Birthdate study of GABAergic neurons in the lumbar spinal cord of the glutamic acid decarboxylase 67-green fluorescent protein knock-in mouse

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INTRODUCTION

Neurons of the dorsal spinal cord relay sensory information, while the ventral spinal cord contains motor neurons and interneurons that coordinate motor output. To establish the correct circuitry, different types of neurons in the spinal cord are formed in a defined spatial and temporal order during development (Caspary and Anderson, 2003; Helms and Johnson, 2003). It is well known that γ-aminobutyric acid (GABA) is a major inhibitory neurotransmitter in the central nervous system, and its physiological roles are well documented in both dorsal and ventral horn of the spinal cord (Schneider and Fyffe, 1992; Malcangio and Bowery, 1996). Previous investigations have revealed that early in development, GABA acts as an excitatory neurotransmitter and a trophic factor to influence growth, synapse maturation, and cell death (Owens and Kriegstein, 2002; Stein et al., 2004; Rivera et al., 2003). GABAergic neurons have been postulated to be a critical component of local circuits in the adult spinal cord, which may influence the primary afferent terminals, projection neurons, other interneurons, and terminals of the descending inhibitory system in the superficial laminae (Millan, 2002). To better understand the role of GABAergic neurons in the adult spinal cord, the expression pattern and time course of generation of the major cell types need to be established. These studies will help define and interpret patterns of gene expression, waves of differentiation, timing and extent of competence, and many other processes involved.

Indeed, the spatial and temporal development of GABA immunoreactivity has been studied in the embryonic rat (Ma et al., 1992; Tran and Phelps, 2000; Tran et al., 2003) and mouse spinal cord (Allain et al., 2004; Huang et al., 2007) both in vivo and in vitro. Antibodies to GABA (Todd and McKenzie, 1989; Todd and Sullivan, 1990; Proudlock et al., 1993) or its synthetic enzyme glutamic acid decarboxylase (GAD; McLaughlin et al., 1975; Tillikainen et al., 2006; Mackie et al., 2003) have been used for immunohistochemical analysis. By using the GAD67-GFP knock-in mouse, we previously observed the expression pattern of GABAergic neurons in the developing spinal cord (Huang et al., 2007). The results revealed that the GABAergic population followed a rostro-caudal and ventro-dorsal gradient of maturation. The birthdates of GABA-immunoreactive neurons have been described in different areas of the nervous system, such as the developing rat dentate gyrus (Dupuy and Houser, 1997), rat
Table 1 | Time and animal numbers designed for BrdU injections.

| Age  | Animals analyzed (litters) |
|------|---------------------------|
| E10.5| 6 (3)                     |
| E11.5| 6 (3)                     |
| E12.5| 6 (3)                     |
| E13.5| 6 (3)                     |
| E14.5| 6 (3)                     |
| E15.5| 6 (2)                     |
| E16.5| 6 (2)                     |
| E17.5| 4 (2)                     |

The table lists the number of mice analyzed for each given injection time point. The number of independent litters from which animals were derived is indicated in parentheses.
FIGURE 1 | Double-labeling of GFP (green) and BrdU (red) in the spinal cord of the GAD67-GFP knock-in mice following injection of BrdU at E10.5 and E11.5. (A,F) Schematic drawings showing the distribution of BrdU-positive cells and double-labeled neurons in the lumbar spinal cord, taken from P60 mice that had received a single pulse of BrdU at E10.5 (A) and E11.5 (F). (A) The schematic drawing represents a single section and it corresponds to the same section illustrated in (B). (F) The schematic drawing represents a single section and it corresponds to the same section illustrated in (D). Red circles correspond to BrdU-positive cells and blue circles correspond to double-labeled neurons. (B) Note the distribution of BrdU-positive cells after injection of BrdU at E10.5. (C) Higher magnification obtained from a different section, in the approximate position indicated by the rectangle in (A). Double-labeled cells for GFP and BrdU were distributed in the ventral horn (arrowheads). (D) Distribution of E11.5-birthdated BrdU-positive nuclei in the spinal cord. (E,G–I) Double-labeled cells for GFP and BrdU were highly concentrated in the spinal dorsal horn after injection of BrdU at E11.5. Double-labeled cells are indicated by white arrowheads. The borders of the spinal cord laminae are depicted as dashed lines. In (A), “d” is dorsal, “v” ventral, and “cc” indicates the central canal. The same section orientation is maintained in the following figures. Scale bars = 100 μm in (B,D,E) and 10 μm in (B,I) (applies to G–I).

RESULTS

The co-localization of GFP and BrdU was examined within neurons of the lumbar spinal cord taken from P60 GAD67-GFP knock-in mice, which had been injected with BrdU at different embryonic stages. BrdU-positive cells were clearly classifiable as either GFP-positive or -negative populations. Double-labeled cells for GFP and BrdU represented GABAergic neurons that were undergoing DNA synthesis during the last cell cycle when BrdU was injected. These double-labeled cells were easily recognized by the large green cell body surrounding a BrdU-labeled red nucleus (Figure 1). Cells labeled with BrdU were sparsely distributed in the gray matter of the spinal cord after injections of BrdU at E10.5 (Figures 1A,B). The number of double-labeled cells was exiguous, suggesting that GABAergic neurogenesis was certainly minor at E10.5.

After injections of BrdU at E11.5, BrdU-labeled cells were spread widely throughout the spinal cord. Most of the BrdU-labeled cells were found in the superficial dorsal horn, the medial part of the deep dorsal horn, and in the area around the central canal (Figures 1D,F), which was a pattern of GABAergic neuronal distribution similar to the one found in the adult GAD67-GFP knock-in mouse (Figure 1E). Intense double-immunoreactivity was observed in the superficial layer...
of the spinal cord and in the area around the central canal (Figures 1E–I).

BrdU+ cells were distributed prominently in the superficial dorsal horn after injections of BrdU at E12.5 (Figures 2A,B). More GFP+/BrdU+ cells were distributed in the deep dorsal horn, and compared with the small number of double-labeled cells remaining in the superficial layer of the spinal cord (Figures 2C,D). These results indicated that the GABAergic neurons generated at E12.5 were mainly present in the deep dorsal horn. After injections of BrdU at E13.5, BrdU+ cells were scattered throughout most of the spinal cord, but tended to be more common in the dorsal horn and the area around the central canal (Figures 2E,F). Double-labeled cells were found in the dorsal horn as well as in the area around the central canal (Figures 2E,G), suggesting that part of GABAergic neurons of the superficial spinal cord were generated at E13.5.

After injection of BrdU at E14.5, only few BrdU+ cells were detected within the lumbar spinal cord (Figure 3). This result indicated that most neurons were born at or before E14.5 and that a very small fraction of GABAergic neurons was generated after E14.5. Cells labeled after E16.5, in addition to being few in number (Figure 3), were also scattered throughout the gray matter of the spinal cord (Figure 2H).

This birthdate study revealed that the overall production of GABAergic neurons was mainly limited during early neurogenesis (E10.5–E14.5), especially during E11.5 and E13.5 (Figure 3B). A few GABAergic neurons were labeled with BrdU as early as E10.5 (Figure 3B). The number of GFP+/BrdU+ cells following BrdU injection at E11.5 was remarkably increased compared to that at E10.5, accounting for nearly 37.1 (32.18–41.98)% of the overall GFP-positive neurons. Neurogenesis reached peaks at E11.5 and E13.5, and then rapidly tapered off at E14.5, with very little neurogenesis occurring after E16.5 (Figure 3).

Since GFP fluorescence was highly concentrated in the superficial dorsal horn of the GAD67-GFP knock-in mouse, we focused on the neurogenesis of GABAergic neurons in laminae I–III of the spinal cord. Figure 4 shows the GFP and BrdU+ double-labeled cells in spinal cords of mice injected with BrdU at different embryonic stages. The peaks of GABAergic neurogenesis in lamina II were at E11.5 and E13.5 (Figures 4B,D,H,I), while in lamina III, the majority of the GABAergic neurons were produced at E12.5 (Figures 4C,G,L), and in lamina I, the peak was at E13.5 (Figures 4D,J,L). These results indicated that laminar distribution of the GABAergic neurons may be related to the neurogenesis stage.

**DISCUSSION**

In the present study, the generation timetable of GABAergic neurons during certain developmental periods in the lumbar spinal cord was investigated by using the GAD67-GFP knock-in mouse. Our results clearly demonstrated that birthdates of GABAergic neurons vary and follow a specific temporal sequence.
Previous studies showed that neurons in the mouse spinal cord are largely born between E10 to E14 (Nornes and Carry, 1978; Caspary and Anderson, 2003; Helms and Johnson, 2003). The neurons of the dorsal horn originated from E12 to E14, while the ones in the intermediate gray region originated from E11 to E14, as well as the motor neurons in the ventral horn originated on E10 and E11 (Nornes and Carry, 1978). Consistent with previous studies, the present study demonstrated that the overall neurogenesis in the lumbar spinal cord was mainly limited to the interval between E10.5 and E14.5, and the labeling pattern of BrdU varied according to the developmental stages. Although these analyses provided an overview of the time and sites of origin, the detailed generation timetable of the GABAergic neurons in the spinal cord is still unknown.

Our birthdates study showed that the vast majority of GABAergic neurons were generated before E14.5 and the birthdates of GABAergic neurons in each lamina were different. In mice, neurons in the dorsal horn are generated in two phases: an early phase (E10–E11.5) that generates the relay neurons and sensory interneurons of the deep dorsal horn, while the late-born neurons (dILA and dILB) migrate along a dorsolateral direction and form the superficial dorsal horn (Altman and Bayer, 1984; Gross et al., 2003; Muller et al., 2002; Caspary and Anderson, 2003; Helms and Johnson, 2003). In previous studies, two early-born cell types, dI4 and dI5 neurons, generate sensory interneurons that predominantly settle in the deep dorsal horn, while the late-born neurons (dILA and dILB) migrate along a dorsolateral direction and form the superficial dorsal horn (Altman and Bayer, 1984; Gross et al., 2003; Muller et al., 2002; Caspary and Anderson, 2003; Helms and Johnson, 2003). dI4 and dI5 neurons differentiate as GABAergic neurons that express the HD transcription factors, while the cells born on E15.5 exhibited only one peak distribution in layer II/III (Yozu et al., 2004). This phenomenon may be attributed to the different birthdates and distribution of the subpopulation of GABAergic neurons in the spinal cord, such as, the subtypes of Ca²⁺ binding proteins and neuropeptides (Tamamaki et al., 2001; Heinske et al., 2004).

In summary, the present study demonstrates that the overall neurogenesis of GABAergic neurons is mainly limited to E10.5–E14.5 in the mouse lumbar spinal cord. The laminar distribution of the GABAergic neurons was related to the developmental stage. A previous study showed that the mouse embryonic...
GABAergic population in the cervical and lumbar spinal cord matures with similar pattern following a rostro-caudal gradient (Allain et al., 2004). Although additional studies are still required, we propose that the birthdates of GABAergic neurons in other spinal cord segments have a developmental pattern similar to that of the lumbar spinal cord.

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