Short communication

Dorsal myelotomy in E15–E16 fetal rat: A promising paradigm in regeneration research, with serendipitous transcriptomic effects on development, of the primary afferent system

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ABSTRACT

Background: The applied multidisciplinary approach in neuroscience has tackled the regeneration enigma in the central nervous system for decades. Reported regeneration potential in mammals is lacking confirmation without a suitable paradigm. In vivo fetal experimentation offers an almost insurmountable drawback, though its feasibility has been shown long ago. New method: Following two former technical papers on fetal surgery, here we present dorsal myelotomy in fetal rat and antegrade HRP-tracing as a suitable paradigm to reveal the intrinsic regenerative program in DRG neurons. Moreover, disclosing the spatio-temporal development of the primary afferent system appeared as an unexpected feature of the design. Conclusion: this paper underlines the feasibility that fetal experimentation may offer a new venue for research into the rat transcriptome.

1. Introduction

Regeneration research into the central nervous system was focused on fetal spinal cord transection almost a century ago. A potential beneficial effect, if any, on recovery of lesioned central axons might be attributable to the immaturity of the spinal cord tissue. In a comprehensive study, Hooker demonstrated the feasibility of fetal experimentation in the rat (Hooker and Nicholas, 1930). No sign of a regenerating capability of central axons was detected. In subsequent decades, scarcely published technical reports have saved this very subject from oblivion (DeMyer and Baird, 1973; Albright, 1987; De Beer et al., 1988, 1992). A supposedly hidden regenerative potentiality in the central nervous system in rats had never been deciphered.

Innate regenerative capacity has a phylogenetic relationship ranked in invertebrates up to higher mammals with opossum and mouse in the upper rank (Tran et al., 2021). Marsupials even express healing capacity after total transection of the immature spinal cord (Saunders et al., 1998). Extensive research into organisms of lower species and their development has attributed tremendously to the insight of molecular processes and biological mechanisms in neuronal networks of the mammalian central nervous system (Bradke and Marin, 2014). Scientific understanding has vastly expanded with the usage of tools like gene targeting in mice, as well. Many aspects of transcriptional mechanisms have been revealed that underline their spatial-temporal intricacy in the transcriptome at early development (Qian and Zhou, 2020). Nonetheless, the inability of central axons to regenerate in higher mammals can be considered as an enigmatic axiom (Tran et al., 2018). Therefore, experimentation in the fetal rat may hold a promise. The primary steps into fetal experimentation commenced with a focus on reduced spillage of amnion fluid that was thought to be a conditional step in achieving lower mortality rates (De Beer et al., 1988, 1992). Just recently a comprehensive analysis of archived raw data from subsequent tracing experiments has been accomplished, and parts are presented in this paper. The tracing features of the developing afferent axons enabled the localization of station-hubs at the assembly line of the primary afferent system uncovering the spatial-temporal development. The capacity for transcriptome modification is illustrated, in this paper. An interesting perspective is to enable future research into the regenerative program by applying combinatory strategies (Griffin and Bradke, 2020). A case specimen presents a duplication of Hooker’s findings illustrating the
vulnerability of the very immature spinal cord (Hooker and Nicholas, 1930). Our paradigm underlines the potential buoyancy in the CNS at proper conditions exemplified by adequate tracing with Horse Radish Peroxidase (HRP).

2. Material and methods

The work presented in this paper pre-dated the European legislation on animal welfare and institutional ethic board approval. For details about the conception, technical details, and the potential complications the reader is referred to the former papers (De Beer et al., 1988, 1992).

2.1. Surgical procedure

In short, the spontaneous breathing anesthetized timed-pregnant rat was operated upon in a supine position. A laparotomy enabled the eventration of one carefully selected fetus that was stabilized in a proper position in the bicuspid holder.

The uterine wall, yolk, and amniotic sacs were opened layer by layer under the microscope. The dorsal midline of the fetus was visually centered in the bulging amniotic sac protruding through the opened yolk sac. The delicacy of tissues is a real challenge to accomplish these subsequent steps in the procedure. Any adjustment in the position of the fetus after the uterotomy is best to be minimized for the sake of preserving the amniotic fluid content.

The tiny amniotic fluid bell captured between the dorsal skin and the amniotic sac drains when cut. Thereafter, the dorsal skin covering the aligned spinal cord settles into the opening due to the intra-uterine pressure. Both the longitudinal incision of the skin and the transverse cut through the dura into the dorsal half of the spinal cord were performed with a 15° knife (Superblade®) under direct vision through the microscope. A thoracic level was aimed at, but a midline vessel at the spinal cord might press to another level when possible. Any muscular response in the fetus’ body had never been noticed. The variable amount of ensuing swelling at the lesion site had been noticed regularly. The constant fluid drip ensured diligent hemostasis. The amniotic sac was closed together with the dorsal skin with a running suture (Ethilon® 12.0). From the opening to the closure of the uterine wall the whole procedure took about half an hour, in general (Fig. 1).

2.2. Eligibility for tracing

Complete litters may offer certainty about an identifiable operated rat only to a certain extent. The identification process could face difficulty as birthmarks of the dorsal operations showed a tendency to become indiscernible at birth or very soon thereafter. Marks on littermates could make them indistinguishable from operates whenever the mother rat had taken them by the scruff of the neck. Moreover, the absence of one neonate appeared a regular finding. In 6% of all litters, a first stillborn was documented scavenged soon after birth. Therefore, the sooner inspection of the litter had been done after delivery, the more successful the identification could be. Except for an infrequenly visible skin stitch, certainty about the operates identity gradually declined to keep pace with augmented standardization.

The neonate selected as a candidate for follow-up was marked by shortening the tail tip with liquid N₂O, or just by sexing the other littermates up to a total of six or seven neonates. After weaning at three weeks the candidate for the tracing study was kept in its cage for as long as desirable. Several long survivors were lost for follow-up due to physical deterioration, which had been observed in a few to be caused by an abdominal swelling or a neoplasm in the posterior fossa and hypophyseal region.

2.3. HRP Tracing procedure on the left

2.3.1. HRP-tracing in fetus

After finalizing myelotomy, the fetus was placed back again into the bicuspid holder for transmural instillation of 1 µl of HRP into muscles of the left hind paw. Twenty-four hours later the fetus was removed from the dam in a third procedure and perfused transcardially, where after the pregnant dam was sacrificed.

2.3.2. HRP-tracing in neonates

Hypothermia was used for both interventions, i.e., application of a sponge soaked in HRP to the central stump of the transected sciatic nerve, as well as transcardial perfusion 24 h later.

2.3.3. HRP-tracing in adult rats

Anesthetized in a glass bell with di-ethyl ether, a sponge soaked in HRP together with DMSO was applied to the central stump of the sciatic nerve. This method for quick respiratory depression was always used before sacrifice. The spinal cord was dissected and processed for enzyme histochemistry.

2.4. Enzyme histochemistry

Before embedding into gelatin, adult spinal cords were divided into two or three sections to accommodate a plastic container. The medulla was always cut off and placed horizontally. The spinal cord was placed on its left side. The deep-frozen block was mounted on a freezing microtome and cut into 40 µm thick sections. The enzyme histochemistry was applied to 240 µm separated consecutive tissue samples and processed according to the recipe of Mesulam using TMB as a chromogen (Mesulam and Brushart, 1979). The tissue sections were mounted on
chrome-alum subbed slides, counterstained in neutral red, and cover-slipped with Permount® and air-dried overnight.

The preparations were scrutinized through the microscope for the dark label of which the locations were documented on paper within 48 h. Full-color slides (Kodak® Ektachrome 320 (EPJ)) were made of all features through the microscope under darkfield illumination.

2.5. Output

Following standardization after the first and second cohort of experiments (De Beer et al., 1988, 1992), the third cohort was carried out comprising a total of 192 dams and 248 operates delivered in four years. Proper visualization of the three subdivisions of the primary afferent system was appreciated and improved steadily and encouraged to proceed with optimization of the complex HRP-procedure as a tool for reliable evaluation. This series contains 2104 embryos, and the control mortality mounted up to a high number of 324 non-operated neonates partly due to contributions from different subgroups, like maternal deaths for instance. The net surgical mortality rate was 27.3%.

2.5.1. The overall output

Standardization constituted a reliable strategy for recruitment of operated rats eligible for tracing at the expense of almost 50% failure in this series. The overall output including successful HRP-tracing rated at about 1:5. The evaluation of the HRP-tracing by histochemistry was a trial and error experience in coping with technical flaws and faults, whether tracing had been performed in utero or later in life.

2.5.2. Successful tracing studies

The afferent system has been properly traced in forty-five rats with a male/female ratio of 2:1. Complete litters matching the fetus count ranked as high as 80% in this successfully traced series.

2.5.3. Miscellaneous fates

Twenty-one operated fetuses died due to puerperal illness or stress of the dams, excluding six fetuses due to ad hoc maternal deaths. In fourteen litters a stillborn fetus had been documented. Clef palate had been diagnosed twenty-eight times. In complete litters, the identification of the operated fetus failed forty-three times. The tracing procedure, anywhere between HRP-application and the final process of enzym histochemistry, failed in forty-one cases. Forty-three operates lost for follow-up aged between p7 and p930. Thirteen rats had been euthanized due to a Mycoplasma pneumoniae infection recurring in the facility.

2.6. Time window for mating

Since it was thought that the ability for regeneration might be expressed before a developmental switch (see Section 4), experiments were carried out in an earlier, more defined developmental stage. Shortening the time for mating to less than half an hour was thought to pinpoint the developmental stage at the moment of the myelotomy. Surgery was scheduled at E16 and E15, i.e. the sixteenth day of gestation noted like E16-8 h. From the incomprehensible features, dynamics could be inferred underlining that timing at the assumed assembly line of the long primary afferent system is critical.

2.7. New perspective after analysis of the data

The raw data of the third cohort experiments have been assessed recently, and parts of the results are presented in this paper. The apparent durability of the quality of the stored slides was confirmed by the handwritten documentation archived together with each experiment, which enabled a comprehensive investigation into unpublished data. This paper covers data on a crucial relationship that had been overlooked in earlier papers. Refurbishment of the device used in the second series had been neglected for hidden sequelae. After deciphering the data from the tracing experiments, it has become apparent that survival, as well as fetal development, are parameters depending on fetal saturation. The spring-loaded hinged bicuspud holder, which was used in the first cohort, exerted uncontrolled pressure on the fetus’ placenta and vasculature in the ventral mesentery. The second paper was based on similar experiments, but with the use of a refurbished device mounted with a bicuspud holder without a spring-loaded hinge (De Beer et al., 1988, 1992). Therefore, both parameters may have benefitted from pressure reduction by serving attenuation of a skilled “Fingerspitzen Gefühl”.

3. Results

The available candidate rats had been selected from complete litters when flawless identification offered the best gain. These rats were prioritized for the tracing procedure. This strategy rendered 80% successful evaluation of operates. Examples of three operated and four control rats are presented to illustrate the paradigm’s potential. The paradigm’s key feature is the dorsal myelotomy offering a scaffold and a viable route for elongation of cut primary afferents in order not to lose any sign of recuperation, albeit regeneration or not, during primary afferent system development.

3.1. The impact of a total transection of the fetal spinal cord

First, a singular case of an inadvertent transection is presented of a p6 neonate of which the E17 dorsal myelotomy coincided with a significant loss of visible cord fragments during the operation.

Unaware of the “spinal” condition the neonate had been selected for a conditional lesion of the left sciatic nerve, cut at p1, and survived for another four days before the tracing procedure at p5. The intended dorsal myelotomy rendered inadvertently a spinal cord transection, but without obvious signs of neurological deficits. Its stomach full of milk was indicating proper nourishment.

The isolated caudal spinal cord is loaded with the hyperintense label, featuring the long primary afferents in the left dorsal column (DC) as well as the central grey matter with a column of motor neurons in the ventral horn (VH) (Fig. 2A-1). The amount of label is of a remarkable extent compared to the other neonate (Fig. 2A-2). Despite a potentially uplifted regenerative state due to the conditioning lesion, any visible sign of regeneration in the transected primary long afferents in the DC is absent. In this respect, this experiment is regarded as a duplication of Hooker’s trans-spinal transection (Hooker and Nicholas, 1930).

3.2. Distinct impact of developmentally phased dorsal myelotomy

The impact of the dorsal myelotomy in two neonates, the E16 +12h.p11 and the E16-8 h.p8, at low and high thoracic levels respectively, exhibits quite remarkable differences (Fig. 2A-2 and A-3). On the slide of the p11 neonate tissue loss at the dorsal surface marks the lesion site by a visible gap (Fig. 2A-2). Labeled long primary afferents in the DC, as well as enhancement in Clarke’s nucleus, are only featured caudally from the lesion site. The synchronous phased axons exhibit similar features. No dynamic or regeneration signs are present.

The majority of long primary afferents superficially bundled in the p8 neonate terminates into a dorsal hump marking the lesion site (Fig. 2A-3). Next to this class of fibers, individual axons are visible beyond that level. These concomitant afferents are classed as younger, i.e., originating somewhat later than the bundled afferents. This minority of axons is thought to constitute the younger primary long afferents present in the left gracile nucleus (Fig. 2A-6). Both classes are considered to be perceptible out of phase. Moreover, the excess of tissue is regarded as a sign of tissue augmentation, indicating the rather early stage in neurogenesis underpinning a plausible delay between both fiber classes, which may well differ in transcriptomic signature (see Section 4). Axon sprouting into the dorsal hump is highlighted (Fig. 2A-4). A residual part
Fig. 2. A. Operated fetuses. 1. E17/p1 left sciatic nerve transection/p5-HRP/p6. 2. E16 + 12h/p10-HRP/p11. Features of the primary afferent system on display on sagittal slides from a p6 and a p11 neonate. These fetuses had been operated upon at E17 and E16 + 12 h, respectively (see text). 1. At the low thoracic level of the total transection the parasagittally located DC is depicted with collaterals to the motor neurons in the VH. The empty dorsal horn may be due to spinal cord obliquity. 2. The p11 lesion site at a low thoracic myelotomy features long primary afferents in the DC in combination with collaterals into Clarke’s nucleus at levels caudally from the visible gap at the dorsal surface. Scale bar: 100 µμ. 3–6. E16-8h/p6-HRP/p8. 3. The p8 lesion site at the high thoracic level myelotomy is marked with an obvious surplus of tissue at the dorsal surface created at the stage of neurogenesis. An oblique cut through the DC exhibits the bundled developing primary long afferent terminations, which reside in the DC, apparently. The dystopic level is indicated as the FE-switch (see Section 4). Elongating primary long afferents circumvent the lesion site by traversing the central gray. 4. Regenerative sprouting into the lesion hump and deviate axons bypassing ventrally to the hump is shown in greater detail. 5. Some residue of afferent axons has elongated up to the medullary level. 6. The label in the left gracile nucleus is depicted by afferents that penetrated into the nucleus after the FE-switch. Scale bar in all slides: 100 µμ. B. Control rats. 1. p6-HRP/p7. 2. p218-HRP/p220. 3. p2-HRP/p3. 4. p240-HRP/p242. Features of the primary afferent system in two neonates and two adult rats on horizontal slides. 1. A p7 bifurcation zone with dorsal rootlets features label enhancement of the primary afferent system at the left DREZ. 2. At lower thoracic levels the DC’s neighboring Clarke’s nucleus is labeled by primary afferents of the intermediate subdivision in a p220 rat. At this age, the long afferents in the DC show delicate label. 3. Left and right gracile nuclei have been labeled. 4. Labeling of the contra-lateral gracile nucleus is found a standard feature. Scale bar: 100 µμ.
of primary long axons has reached the medulla (Fig. 2A-5). After penetration of the gracile nucleus, the inside label becomes visible (Fig. 2A-6).

3.3. Tracing of control rats

The tracing features of the three subdivisions of the primary afferent system are displayed in horizontal slides. Features of the short subdivision are depicted in the dorsal root entry zone (DREZ) and bifurcation zone of a p7 control rat (Fig. 2B-1). Axons of the intermediate subdivision have labeled Clarke’s nucleus at lower thoracic levels in a p220 control rat (Fig. 2B-2). The long afferent subdivision is represented by the labeled axons in the left DC of which the axons have penetrated the nuclei at the left as well as at the right side (Fig. 2B-3 and B-4).

4. Discussion

Hooker’s issue about the detrimental effect of fetal injury (Hooker and Nicholas, 1930) is reiterated in an equivalent experiment illustrating the non-existence of regenerative potential and the missing resilience to injury after fetal spinal cord transaction at inappropriate conditions. In contrast, this paradigm facilitates elongation of primary afferents after dorsal myelotomy due to scaffolding. The lesion impacts in the p8 and p11 neonates differ markedly. The difference is ascribed to dissimilar developmental stages. So, the actual stage is crucial for the impact the lesion made. The myelotomies in the p5 and p11 neonates are considered to exhibit the impact of lesions from beyond the developmental capacity of the afferent system. A so-called developmental switch delineates that moment in-between. The very immature developmental stage of the primary afferent system of the p8 neonate coincides with neurogenesis. The neuropoetic stage is exemplified by the lesion hump. Moreover, we discern a particular elongation switch, set at E15.

4.1. Fast elongation switch

The switch for corticospinal axon elongation had been documented to occur in neonates (Iwashita et al., 1994). Research into the dependency of developmental switches in the CNS on the lesion impact has a long track record in neuroscience worldwide in many taxaons (Schwab and Bartholdi, 1996). The two distinct features of the primary afferent system development are understandable, so far. Superimposed, a strange effect had been noticed featuring desynchronization of elongating primary afferent axons with multilevel terminations throughout the DC. This perturbed development is depicted by the dystopic front-stop in the p8 neonate. The bundled afferents terminating at the hump are considered to have gone out of phase related to those elongating afferents that have reached the gracile nucleus. Elongation is blocked at a virtual station hub on the assembly line. The axons may have accomplished a fast elongation switch (FE-switch). This FE-switch is conditionally confined to the pioneering primary long afferents, that have been elongated into or in close vicinity of the medulla. The bundled axon configuration may bear an imaginable resemblance with the hypothetical station-hub at the assembly line when pioneering axons have reached their destinations at the medulla. The subsequent axon penetration into the gracile nuclei may become preceded by adapting elongation speed to a tempo of slower growth of the spinal cord proper, i.e., the FE-switch. Additional dystopic features along the assembly line will be addressed in an upcoming paper. Here, the perturbations in the afferent system development are attributed to the effect of hypoxia in the operated fetus.

4.2. Transcriptomic signature

Improvement of peroperative oxygenation had been suggested to eliminate puerperal maternal death (De Beer et al., 1992). In contrast, suboptimal saturation in embryogenesis has been widely researched utilizing an experimental model known for effective preconditioning (Cox-Limpens et al., 2015). Complete clamping of uterine and ovarian arteries for thirty minutes in E17 pregnant rats rendered 14.2% fetal mortality after a full-term pregnancy. Extensively down-regulated transcripts in brain tissue and up-regulated transcripts in cell nuclei had been documented sequelae (Cox-Limpens et al., 2013). Taken together, relative hypoxia induced by suboptimal saturation depending on varying pressure of the bicuspid holder exerted onto the placenta lends our paradigm an unexpected view at a plausible transcriptomic effect upon the developing afferent system in the spinal cord. The deviate configuration in the p8 neonate underlines this issue of seeming delay. This feature, lasting for thirteen days after the myelotomy, is indicative of an altered transcriptome. The fetal susceptibility is found to be transiently present, i.e. before the developmental switch. In the p5 and p11 neonates, these features are lacking. Likely, their afferents had been lesioned beyond the developmental switch.

4.3. Conclusion

The surgical procedure proper does not warrant the revealed signs of delay between developing afferent axons. The transcriptomic modification, held responsible for the uncovered effect, was deduced from the data only after this recent analysis was completed. So, the paradigm may offer a way to unravel enigmatic features of the spatial-temporal development of the long primary afferent system and value in disclosing a potential intrinsic regenerative program in DRG neurons. Whenever demonstrable, that might be scheduled before the FE-switch. Last but not least, the paradigm might convey preclinical research, for instance regarding congenital indifference to pain, to scratch the surface. Whatever the full picture may look like, the serendipity of our findings prompted this paper, completing a triad on the technical description of the paradigm with additional information relevant for a proper appreciation of the unforeseen characteristic of the device.

Disclosure

The author devoted over ten years early in his career to fetal experimentation in combination with full-time clinical employment in the academic hospital, Leyden University. After retirement from the neurosurgical private practice, the author digitized the unpublished acquired data which this paper is based upon. After almost thirty years the unencumbered content is regarded as free for communication on a personal basis exerting the rights of private intellectual property.

CRediT authorship contribution statement

The author is responsible for the content presented in all aspects.

Declaration of conflict of interest

The author declares no conflict of interest.

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