The results of our study thus reveal novel roles for STAT5 in the development of the neocortex and spinal cord.

Results

pSTAT5 is active in newly born post-mitotic neurons and developing axon tracts in the embryonic telencephalon

As a first step towards elucidating a potential functional role for STAT5 in forebrain development we examined the spatial distribution of pSTAT5 (including pSTAT5a/5b) in the embryonic telencephalon. The telencephalon is subdivided into distinct dorsal and ventral regions that give rise to the neocortex/hippocampus and striatum/globus pallidus, respectively. At embryonic day (E) 10.5, immunoreactivity for pSTAT5 was observed in the mantle zone of the lateral (lge) and medial (mge) ganglionic eminences, embryonic subdivisions of the ventral telencephalon (Fig. 1A). Conventionally it is thought that STAT5 is phosphorylated in the cytoplasm where it is then transported to the nucleus to affect gene transcription. To examine the subcellular localization of pSTAT5, high magnification examination of pSTAT5/Hoechst labeled nuclei revealed predominant cytoplasmic staining as well as a speckled pattern of pSTAT5 immunoreactivity within the nucleus (inset in Fig. 1A), consistent with the idea that some pSTAT5 protein may be transcriptionally active in neurons *in vivo*.

The mantle zone of the lge and mge contains the earliest-born ventral telencephalic neurons, whereas the ventricular zone (VZ), where pSTAT5 immunostaining was not detected, is populated by dividing progenitor cells. To confirm that pSTAT5 was indeed expressed in neurons, we showed co-localization with the early neuronal marker TUJ1 (neuronal-specific βIII-tubulin; Fig. 1B) and doublecortin (DCX; Fig. 1C), a microtubule associated protein that labels immature migrating neurons. pSTAT5 is thus expressed in newly born, migrating neurons in the ventral telencephalon.

While neuronal differentiation is already evident in the ventral telencephalon by E10, differentiation is delayed dorsally, with the first post-mitotic neurons appearing slightly later. At E12.5, predominantly cytoplasmic and lower levels of nuclear pSTAT5 immunoreactivity were observed in the developing preplate of the dorsal telencephalon, where the earliest-born cortical neurons migrate, but were excluded from the cortical VZ (Figs. 1D–F). Notably, pSTAT5 expression was also evident in the cortical hem, the medial-most aspect of the dorsal telencephalon that serves as an important signaling center for neocortical development (Fig. 1E; Grove and Tole, 1999). In contrast, by E12.5, pSTAT5 expression was much reduced in the mantle zone of the ventral telencephalon, with immunostaining restricted to the subventricular zone (SVZ) of the lge (Fig. 1D). Co-labeling with anti-TUJ1 (Fig. 1E) and anti-DCX (Fig. 1F) confirmed that pSTAT5 was expressed in neuronal cells in both the dorsal and ventral telencephalon at E12.5.

By E14.5, neurons that will form the cortical plate proper have begun to differentiate, migrating out of the germinal zones to split the cortical preplate into an overlying marginal zone and underlying subplate layer. In addition, corticofugal axons have begun to project subcortically and are evident in the intermediate zone, extending ventrally towards the internal capsule. At E14.5, pSTAT5 was expressed at high levels in the marginal zone, as well as in cortical axon tracts coursing through the intermediate zone (Figs. 1G–I). pSTAT5 co-labeled with TUJ1 (Fig. 1H) and DCX (Fig. 1I) in axonal tracts in the cortical intermediate zone and neurons in the marginal zone. In contrast, pSTAT5 expression was no longer detected in the ventral telencephalon at E14.5 and by E16.5 little to no pSTAT5 immunoreactivity was detected in either the dorsal or ventral telencephalon (Figs. S1A, B). This finding was supported by Western blotting that showed a significant decrease in pSTAT5 levels at E16.5 compared to earlier embryonic stages (Fig. S1C).

To confirm the specificity of our staining pattern, immunolabeling was performed with a second commercial source of pSTAT5 antiserum, revealing an identical profile of expression (data not shown). In addition, E14.5 *Stat5* mutant brains were immunolabeled with anti-pSTAT5, which resulted in a lack of specific staining (Fig. S1D). Finally, we also examined E14.5 brains using an antibody against STAT5 which recognizes both phosphorylated and unphosphorylated forms. As expected, the pan-STAT5 antibody showed a broader expression pattern than that observed with pSTAT5, with immunolabeling detected throughout the forebrain (Fig. S1E).

pSTAT5 is expressed by differentiating neurons in vitro

Our data demonstrated that STAT5 was activated in differentiating neurons during early neurogenesis *in vivo*. To determine if this was also true of neurons cultured *in vitro*, dissociated cells derived from the E14.5 dorsal telencephalon were grown for 3 or 5 days without growth factor support to promote neuronal differentiation. In these cultures, pSTAT5 and TUJ1 were co-expressed in cells with a neuronal morphology, with activated pSTAT5 evident in neuronal nuclei and cell bodies (Figs. 1J–L). Punctate pSTAT5 staining was also observed in neuronal projections, suggesting that STAT5 activation may play a role in signal transduction in the dendrites and axons (Figs. 1K, L). To more conclusively identify the subcellular localization of pSTAT5 in neurons we undertook double labeling studies, showing co-localization of pSTAT5 with the dendritic marker MAP2 (Figs. 1M, O) and the axonal marker Tau (Figs. 1N, P), demonstrating that pSTAT5 is expressed in both neuronal dendrites and axons *in vitro*.

Cortical plate neurons differentiate normally in Stat5 null mutants

The expression of pSTAT5 in newly born neurons in the telencephalon was suggestive of a role in neuronal differentiation. To examine this possibility, we analyzed the dorsal telencephalon of *Stat5a/b* double mutants (hereafter referred to as *Stat5* mutants; Teglund et al., 1998). At E15.5, *Tbr1*, encoding a T-box transcription factor that is highly expressed in cortical layer VI and subplate neurons (Fig. 2A; Rubenstein et al., 1999), was expressed in *Stat5* mutants (Fig. 2B) in a pattern indistinguishable from wild-