

A New Technique for Collection of Cerebrospinal Fluid in Rat Pups

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ABSTRACT

BACKGROUND: Neuroprotective strategies to prevent or decrease brain injury in hypoxic ischemic newborns are one of the main research lines in neonatology. Animal models have been used to assess the efficiency of new therapeutic strategies. Brain damage biomarkers in cerebrospinal fluid (CSF) are frequently used to evaluate the outcome at the bedside. Despite the importance of this approach in clinical practice, there are many difficulties in using it in small animals. The aim of this paper was to describe a new technique for collecting CSF in rat pups. Furthermore the reference values of S100 β protein levels, commonly used in common clinical practice, were analyzed in animals between 7 to 12 days.

METHODS: 42 Wistar rat pups aged 7 to 12 days were used. CSF was obtained by direct puncture of the cisterna magna with a 24-gauge needle. S100 β protein levels were determined with enzyme-linked immunosorbent assay (ELISA).

RESULTS: CSF was successfully obtained in 96% of the cases, with an average amount of 21.28 μ l (5–40 μ l). Normal values for S100 β were described. HI animals presented higher S100 β values than controls.

CONCLUSIONS: A simple, reproducible technique for CSF collection in rat pups has been described. This new method will allow study of brain injury biomarkers in newborn hypoxic ischemic animal models.

KEYWORDS: cerebrospinal fluid collection, rat pups, S100B-protein, hypoxic-ischemic

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Introduction

Cerebrospinal fluid (CSF), produced mainly by the choroid plexuses and the ependymal layer of the ventricular system, offers an indirect window on the biological reactions in the central nervous system (CNS). Its analysis has been used for years to detect CNS pathology.¹ Cellularity and molecular characteristics of the fluid, as well as some biomarkers, have been investigated to identify variations in the CNS in response to aggression and neuroprotective therapies.²

Rats are among the most commonly employed species in neuroscience laboratories. However, technical difficulties in CSF extraction and the low volumes obtained have limited the use of this biological fluid in experimental studies. Despite this, different methods for obtaining CSF in adult rats have been described, such as the insertion of a catheter into the cisterna magna,³ cisterna magna direct puncture,⁴ and lumbar puncture on anesthetized animals.⁵ Cisterna magna puncture is the most frequently reported technique, and it has been reported that one can obtain 190 μ L of the fluid in adult rats.⁶ All these methods have been proven in adult rats but they require training and precision. Their most important handicap is blood contamination of the CSF. This problem is common in most of them, which alters the fluid's biomarker values.⁷

Although many animal models have been used to evaluate neuroprotective strategies for newborn brain damage, there are only few studies that explain how to collect CSF in immature rats,^{8–10} because obtaining sufficient quantity of CSF in these animals to determine biochemical parameters is extremely complex.

The protein S100 β (S100 β P) is a great biomarker of brain damage and its levels are increased in blood and CSF in patients with acute CNS injury.¹¹ It is a calcium-binding protein produced by astrocytes¹² and has multiple functions from cytoskeleton and neuron to astrocyte cycle regulation.¹³ S100 β P concentration, particularly in serum and CSF, is used as a neuronal and astrocyte damage marker^{14,15} as well as for the measurement of the effectiveness of neuroprotection strategies.¹⁶ Although S100 β P determination in blood and CSF is commonly used in clinical practice including newborn patients with hypoxic-ischemic encephalopathy (HIE),¹⁷ to our knowledge there is only one study that has analyzed this biomarker in the CSF of rats pups.¹⁰

The aim of our study was to describe and validate a new technique to obtain CSF in rat pups (p7–p12) in order to extract enough amount of fluid to enable the determination of



biomarkers. Moreover, in order to prove its usefulness, we also validate normal S100 β P values in rats pups.

Material and Methods

Animals. Wistar pups aged between 7 and 12 days were used. After birth, the animals were kept with their mothers in cages with 12-hour light/dark cycles at a constant temperature of $22 \pm 1^\circ\text{C}$ with free access to food and water. The animals were divided into seven groups according to their age, and another group with brain damage (p7–p12 and HIE, see Table 1).

The experimental procedures were approved by the local ethical committee of the University of Barcelona, following European (2010/63/UE) and Spanish (RD 53/2013) regulations for the care and use of laboratory animals.

CSF collection technique. Animals were placed on a sterile surface and anesthetized with inhaled isoflurane 4%. Once the pup was anesthetized (Fig. 1A), its neck was flexed by placing the head at an angle of 90° allowing visualization of a diamond-shaped surface between the occipital bone and the start of the cervical column (Fig. 1B, C). A 24-G needle was perpendicularly inserted (between 0.5 and 1 mm) with the beveled edge facing upward (Fig. 1). Two investigators performed this procedure, one maintaining the animal in the correct position, and the other inserting the needle.

CSF was collected with an automatic pipette (Fig. 1D). Following extraction, the animals were sacrificed. The CSF was transferred to an Eppendorf tube, and kept frozen at -80°C .

Brain damage animal model. Another group of animals underwent an HIE model using the Rice–Vannucci method.¹⁸

For this procedure, pups were anesthetized with isoflurane (4% induction, 2% maintenance). After this, the rat pups were subjected to left common carotid artery ligation with doubled silk sutures (4.0).

Following the above procedure, rat pups were allowed to recover with the dams for 15 minutes and then were exposed to a hypoxic environment (8% O₂, 92% N₂) for 90 minutes. During this period, all of them were maintained at 36.5°C . In this group of animals, CSF samples were collected 6 hours after the end of hypoxia.

S100 β protein determination. Enzyme-linked immunosorbent assay kit for S100 calcium binding protein S100 from USCN Life Science Inc. was used to determine the levels of S100 β protein at a dilution of 1/20.

Results

Sample volume. Forty-two pups (54% females) were used. They were divided into seven groups depending on their age [from 7 to 12 days (p7–p12) and one damage group named HIE]. The mean weight of the animals was 20.22 g (14.35–24.65 g) and the brain weight was 0.81 g (0.58–1.07). The duration of anesthesia was 120.39 s (± 19.82). CSF was obtained successfully in 96% of the cases, with an average amount of 21.28 μL (5–40 μL). The aspiration rate was difficult to assess,

Table 1. Data summary of the animals used for the experiment and the results.

	p7	p8	p9	p10	p11	p12	HIE (p10)
Animals number (n)	10	10	10	14	14	10	5
Animal weight (g)	15.07 (13.21–18.3)	15.1 (11.57–18)	17.7 (13.3–20.16)	18.23 (14.83–22.9)	19.8 (17.4–21.2)	22.92 (21.4–23.5)	19.7 (15.85–23)
Anaesthesia time (s)	118 (90–150)	137 (128–150)	127 (107–137)	117 (97–124)	111 (102–125)	116 (90–118)	120 (105–133)
Brain weight (g)	0.64 (0.58–0.74)	0.69 (0.5–0.79)	0.75 (0.68–0.82)	0.8 (0.72–0.89)	0.94 (0.9–1.04)	1.04 (0.98–1.07)	0.83 (0.80–0.85)
CSF amount (μL)	15 (0–25)	20 (5–40)	30 (15–40)	25 (0–40)	20 (5–40)	10 (5–40)	25 (15–40)
% Successes	80	100	100	90	100	100	100
% Contaminated samples	16.6	16.6	0	0	0	0	0
S100B protein values	8.1 (6.9–9)	17 (6.3–19)	10 (7.5–15)	10.2 (4.5–16.3)	8.5 (5–10)	6 (4–8.5)	45 (40–50)

Note: Data expressed in median and range.

Abbreviations: p, Days of live; HIE, Hypoxic-ischemic encephalopathy; g, grams; s, seconds.

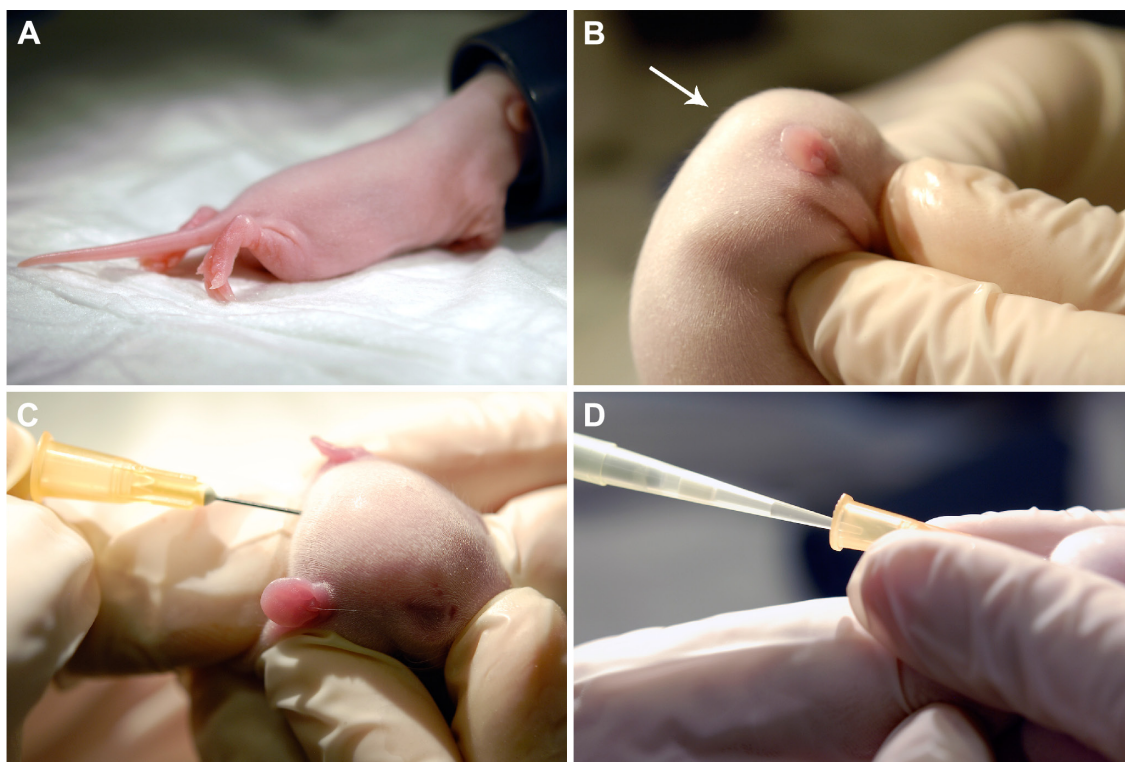


Figure 1. Cerebral spine fluid collection technique. (A, B) After being anesthetized (isofluorane 4%), a rat pup is held by one investigator with the head positioned at 90° angle. The arrow points to the puncture place. The 24 G needle with the bevel up is inserted perpendicularly (B). (C, D) CSF output flows out spontaneously and is collected with a pipette in 20- μ L aliquots and later kept in Eppendorf tubes at -80°C .

but it was less than 20 seconds. The largest amount of CSF was obtained from those animals with higher weights ($P = 0.042$). The CSF amount did not correlate with the animals' age or brain weight ($P > 0.05$). The blood contamination rate in the samples was 4.7%. In Table 1, there is a description of the sample characteristics depending on the group.

S100 β P values in CSF. Blood-contaminated samples were excluded from the analysis. S100 β P values in normal animals ranged between 5 and 25 ng/mL. There were no differences in the time of anesthesia, so this does not seem to influence the S100 β P values.

HIE brain damage was carried out in some animals of 10 days of life, using the Rice–Vannucci model¹⁸ to obtain a damage control group.

The S100 β P values in CSF of HIE animals were significantly higher than those of the control group (9.8 vs 45.2 ng/mL, $P < 0.01$).

The S100 β P values in the first days of life are presented in the graphics (Fig. 2).

Discussion

Clinical use of CSF biomarkers such as S100 β and neuron-specific enolase (NSE)¹⁹ in neurocritical patients has become a standard of management. These biological variables have been validated as great tools to assess the prognosis at the bedside of this group of patients.¹⁷ Although they have been used in

many animal species including adult rats, there have been few studies by including these markers in the CSF of small pups because it was technically very laborious.

The S100 β protein belongs to a multigenic family of low molecular weight (9–13 kDa) calcium-binding S100 proteins.²⁰ It is abundant in the glial cells of the CNS and is actively secreted from astroglia. Intracellularly, S100 β P is involved in signal

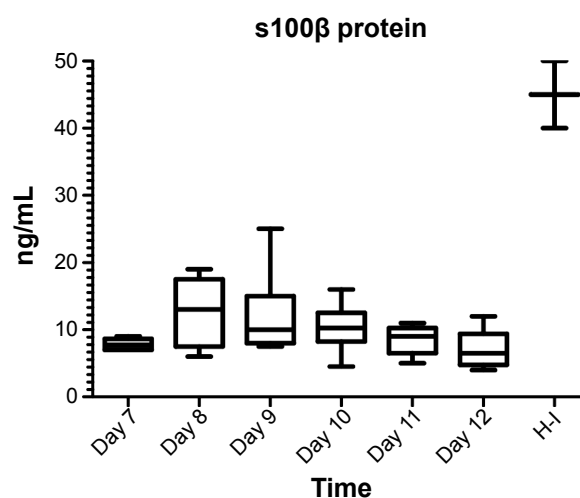


Figure 2. S100 β P normal values in CSF and in HI animals. Normal levels of S100 β from 7 to 12 days of life in control rat pups. HI levels in 10-day-old damaged animals.



transduction via the inhibition of protein phosphorylation and regulation of enzyme activity, and by affecting calcium homeostasis as well as cell morphology by interaction with elements of the cytoskeleton.²¹ At nanomolar concentrations, it exerts a neuroprotective and neurotrophic influence, but at micromolar concentrations it leads to astrocytic death.²² In clinical practice, S100 β P concentrations, particularly in CSF and blood serum, have been used as a parameter of glial activation or death in several situations of brain injury.²³ In the past decade, a number of studies have demonstrated the potential use of S100 β P in detecting brain damage in asphyxiated newborns.²⁴

There have been several papers reporting S100 β P levels in CSF in different rodent models of brain damage.²⁵ Most of these have been in adult rats, including models of trauma shock²⁶ and sepsis,²⁷ but to our knowledge there are no published studies with S100 β P levels and HIE in a rat model. Only Huang and colleagues had described normal S100 β P values in adult rats.⁶

In the present study, we have described a new procedure to obtain CSF samples from small rats using direct cisterna magna puncture. Several methods have been employed for the collection of CSF from adult rats.^{28,29} The most recently published methods involved the exposure of the atlantooccipital membrane or the duramater³⁰ and a very sophisticated technique using ultrasound-guided puncture.³¹ All of these have been validated and used in many adult animal studies. But none of them has been described as a good technique to obtain CSF from small rodents or rat pups.

Our new procedure has been designed to be used in very small animals (less than 20 g) (7–12 days), which are the ones used in models of newborn brain damage.¹⁸ With this technique, enough CSF could be obtained and damage biomarkers could be determined. This will improve the evaluation approach of new strategies for neuroprotection, offering a new perspective, similar to that offered in normal clinical evaluation.

Other techniques described in adult rats involve implanting a permanent catheter³² or else require complex surgical techniques³³ that are not viable in small animals. The new technique does not involve the construction of any special cannula or catheter, and does not require extensive knowledge of stereotactic surgery. It is an inexpensive and easily reproducible procedure even without much training.⁷ Moreover, the new procedure is quicker (taking <1 min/sample) than other described methods.²⁵

Aside from all these important points, the most useful advantage of this new technique is the amount of CSF obtained. Ten to 40 μ L per sample should be enough to perform biological determinations as we have proven in our study.

In addition, direct visualization of the output allows the exclusion of blood-contaminated samples. This is essential to avoid blood cell interference, although the contamination rate of the samples is low (<10%).^{34,35}

Normal values of S100 β P obtained in our study are similar to those described in other studies with adult rats. There is

only one study using rat pups with a small number of animals, so it is difficult to draw conclusions.¹⁰

In control animals, no differences in S100 β P values from P7 to P12 were observed. When S100 β P was evaluated in HIE animals, the values were significantly higher than in controls. The S100 β P values were about 4 times higher in animals with HIE than in healthy animals. No control animal had S100 β P values >25 ng/mL. Moreover, when we compared the values between p10 controls and HIE p10 animals, we could see that there were statistically significant differences (10.5 vs 45.2 ng/mL, $P < 0.01$). All these results are similar to those described in humans.³⁶

Although the procedure is really good to obtain CSF in small rats, its main limitation is that the animals have to be sacrificed after the procedure; therefore, this method is not useful for multiple CSF collections at different time points.

Conclusions

In summary, a simple and reliable procedure collecting CSF from the cisterna magna has been described in rat pups. Compared to the existing protocols, this procedure provides not only the advantage of being performed in small animals but also accurately obtaining uncontaminated CSF, which may facilitate the determination of biological markers in the CSF such as S100 β P. A part from that, the normal values of this neural damage biomarker in small pups have been provided.

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Author Contributions

Conceived and designed the experiments: JRF, CDF, MCC. Analyzed the data: JRF, CDF, MCC. Wrote the first draft of the manuscript: JRF, MCC. Contributed to the writing of the manuscript: JRF, CDF, MCC. Agree with manuscript results and conclusions: JRF, CDF, MCC. Jointly developed the structure and arguments for the paper: JRF, CDF, MCC. Made critical revisions and approved final version: JRF, CDF, MCC. All authors reviewed and approved of the final manuscript.

REFERENCES

1. Nagdyman N, Kömen W, Ko HK, Müller C, Obladen M. Early biochemical indicators of hypoxic-ischemic encephalopathy after birth asphyxia. *Pediatr Res*. 2001;49:502–506.
2. Van den Berg W, Romeijn S, Verhoef J, Merkus F. Serial cerebrospinal fluid sampling in a rat model to study drug uptake from the nasal cavity. *J Neurosci Methods*. 2002;116:99–107.
3. Westergren I, Johansson B. Changes in physiological parameters of rat cerebrospinal fluid during chronic sampling: evaluation of tow sampling methods. *Brain Res Bull*. 1991;27:283–286.
4. Pegg C, He C, Stroink A, Kattner K, Xu Wang C. Technique for collection of cerebrospinal fluid from the cisterna magna in rat. *J Neurosci Methods*. 2010;187:8–12.
5. De la Calle JL, Paino CL. A procedure for direct lumbar puncture in rats. *Brain Res Bull*. 2002;59:245–250.
6. Huang Y, Saljo A, Suneson A, Hansson A. Comparison among different approaches for sampling cerebrospinal fluid in rats. *Brain Res Bull*. 1996;41:273–279.

7. Consiglio AR, Lucion AB. Technique for collecting cerebrospinal fluid in the cisterna magna of non-anesthetized rats. *Brain Res Brain Res Protoc.* 2000;5:109–114.
8. Ohki S, Togari H, Sobajima H, Fujimoto S, Kobayashi M, Hyodo J. Lactate attenuates neuron specific enolase elevation in newborn rats. *Pediatr Neurol.* 1999;21:543–547.
9. Vannuci R, Brucklacher R, Vannucci S. The effect of hyperglycemia on cerebral metabolism during hypoxia-ischemia in the immature rat. *J Cereb Blood Flow Metab.* 1996;5:1026–1033.
10. Vicente E, Tramontina F, Leite MC, et al. S100 β levels in the cerebrospinal fluid of rats are sex and anaesthetic dependent. *Clin Exp Pharmacol Physiol.* 2007;34:1126–1130.
11. Fink EL, Berger RP, Clark RS, et al. Serum biomarkers of brain injury to classify outcome after pediatric cardiac arrest. *Crit Care Med.* 2014;42:664–674.
12. Donato R. S100: a multigenic family of calcium-modulated proteins of the EF-hand type with intracellular and extracellular functional roles. *Int J Biochem Cell Biol.* 2001;33:637–668.
13. Van Eldik LJ, Wainwright MS. The Janus face of gliar-derived PS100 β : beneficial and detrimental functions in the brain. *Restor Neurol Neurosci.* 2003;21:97–108.
14. Kögel D, Peters M, König HG, et al. PS100 β potently activates p65/c-Rel transcriptional complexes in hippocampal neurons: clinical implications for the role of PS100 β in excitotoxic brain injury. *Neuroscience.* 2004;127:913–920.
15. Böhmer AE, Oses JP, Schmidt AP, et al. Neuron-specific enolase, S100 β , and glial fibrillary acidic protein levels as outcome predictors in patients with severe traumatic brain injury. *Neurosurgery.* 2011;68:1624–1630.
16. Vos PE, Lamers KJ, Hendriks JC, et al. Glial and neuronal proteins in serum predict outcome after severe traumatic brain injury. *Neurology.* 2004;27:1303–1310.
17. Sun J, Li J, Cheng G, Sha B, Zhou W. Effects of hypothermia on NSE and PS100 β protein levels in CSF in neonates following hypoxic/ischaemic brain damage. *Acta Paediatr.* 2012;8:316–320.
18. Vannucci R, Vannucci S. Perinatal hypoxic-ischemic brain damage: evolution of an animal model. *Dev Neurosci.* 2005;27:81–86.
19. Bennet L, Booth L, Gunn AJ. Potential biomarkers for hypoxic-ischemic encephalopathy. *Semin Fetal Neonatal Med.* 2010;15:253–260.
20. Wilder PT, Rustandi RR, Drohat AC, Weber DJ. S100B inhibits the protein kinase C-dependent phosphorylation of a peptide derived from p53 in a calcium dependent manner. *Protein Sci.* 1998;7:794–798.
21. Huttunen HJ, Kuja-Panula J, Sorci G, et al. Coregulation of neurite outgrowth and cell survival by amphoterinans 100 proteins through receptor for advanced glycation en products (RAGE) activation. *J Biol Chem.* 2000;275(51):40096–40105.
22. Rothermundt M, Peters M, Pren J, Arolt V. S100B in brain damage and neurodegeneration. *Microsc Res Tech.* 2003;60:614–632.
23. Beharier O, Khan J, Susterman E, Sheiner E. S100B a potential biomarker for early detection of neonatal brain damage. *J Matern Fetal Neonatal Med.* 2012; 25:1523–1528.
24. Florio P, Abella R, Marinoni E, et al. Biochemical markers of perinatal brain damage. *Front Biosci (Schol Ed).* 2010;2:47–72.
25. Cao F, Yang XF, Liu WG, et al. Elevation of neuron-specific enolase and S-100B protein level in experimental acute spinal cord injury. *J Clin Neurosci.* 2008;15:541–544.
26. Pelinka L, Bahrami S, Szalav L, Umar F, Redl H. Hemorrhagic shock induces an S100B increase associated with shock severity. *Shock.* 2003;19:422–426.
27. Rosengarten B, Hecht M, Auch D, et al. Microcirculatory dysfunction in the brain precedes changes in evoked potentials in endotoxin-induced sepsis syndrome in rats. *Cerebrovasc Dis.* 2007;23(2–3):140–147.
28. Boumman H, Van Wimersma T. A rapid and simple cannulation technique for repeated sampling of cerebrospinal fluid in freely moving rats. *Brain Res Bull.* 1979;4:575–577.
29. LoPachin R, Rudy T, Yaksh T. An improved method for chronic catheterization of the rat spinal subarachnoid space. *Physiol Behav.* 1981;27:559–561.
30. Liu M, Shen L, Begg DP, D'Alessio DA, Woods SC. Insulin increases central apolipoprotein E levels as revealed by and improved technique for collection of cerebrospinal fluid from rats. *J Neurosci Methods.* 2012;209:106–112.
31. Lu YG, Wei W, Wang L, et al. Ultrasound-guided cerebrospinal fluid collection from rats. *J Neurosci Methods.* 2013;215:218–223.
32. Ylitalo P, Heikkinen ER, Myllylä VV. Evaluation of successive collections of cisternal cerebrospinal fluid in rats, rabbits, and cats. *Exp Neurol.* 1976;50:330–336.
33. Huang Y, Säljö A, Suneson A, Hansson H. A new approach for multiple sampling of cisternal cerebrospinal fluid in rodents with minimal trauma and inflammation. *J Neurosci Methods.* 1995;63:13–22.
34. Lai YL, Smith PM, Lamm WJE, Kilde-Brandt J. Sampling and analysis of cerebrospinal fluid for chronic studies in awake rats. *J Appl Physiol Respir Environ Exerc Physiol.* 1983;54:1754–1757.
35. Lebedev SV, Blinov DV, Petrov SV. Spatial characteristics in rats and novel technique for puncture with a stereotactic manipulator. *Bull Exp Biol Med.* 2004; 137:635–638.
36. Aridas JD, Yawno T, Sutherland AE, et al. Detecting brain injury in neonatal hypoxic ischemic encephalopathy: closing the gap between experimental and clinical research. *Exp Neurol.* 2014;261:281–290.