Elimination of muscle afferent boutons from the cuneate nucleus of the rat medulla during development

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Section Editor: Weimin Zhong
**Abbreviations**

P: Postnatal day  
EDC: Extensor digitorum communis  
CTB: Cholera toxin B sub unit  
μl: microlitre  
PBS: Phosphate buffered saline  
PBST: Phosphate buffered saline with Triton X-100  
DRG: Dorsal root ganglion  
μm: micrometre  
UK: the United Kingdom of Great Britain and Northern Ireland
Abstract

There is developmental refinement of the proprioreceptive muscle afferent input to the rat ventral horn. This study explored the extent to which this occurs in the medulla. Muscle afferents were transganglionically labelled from the extensor digitorum communis forelimb muscle with cholera toxin B sub unit. Tracer amounts and transport times were adjusted for animal size. Immunohistochemistry revealed tracer localisation in the medulla and dorsal root ganglia. Labelled muscle afferent boutons were counted in the cuneate nucleus between postnatal days 7 and 42, during which time a large decrease in the density of labelled boutons was observed qualitatively. Localisation of input to dorsolateral parts of the nucleus remained broadly the same at different ages, although disappearance of a marked innervation of ventromedial regions in more caudal sections was observed. Bouton counts were corrected for growth of the medulla with age, and any spread of tracer to adjacent muscles indicated by counts of labelled dorsal root ganglion neurons. There was a statistically significant, approximately 40% reduction in the number of muscle afferent boutons in the cuneate nucleus during this developmental period. Previous studies suggest that perturbations to the corticospinal input during a developmental critical period influence the eventual size of the muscle afferent input to the ventral horn. Corticocuneate fibres invade the nucleus during the same period and may influence reorganisation of its muscle afferent input, making it another potential site for aberrant reflex development in cerebral palsy.

Keywords: Cerebral palsy, cholera toxin B, proprioreception, sensorimotor pathways, Synaptic plasticity, transganglionic tracing.
Crucial to the understanding of the sensorimotor deficits observed in spastic cerebral palsy is the observation that perinatal lesions of the corticospinal tract can lead to abnormal development of spinal reflexes, evolving over months and years. This includes retention and reinforcement of developmental features, such as low threshold responses and excitatory connections to antagonist muscle groups, that do not emerge in adult stroke victims, even though they also suffer from spasticity (Myklebust et al., 1987; Leonard et al., 1991; O’Sullivan et al., 1998). Muscle afferents invade the ventral horn very early in development; by 8.5-9 post-conceptional weeks in human (Konstantinidou et al., 1995; Clowry et al., 2005) and by E17-19 in rats (Kudo and Yamada, 1985; Kudo and Yamada, 1987; Ziskind-Conhaim, 1990). However, morphological evidence for a reduction in the muscle afferent input to the ventral horn later in development has been provided in the rat both by transganglionic tracing with cholera toxin B (Gibson and Clowry, 1999; Clowry 2007) and immunohistochemical detection of parvalbumin positive proprioceptive afferents (Dekkers et al., 2002). There is a considerable reduction in the number of muscle afferents in general in the ventral horn and in particular those contacting the somata and proximal dendrites of homonymous motoneurons between P7 and maturity.

This refinement of segmental circuitry coincides with the development of corticospinal innervation (reviewed by Clowry, 2007) and we have hypothesised that activity dependent development of segmental circuitry is dependent upon corticospinal input. Perturbations to the corticospinal input during development, but not in mature animals, lead to permanent changes in the density of muscle afferent innervation of the ventral horn and activity dependent expression of the proteins parvalbumin and c-Jun (Gibson et al., 2000; Clowry et al., 2004). However, in addition to segmental reflexes, long loop reflexes via the cortex are also elicited in response to stimulation of muscle afferents (Baker et al., 2006). In the rodent, innervation of the dorsal column nuclei by sensory afferents begins before birth (Chimelli and Scaravilli, 1987; Wessels et al, 1991) whereas the cortical innervation of the dorsal column nuclei begins a few days postnatally and extends into the second postnatal week (Reh and Kalil, 1981; Chimelli et al, 1994). In the present study, we have investigated, in the rat, whether there is also a refinement of the muscle afferent projection to the cuneate nucleus involving the elimination of putative synaptic boutons at a later stage of development, as a first step towards
investigating whether formation of aberrant long latency reflexes is a component of the motor disorder observed in cerebral palsy.

**Experimental Procedures**

Fifteen Wistar rats (Charles River, UK) were used and the day of birth was called P0. The ages at the time of perfusion were postnatal day 7 (P7, 5 animals), P14 (3) P21 (2) and P42 (5). Under halothane anaesthesia and in aseptic conditions the extensor digitorum communis muscle (EDC) was exposed in the right forelimb and injected with 0.5% cholera toxin B sub unit (CTB, Sigma-Aldrich, Poole, UK) in saline using a Hamilton syringe, taking care to prevent leakage into surrounding tissues. A volume of 0.5μl was injected at P5 and this increased proportionally with age and size of the animals up to 4μl at P39. The overlying skin was sutured and carbofen administered for pain relief before the removal of anaesthesia. To allow for axonal transport of CTB the animals survived for 2 days (P7, P14) or 3 days (P21, P42) prior to transcardial perfusion with saline followed by buffered 4% paraformaldehyde solution (1ml/g body weight) under general anaesthesia induced by a lethal i.p. injection of sodium pentobarbitone. The hindbrain and right C7 dorsal root ganglion was removed, postfixed in the same solution for 2 hours and placed in 30% sucrose in 0.1M phosphate buffered saline (PBS) overnight. The length of the medulla was measured. All surgical procedures were approved by the Newcastle University Ethical Review Committee and the UK Government Home Office.

Frozen sections (50 μm) of hindbrain were cut with a freezing microtome and placed into PBS. Cryostat sections (20 μm) of C7 DRG were collected on gelatinised slides. The brain sections were incubated free floating, DRG sections on the slide in a humid chamber, in PBS with 0.1% Triton X-100 (PBST) with 3% normal serum and goat anti CTB primary antibody (Quadratech, Epsom, UK) diluted 1 in 10 000, for 24 hours at 4°C, on a slowly rotating mixer. Then, sections were incubated with the appropriate biotinylated secondary antibodies (Vector Laboratories, Peterborough, UK; 0.5% in PBST) for 2 hours at room temperature, then in streptavidin-HRP (Vector Labs, 0.5% in PBST) for 1 hour. Sections were finally reacted with 0.05% diaminobenzidine/ 0.003% hydrogen peroxide in PBS. Washing with PBS was carried out between incubations. Hindbrain sections were mounted onto
gelatinised slides. All slides were air-dried, dehydrated, cleared, mounted in Entellan (Merck, Loughborough, UK) and coverslipped.

One section from five different levels evenly spaced along the length of the medulla was analysed per animal (Fig. 1A-E). The cross sectional area of the cuneate nucleus at each level was measured from digital images using Scion image analysis software [http://www.scioncorp.com/pages/scion_image_windows.htm](http://www.scioncorp.com/pages/scion_image_windows.htm). No difference in area was found between P14, P21 and P42 animals, as is the case for cervical spinal cord grey matter (Gibson and Clowry, 1999) but at P7, the cross-sectional area of the nucleus was found to be 10% smaller. A counting grid was projected over nearly all the nucleus using a *camera lucida*, anchored to the dorsomedial borders and avoiding the external cuneate nucleus located dorsolateral to the cuneate nucleus at some levels (Fig. 1F). To account for the growth of the nucleus a rectangular grid of 30 squares of side 40 μm (when viewed through microscope) was used at the older ages and of side 37.5 μm at P7. Boutons were counted in alternate squares of the grid only, to prevent biased placement of the grid, viewed directly through the microscope whilst adjusting the depth of focus. Boutons were identified as densely stained spheroids often joined by thin, presumably axonal, processes (Fig. 3). Account was taken for the growth in length of the medulla, which was more marked than the growth in cross-sectional area, increasing by a factor of 1.4 from P7 to P42. The longitudinal length of the cuneate nucleus was divided by the thickness of a single section, to give an estimate of the total number of sections containing cuneate nucleus (n). The number of boutons counted in the five selected sections were scaled up, first by multiplying n/5, and then doubled to account for only counting in alternate squares, giving an estimate of the total number of muscle afferent boutons within a rectangular column containing the cuneate nucleus, covered by the counting grid.

Three sections from the middle 50% of the C7 DRG of each animal were analysed, avoiding the two poles of ganglion and adjacent sections. The C7 DRG was chosen as this could be expected to contain the largest number of sensory neurones projecting to both the EDC and adjacent muscles. All neurons, and all CTB positive neurons, in the section were counted and the proportion of CTB
positive cells was calculated. Labelled neurones displayed intense immunoreactivity throughout their cytoplasm, whilst unlabelled neurons picked up a light background staining that nevertheless made them identifiable as neurons by their large, round pale nucleus. Only cells that showed an unequivocal level of immunoreactivity above background staining were counted as CTB positive and care was taken to apply consistent counting criteria across experimental animals. Using proportions of cells is appropriate as there is no evidence for any decrease in the number of dorsal root ganglion neurons between postnatal day 7 and maturity (Popken and Farel, 1987). The total number of boutons estimated for each animal was multiplied by the proportion of CTB labelled cells in that animal/mean proportion of CTB labelled cells for all animals of all ages.

Images were captured with a Zeiss Axiocam digital camera and figures prepared using Adobe Photoshop software, which was primarily used to adjust brightness, contrast and sharpness, and only occasionally to remove dirt or other artefacts.

Results

CTB injected into the right EDC muscle retrogradely labelled neurons in the dorsal root ganglion (Fig. 2) and transganglionically labelled axonal projections to the cuneate nucleus (Figs. 1 and 3), including axonal boutons with the appearance of synaptic terminals (Figs. 3B,D,F,H.). Varicose axonal labelling was also seen in the external cuneate nucleus in more rostral sections, which is distinct from the cuneate nucleus. Any differences in retrograde labelling between dorsal root ganglia at P7 and older ages were not obvious (Fig. 2) although at older ages the ganglia had a larger cross sectional area and lower density of neurons, due to the presence of larger, myelinated axon bundles. The proportion of labelled cells was slightly larger at P7 (mean 11.2% ± 1.2%; 95% confidence limits) compared to P42 (9.6% ±0.8%) but the difference was not statistically significant (Student’s t-test, 2 tailed, corrected for difference in variance between samples).
The appearance of axonal labelling in the cuneate nucleus changed with age. At P7 in particular, labelling was dense and it was hard to discern individual boutons at lower magnifications (Fig. 3A). By P42, labelling was much sparser and in most cases both individual varicose axons and clouds of boutons are easily discernible (Fig 3). The regions of the cuneate nucleus in which axonal boutons were found to be broadly the same at all ages, occupying the dorsal and lateral borders of the nucleus (Figs. 1 and 3) as has been previously described for labelling from the entire radial nerve (Beck, 1981). The centre of the nucleus was relatively free of labelled axons. However, there was, in more caudal sections, a denser innervation of ventromedial areas at P7 and P14 than seen at older ages (Fig. 3A).

Bouton counts, adjusted for both the growth of the medulla and changes and variations in transganglionic labelling efficiency, showed a statistically significant negative correlation by linear regression with age ($r = -0.616, P<0.02$, Fig 4A). There were significantly more labelled boutons present at P7 compared with P42 (Fig. 4B).

**Discussion**

As was previously shown in the spinal cord ventral horn (Clowry and Gibson, 1999) we provide anatomical evidence that a larger number of presumptive synaptic boutons are formed in the cuneate nucleus by muscle afferent axons during development than are observed in mature animals. That there is a change of density of boutons with age is obvious (Fig. 3) but it has to be proved that this involves elimination of boutons, rather than simply a reduction in density of boutons caused by growth of medulla. There are a number of methodological considerations to be made when conducting this type of study. Firstly there may have been observer bias in counting, as it was not feasible to blind the observer to the age of the specimen being examined as staining patterns were so different between ages. It might be preferable to use immunofluorescent staining for CTB, perhaps combining double staining for a synaptic marker, collect images by confocal microscopy at fixed levels through the section and use automated counting, however, our preliminary experiments have not produced immunofluorescent staining of CTB as sensitive as our immunoperoxidase staining. In the meantime
we have relied upon the likelihood that such a large difference in bouton number is unlikely to be explained by unconscious bias.

Secondly, larger numbers of proprioreceptive sensory neurons could be labelled in young animals, due to spread of tracer to adjacent muscles. This is because muscle compartments are smaller and connective tissue fascia less well developed in neonatal animals, as explored by Lowry et al (2001) who concluded that retrograde labelling studies that suggested motoneuron numbers in the spinal cord decrease postnatally were flawed for this reason. In this study, as in our previous study in the ventral horn (Gibson and Clowry, 1999) we have estimated the number of boutons in a defined region, taking into account the growth in grey matter volume with age. In addition, we have corrected our counts for any spread of tracer by taking into account the number of dorsal root ganglion neurons labelled in the C7 ganglion. Although the posterior extensor compartment of the forearm will receive innervation from several DRG, the C7 ganglion should make a major contribution (Gibson & Clowry, 1999) and the degree of spread should not differ greatly between ganglia as all these muscles are radial nerve innervated and will presumably have their sensory innervations arise from the same dorsal root ganglia. The proportion of DRG neurons labelled remained similar across ages; a reduction of about 15% between P7 and P42 was found but this was not statistically significant. We have assumed that the number of DRG neurons does not decrease between P7 and P42 making use of proportions of cells labelled valid; indeed, best evidence suggests that DRG neuron numbers may increase slightly (Popken and Farel, 1997) so our correction of counts may have overestimated the number of boutons present at P42. Therefore, by allowing for a possible, small overlabelling of DRG neurons in younger animals in the present study, it can be safely concluded that there is at least a 40% reduction overall in muscle afferent boutons from the EDC muscle during postnatal development in the cuneate nucleus. Furthermore, our original study which showed a fourfold decrease in EDC muscle afferent boutons in the ventral horn during development (Gibson and Clowry, 1999) is further validated as such a change is clearly not accounted for by any changes in the extent of transganglionic labelling.
Developmental elimination and remodelling of synapses is a phenomenon that has been observed in a number of diverse projections, for example, from retinal ganglion cells to the dorsal geniculate nucleus (Hooks and Chen, 2006) loss of climbing fibre innervation of Purkinje cells (Kakizawa et al, 2000) elimination of polyneuronal innervation of muscle fibres (Sanes and Lichtman, 1999) and many others. It is presumed to play a role in the final determination of precision and weighting of connections between neurons by activity dependent mechanisms (Zhang and Poo 2001).

Nevertheless, synapse elimination in development may not be universal and experiments in frog, chick and mouse suggest there is precise connectivity between muscle afferents and motoneurons and spinal interneurons from the onset of reflex activity (Frank and Westerfield, 1983; Lee et al, 1988; Mears and Frank, 1997; Wang et al, 2008). However, experiments in rats and humans have contradicted these observations suggesting initial inappropriate heteronymous connections are lost during development (O'Sullivan et al, 1991; Seebach and Ziskind-Conhaim, 1994; McDonough et al, 2001).

In the cuneate nucleus, muscle afferent labelling in the adult animals exhibited somatotopy with the majority of terminals targeting the dorsolateral margins of the nucleus, in agreement with a previous study that employed transganglionic labelling from the radial nerve in rat (Beck, 1981) and avoiding the centre of the nucleus, which, in the middle of the nucleus in a rostro-caudal plane, is targeted by cutaneous afferents from the forelimb digits (Maslany et al, 1991; Crockett et al, 1996). This pattern of innervation was qualitatively the same at younger ages in rostral parts of the nucleus suggesting that synaptic refinement in this case may not necessarily involve elimination of inappropriate heteronymous connections. This is similar to the pattern of development of muscle afferent projections to the dorsal horn of the spinal cord of both rat (Gibson and Clowry, 1999) and cat (Martin and Chakrabarty 2008). A quantitative reduction in homonymous connections in the dorsal horn has been reported in cat (Martin and Chakrabarty, 2008) and in rat the density of dorsal horn innervation at younger ages did appear greater (Gibson and Clowry 1999). However, in more caudal parts of the nucleus, a prominent labelling was observed at P7-P14 in ventromedial parts of the nucleus that disappeared at older ages. In this region, elimination of heteronymous connections seems likely.
In the ventral horn, the reduction in the number of muscle afferents observed included disproportionate elimination of boutons contacting the somata and proximal dendrites of homonymous motoneurons between P7 and maturity (Clowry, 2007) perhaps contributing to the loss of relative strength and increase in threshold of the stretch reflex during development (Kudo and Yamada, 1987). In the medulla, the observed elimination of muscle afferent boutons may reflect a shift in strength and weighting of muscle afferent input to both cuneothalamic neurons and associated interneurons in the nucleus. The role of longer latency reflexes via cuneate, thalamus and cortex is unclear but may involve passing central oscillations around a peripheral loop which could be used to recalibrate the motor system after a movement (Baker, 2007). The cuneate nucleus in the medulla contains the first synapse for all muscle afferent information arising from the upper limb on its way to the cortex and plays an active role in information processing. Local interneurons inhibit cuneothalamic transmission (Aguilar et al, 2002) and descending corticocuneate projections can facilitate or inhibit cuneothalamic neurons selectively (Lue et al, 1997; Palmeri et al, 1999; Nuñez and Buño, 2001; Aguilar et al, 2003).

Muscle afferent projections to the cuneate nucleus undergo a period of remodelling late in development. Therefore, as proposed in the spinal cord (Clowry, 2007) there is scope for both cortical and afferent projections to interact in the maturation of circuitry in the cuneate nucleus and for perinatal lesions of the cortical projection to lead to aberrant development of long latency reflexes. Further studies of development and plasticity in the dorsal column nuclei may be crucial to our understanding of spastic cerebral palsy.

Acknowledgements

We are grateful to the staff of Newcastle University Comparative Biology Centre for their care of the experimental animals, and to Ms Christina Trainor for her help with some of the histological procedures.
References


Figure 1.

This shows representative sections from the five different levels of the medulla from a P14 animal used for bouton counts evenly spaced caudally to rostrally (A to E). In each section labelling is present in the cuneate nucleus (arrow, CN, surrounded by a dashed line) mostly around the dorsal and lateral margins, and also in the external cuneate nucleus in more rostral sections (arrow ECN) with labelling also present in the cuneate fasciculus in between the two structures. F shows a higher power image showing the placement of the counting grid. The grid was always aligned with dorsal and medial borders of the nucleus, even if this left very small portions of the ventral and lateral regions uncovered. V marks the fourth ventricle.

Scale bars A-E = 500 μm, F = 200 μm
Figure 2

This illustrates retrograde labelling of primary sensory neurons in sections from the dorsal root ganglion at C7 at P42 (A) and P7 (B). Both larger labelled neurons (LLN) and smaller labelled neurons (SLN) were observed at both ages amongst many more, large unlabelled (ULN) and smaller unlabelled (SUN) neurons. It can be seen that the proportion of labelled cells is approximately the same at both ages. The dorsal root ganglion is larger in cross sectional area at P42 because bundles of axons (F) are larger at this age.

Scale bar = 2mm

Figure 3

This figure illustrates the differences in immunoreactive labelling of CTB at low and high magnification between P7 (A,B,E,F) and P42 (C,D,G,H) at caudal (A,B,C,D) and rostral (E,F,G,H) levels of the medulla. The cross-sectional centre of the cuneate nucleus is marked in all cases with an asterisk. The external cuneate is marked ECN and the cuneate fasciculus (CF). In the cuneate nucleus, in all cases axonal labelling is concentrated around the dorsal (small D) and lateral (L) margins, but overall it appears much denser at P7. In addition, fairly dense labelling can also be observed in the ventromedial margins of the nucleus at P7 (A, arrow). At higher magnifications varicose axonal processes are clearly seen (e.g. arrow in D) as well as clouds of presumptive synaptic boutons.

Scale bar = 200 μm in A,C, E, G, and 80 μm in B, D, F, H.

(see below)
Figure 4 

This summarises the quantification of muscle afferent innervation at different developmental stages. In both cases number of boutons refers to an estimate of the number of CTB labelled boutons from the EDC within a rectangular column through the medulla containing nearly all the cuneate nucleus, with counts adjusted for differences in size of the medulla at different ages and possible leakage of tracer to nearby muscles or skin. 

A shows a steady and significant decline in the number of boutons with age and B directly compares the number of boutons found at each end of the age range. The asterisk indicates a statistically significant difference (P< 0.04, Student’s t-test corrected for differences in variance between samples).