

## NMDA receptor binding declines differentially in three spinal motor nuclei during postnatal development

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### Abstract

The NMDA subtype of glutamate receptors mediates a variety of neuronal processes involved in the development of dendritic morphology. For example, NMDA receptor antagonism during the early postnatal period attenuates dendritic growth in spinal motoneurons. NMDA receptors are present in high levels in the spinal cord early in the postnatal period and decline during development, a period of extensive dendritic plasticity in the spinal cord. Previous studies have suggested that an adult pattern of distribution of NMDA receptors is established as early as postnatal day (P)21 (day of birth = P1). However, given that dendritic growth in spinal motoneurons is not complete by this age and that NMDA receptor activation is necessary for dendritic growth, we assessed NMDA receptor binding in specific spinal motor nuclei during normal development. NMDA receptors were labeled with [<sup>3</sup>H]MK-801 at P7, P14, P28, P49, and in adult male rats. Receptor binding in the spinal nucleus of the bulbocavernosus (SNB), dorsolateral nucleus (DLN) and retrodorsolateral nucleus (RDLN) was measured using *in vitro* quantitative autoradiography. NMDA receptor binding over the SNB, DLN and RDLN in intact males was initially high, and declined to adult levels. However, the time course of the decline differed across nuclei. The local decline in NMDA receptor binding observed in the SNB and DLN is coincident with the periods of dendritic growth in these nuclei, further supporting a role for NMDA receptors in the development of motoneuron dendritic morphology.

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The role of NMDA receptors in mediating neuronal plasticity and the influence of glutamatergic signaling, including NMDA receptor activation, on dendritic morphology has been well established. Activation of glutamate's ionotropic and metabotropic receptors can lead to calcium influx [24], and changes in intracellular calcium have been linked to changes in dendritic development in a variety of neuronal populations. Activation of different calcium-dependent pathways at different times results in dendritic outgrowth, branching, or regression in a variety of neuronal types. For example, activation of NMDA receptors in granule cells triggers a signaling pathway for dendritic differentiation in Purkinje cells, and blockade of NMDA receptors results in decreased dendritic length and branching in Purkinje cells [15]. In reti-

nal ganglion cells, blockade of NMDA and non-NMDA glutamatergic signaling decreases dendritic length and branch number [31].

NMDA receptor expression (as measured by radioligand binding) in the spinal cord is extremely high in the early postnatal period, and declines to adult levels by P21, when the largest expression is restricted to the substantia gelatinosa [21]. This decline suggests that NMDA receptors are down regulated to adult levels after the early postnatal period, a period marked by extensive dendritic plasticity in spinal motoneurons. During this period, blockade of NMDA-mediated glutamatergic activity via MK-801 inhibits dendritic growth in motoneurons innervating quadriceps muscles in rats [20] and L2–L5 spinal motoneurons in mice [16]. Previous work from our laboratory has also demonstrated that postnatal administration of MK-801 to rats inhibits dendritic growth in motoneu-

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rons of the spinal nucleus of the bulbocavernosus (SNB; [14]).

The SNB is one of several sexually dimorphic nuclei in the lumbar spinal cord of the rat. In male rats, motoneurons in the SNB (also known as the *dorsomedial nucleus* or DM [28]) innervate the perineal muscles bulbocavernosus (BC) and levator ani (LA) as well as the anal sphincter. Motoneurons in the neighboring DLN (or DL in [28,18]) innervate the ischiocavernosus (IC) and the non-dimorphic urethral sphincter [1,28,25]. The BC/LA and IC muscles attach to the base of the penis and are essential for successful copulation and insemination [27,10]. Dendritic development in these two sexually dimorphic nuclei occurs postnatally, but with different patterns and over different periods of time. SNB dendritic development is biphasic, exhibiting an exuberant growth through the first four postnatal weeks, followed by a retraction to mature lengths by 7 weeks of age [7]. On the other hand, DLN dendritic development is monotonic; dendritic growth occurs over the first 7 weeks of age, by which time adult lengths are achieved [8].

Given the demonstrated role of NMDA receptors in dendritic development [21,16,14], these differences in dendritic growth between the SNB and DLN are difficult to reconcile with the reported decline in NMDA receptor binding to adult levels and distribution by P21. In this study, we assessed the developmental expression of NMDA receptors in the spinal cord using a regionally specific quantitative approach. We used *in vitro* quantitative autoradiography (QAR) to examine the developmental time course of NMDA receptor binding in three spinal motor nuclei, the SNB, DLN and RDLN (a non-dimorphic lumbar motor nucleus innervating the intrinsic foot muscle flexor digitorum brevis). The use of QAR allows us to simultaneously assess receptor binding in both pre- and postsynaptic sites, as well as in non-neuronal cell types, all of which contain glutamate receptors and have been implicated in the regulation of dendritic growth. Preliminary results have been published in abstract form [30].

All animals except for adults were obtained from litters of outbred female Sprague–Dawley rats (Harlan Laboratories, Indianapolis, IN). Litters were culled to eight pups when necessary, retaining males preferentially. Adult male Sprague–Dawley rats (approximately 120 days old) were obtained directly from Harlan Laboratories. The rats were maintained on a 14:10 h light/dark cycle, with food and water freely available. All procedures were in accordance with the Indiana University Animal Care and Use Guidelines.

At either P7, P14, P28, P49, or adulthood, male rats ( $n = 4–7$  per age; overall  $n = 28$ ) were decapitated and spinal cords removed via laminectomy, blocked to include L5, L6, and S1, flash frozen on dry ice, and stored at  $-70^{\circ}\text{C}$  until sectioning. Horizontal sections ( $16\ \mu\text{m}$ ) through the spinal cord were cut at  $-13^{\circ}\text{C}$  on a cryostat and thaw-mounted onto chrome-alum gelatin-coated slides. Sections were collected in three alternate series. One series was immediately fixed in paraformaldehyde vapors and then stained with cresylecht violet for use as a reference series to identify sections

containing the SNB, DLN, or RDLN. The remaining series (total and non-specific binding series) were desiccated and stored at  $-70^{\circ}\text{C}$  prior to autoradiography.

To assess changes in NMDA receptor binding, sections containing the SNB, DLN, or RDLN (as identified using the reference series) were labeled with [ $^3\text{H}$ ]MK-801 (dizocilpine maleate) using a procedure similar to that of [17]. Slides were incubated at room temperature for 90 min in a 60 mM EPPS buffer (pH 7.4) containing 10 nM [ $^3\text{H}$ ]MK-801 (specific activity = 21.7 Ci/mmol; NEN Life Sciences, Boston, MA) and glutamate, glycine, spermine, and spermidine ( $100\ \mu\text{M}$  each). Non-specific binding was assessed in anatomically adjacent sections by competing the tritiated ligand against  $200\ \mu\text{M}$  ketamine HCl. After incubation, all slides were rinsed twice in ice-cold EPPS buffer, rapidly fixed in an acetone/gluteraldehyde solution, and dried in a stream of warm air. Slides were fixed additionally under vacuum with paraformaldehyde vapors for two hours, air-dried overnight, placed in autoradiographic cassettes (Hypercassettes; Amersham, Cleveland, OH), opposed to tritium-sensitive film ( $^3\text{H}$  Hyperfilm; Amersham) along with standardized autoradiographic microscapes (Amersham), and stored at  $4^{\circ}\text{C}$  for 6 weeks. The films were developed (Kodak D-19), fixed (Kodak fixer), and air-dried. Slides were then stained with cresylecht violet for subsequent comparison with autoradiograms.

Density of [ $^3\text{H}$ ]MK-801 binding in the resulting autoradiograms was quantified using a computer based image analysis system (MCID; Imaging Research Inc., St. Catharines, Ontario). Slides containing the histological sections were placed on a light box (Northern Light; Imaging Research Inc.) and digitized. The corresponding autoradiograms were then placed on the light box, digitized, and aligned with the histological sections. DLN, SNB, and RDLN motor nuclei (from each animal) were defined on the histological sections and samples taken from the corresponding areas of the autoradiograms on each section in which they occurred (Fig. 1). Across all ages, an average of 4.5 sections were analyzed in the DLN, 3.2 sections in the SNB, and 6.1 sections were analyzed in the RDLN. All measures were standardized against the microscapes included on each film and expressed in fmol/mg wet tissue weight. Specific binding was calculated by subtracting nonspecific binding from total binding for each pair of sections. For all animals, averages of specific binding measures were calculated for each nucleus. Specific binding in the SNB, DLN, and RDLN was compared across ages using one-way ANOVAs with Fisher's least significant difference (LSD) post-hoc analyses.

One-way ANOVA demonstrated a significant effect of age on [ $^3\text{H}$ ]MK-801 binding in the SNB [ $F(4, 23) = 9.875$ ,  $p < .05$ ]. [ $^3\text{H}$ ]MK-801 binding declined 48.2% between P7 and P28 (LSD,  $p < .05$ ) and did not further decline through adulthood (Fig. 2); [ $^3\text{H}$ ]MK-801 binding in the SNB in P7 rats did not differ from P14 rats (LSD, n.s.) nor did [ $^3\text{H}$ ]MK-801 binding in P49 and adult rats differ from P28 rats (LSD, n.s.). One-way ANOVA also demonstrated a significant effect of age on [ $^3\text{H}$ ]MK-801 binding in the DLN [ $F(4, 22) = 11.287$ ,

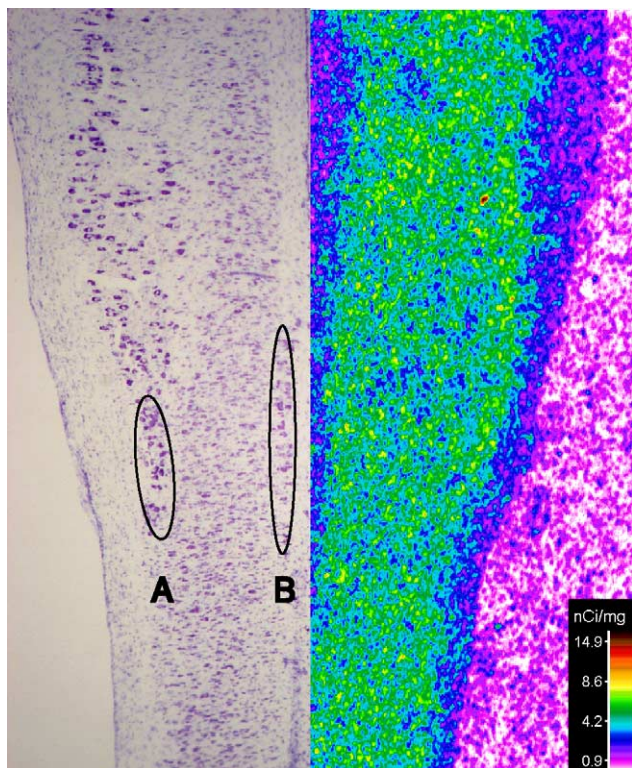


Fig. 1. Horizontal section through lumbar spinal cord showing Nissl staining (left side) and pseudo color image (right side) generated from the autoradiogram of the corresponding Nissl stained section. Sampling area over the DLN (A), SNB (B), and RDLN (not shown) was determined on the Nissl stained section and [ $^3\text{H}$ ]MK-801 binding densities were calculated from serially aligned autoradiograms. Scale bar indicates binding densities in nCi/mg.

$p < .05$ ]. [ $^3\text{H}$ ]MK-801 binding in the DLN declined 54.6% overall between P7 and P49 (LSD,  $p < .05$ ; Fig. 2), however, there were also significant declines from P7 to P14 (20.6%; LSD,  $p < .05$ ) and from P7 to P28 (33.7%; LSD,  $p < .05$ ). After P49 there was no further decline in [ $^3\text{H}$ ]MK-801 binding levels; [ $^3\text{H}$ ]MK-801 binding in the DLN in adult rats did not differ from P49 rats (LSD, n.s.). One-way ANOVA demonstrated a significant effect of age on [ $^3\text{H}$ ]MK-801 binding in the RDLN [ $F(4, 20) = 33.043$ ,  $p < .05$ ]. [ $^3\text{H}$ ]MK-801 binding declined 54.4% between P7 and P28 (LSD,  $p < .05$ ) and did not further decline through adulthood (Fig. 2); [ $^3\text{H}$ ]MK-801 binding in the RDLN in P7 rats did not differ from P14 rats (LSD, n.s.) nor did [ $^3\text{H}$ ]MK-801 binding in P49 and adult rats differ from P28 rats (LSD, n.s.).

Previous QAR analysis of NMDA receptor binding in the spinal cord has only examined broad changes throughout the entire gray matter of the spinal cord. Based on this level of quantitative analysis, declines in NMDA receptor binding were only detected through P21, when it was reported that an adult level and pattern of NMDA receptor binding was achieved [21]. Our results agree with this general pattern of decline, but further show that NMDA receptor binding levels, based on a quantitative regional analysis, decline differentially in the three motor nuclei we examined. NMDA

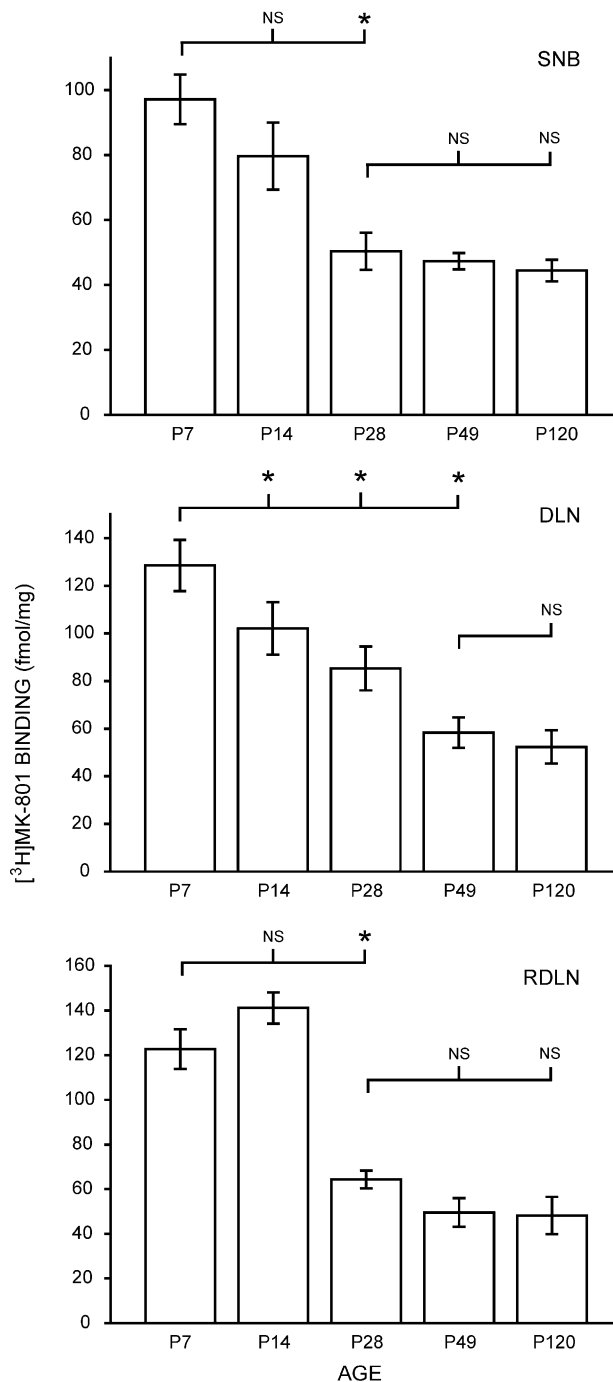


Fig. 2. [ $^3\text{H}$ ]MK-801 binding in normal males over the area of the SNB (top), DLN (middle), and RDLN (bottom). [ $^3\text{H}$ ]MK-801 densities decreased over the SNB and RDLN to adult levels by P28, while binding densities in the DLN decreased through P49 at which point an adult level of binding was achieved. Bar heights represent mean  $\pm$  S.E.M. for four to seven animals per group. NS,  $p > .05$ ; \* $p < .05$ .

receptor binding in the SNB declined after P7, and by P28 was reduced by 48.2%; no further decline in receptor binding was observed after P28. In the DLN, NMDA receptor binding declined after P7, but unlike the SNB had declined 33.7% by P28, with a further decline of 31.6% by P49. After P49 no

further decline in NMDA receptor binding was observed in the DLN. NMDA receptor binding in the RDLN remained high from P7 to P14, and declined 54.4% between P14 and P28. P28 NMDA receptor binding levels in the RDLN were similar to those seen at P49 and in adult animals.

As previously mentioned, SNB dendritic growth is biphasic: SNB dendrites grow exuberantly from P7 through P28, followed by a subsequent retraction to adult lengths by P49 [7]. It is during the initial growth phase (P7–P28) that we observed an elevated level of NMDA receptor binding in the SNB, which declined to adult levels by P28, the age at which dendritic growth is complete. In the DLN, dendritic growth is monotonic: DLN dendrites grow from P7 to P49, at which point an adult pattern of dendritic morphology is established [8]. Similar to the SNB, in the DLN there were high levels of NMDA receptor binding during the dendritic growth phase that declined to adult levels by P49, the same point at which DLN dendritic growth is complete. Thus, the differential pattern of decline in NMDA receptor binding observed in the SNB and DLN coincides with the period of dendritic growth across each of these nuclei, and the time point at which an adult level and pattern of NMDA receptor binding is established in each nucleus corresponds to the cessation of dendritic growth.

The pattern of dendritic development in the RDLN has not been examined to date. However, we did observe a similar decline in NMDA receptor binding from high levels at P7 and P14 to a lower, adult level by P28. We would hypothesize, based on the dendritic growth profiles in the SNB and DLN and the regulation of dendritic morphology via NMDA activation in other spinal motoneurons, that the period of dendritic growth in the RDLN should occur between P7 and P28.

While the QAR method employed here revealed temporally and regionally distinct changes in NMDA receptor binding, an important next step will be to identify in which particular cell types this down-regulation occurs, as well as the mechanism(s) that regulate it. For the two sexually dimorphic motor nuclei, a likely candidate for regulating NMDA receptor expression is gonadal hormones.

During the perinatal period, gonadal hormones influence a variety of processes in the SNB and DLN, including normally occurring motoneuron death [26], somal growth [2,3,7,8], and dendritic development [7,8]. In male rats castrated on P7, SNB and DLN dendrites never grow beyond precastration lengths, whereas dendritic lengths of castrates receiving testosterone (T) replacement are equivalent to those of intact males by 4 weeks of age in the SNB and by 7 weeks of age in the DLN [7,8]). Administration of estradiol (E) to castrated males partially supports SNB dendritic growth through the first four postnatal weeks [9] and fully supports DLN dendritic growth over the same time period [11]. Furthermore, blocking E synthesis during this time period results in a significant attenuation of SNB dendritic growth [4]. The morphology of SNB motoneurons is insensitive to estrogens after 4 weeks of age [9,12,13,5,29]).

Interestingly, the early postnatal influence of gonadal hormones on dendritic morphology in the SNB and DLN is coincident with the period in which high levels of NMDA receptors are expressed in the spinal cord. Furthermore, evidence exists for the interaction of gonadal hormones and NMDA receptor expression. Previous studies have demonstrated that gonadal hormones modulate NMDA receptor binding [22], as well as NMDA receptor subunit mRNA and protein expression [6] in hippocampus. In addition, gonadal hormones have been shown to modulate NMDA receptor binding and mRNA expression in lateral septum [23]. In adult males, T regulates NMDAR1 mRNA expression in SNB motoneurons; castration reduces NMDAR1 mRNA expression and T replacement restores expression to that of normal animals [19]. In addition, NMDA receptor activation is necessary for estrogenic support of SNB dendritic growth [14] and androgenic regulation of SNB soma size [19]. Given that gonadal hormone and NMDA receptor-mediated effects on dendritic growth in the spinal cord temporally coincide and that gonadal hormones have been shown to modulate NMDA receptor expression, we would predict that gonadal hormones should regulate NMDA receptor expression in the developing spinal cord.

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