Reversal of sensory deficit through sacral neuromodulation in an animal model of fecal incontinence

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Abstract

Background

Sacral neuromodulation (SNM) is a treatment option for intractable fecal incontinence. The mechanism of action is unclear, however, increasing evidence for afferent somatosensory effects exists. This study's aim was to elucidate effects of acute SNM on the cerebral cortex in a rodent model of pudendal nerve injury.

Methods

The effects of 14 Hz and 2 Hz SNM on sensory cortical activation were studied. In 32 anesthetized rats, anal canal evoked potentials (EPs) were recorded over the primary somatosensory cortex. Pudendal nerve injury was produced by 1-hour inflation of two intra-pelvic balloons. Four groups were studied: balloon injury, balloon injury plus either 14 Hz or 2 Hz SNM, sham operation. Immunohistochemistry for the neural plasticity marker polysialylated neural cell adhesion molecule (PSA-NCAM) positive cells (numerical density and location) in the somatosensory cortex was performed.

Key Results

Anal EP amplitudes diminished during balloon inflation; 14 Hz SNM restored diminished anal EPs to initial levels and 2 Hz SNM to above initial levels. Evoked potential latencies were prolonged during balloon inflation. The numerical density of PSA-NCAM positive cells increased in the SNM groups, but not in sham or balloon injury without SNM. Stimulated cortices showed clusters of PSA-NCAM positive cells in layers II, IV, and V. Post SNM changes were similar in both SNM groups.

Conclusions & Inferences

Sacral neuromodulation augments anal representation in the sensory cortex and restores afferent pathways following injury. PSA-NCAM positive cell density is increased in stimulated cortices and positive cells are clustered in layers II, IV, and V.

Key Messages

• A better understanding of the mechanism of action of sacral neuromodulation is needed for improved patient selection and treatment optimization.

• The aim of this study was to elucidate the effects of acute sacral neuromodulation on the cerebral cortex in a rodent model of fecal incontinence.

• Thirty-two Wistar rats were studied in acute experiments involving cortical evoked potential recordings, a model of fecal incontinence and sacral neuromodulation at two frequencies (14 Hz and 2 Hz). PSA-NCAM expression was then studied in harvested brains using immunohistology.

• Sacral neuromodulation restored diminished anal evoked potentials and 2 Hz stimulation was more effective than 14 Hz stimulation. Sacral neuromodulation increased PSA-NCAM expression in the somatosensory cortex, especially in layer II, which relays information to intra-hemispherical association centers.

INTRODUCTION

Fecal incontinence (FI) is a common and socially disabling condition with significant unmet clinical needs.1,2 Pathophysiology is multifactorial, but in women commonly involves pelvic neuronal and structural trauma secondary to obstetric injury.[3,4] Sacral neuromodulation (SNM) is now the first line surgical intervention for patients with severe FI symptoms unresponsive to conservative treatment in the absence of significant anal sphincter disruption.[5] The mechanism by which SNM augments continence is not fully understood, however, recent human and animal data indicate afferent somatosensory effects and changes in sensory cortical function.[6] Our group has previously studied the effect of SNM on afferent pathways in rats using anal evoked potential (EP) recordings over the primary somatosensory cortex. Acute SNM was shown to elicit a long-term potentiation (LTP) like phenomenon in the somatosensory cortex in healthy animals.[7,8] A study of the effect of stimulation frequency in SNM found that 2 Hz stimulation elicited maximal EP potentiation.[8] Electrophysiological findings were supported by immunocytochemical evidence of increased levels of polysialylated

neuronal cell adhesion molecule (PSA-NCAM) in the primary somatosensory cortex after SNM.7 PSA-NCAM is a marker of neuronal plasticity, which is routinely used in the evaluation of hippocampal LTP and is present in the neocortex.[9] The aim of this study was to elucidate effects of SNM on the cerebral cortex in a rodent model of pudendal nerve injury using electrophysiological and immunohistological analysis. First, a rodent model of intra-pelvic balloon inflation, designed to mimic the effects of childbirth,[10] was applied to address the effect of SNM on diminished sensory cortical function. Second, the clinically used frequency (14 Hz) and the experimentally derived optimal frequency (2 Hz)[8] were compared. Third, the numerical density and location of PSA-NCAM positive cells within the primary somatosensory cortex was investigated.

METHODS

Electrophysiological experiments

Ethics and general surgical preparation Experiments were approved by the Animal Research Ethics Committee in University College Dublin, and licensed by the Department of Health and Children (licence no: B100/4435). Thirty-five nulliparous female Wistar rats (200–260 g) were used. Animals were kept at a 12/12 h light/dark cycle and had access to water and rodent standard diet ad libitum.

For electrophysiological experiments, rats were anesthetically induced with isoflurane (4%; Piramal Healthcare, Northumberland, UK) in oxygen (1 L/min) and then surgically anesthetized using urethane (Sigma, Arklow, Ireland) in a 20% solution at a dose of 1.5 g/kg i.p. The level of anesthesia was monitored regularly throughout the procedure by testing the pedal withdrawal reflex to toe pinch and the corneal reflex. Rats rested on a homeostatic warming blanket (Harvard Apparatus, Kent, UK) to maintain a body temperature of 37 °C. The femoral vein was cannulated to administer supplemental anesthesia/fluids as needed, and the femoral artery was cannulated to monitor blood pressure. Tracheostomy and intubation were performed to ensure airway patency. On conclusion of each experiment, the rat was euthanized by an anesthetic overdose. Rats used for immunohistological control experiments only, were anesthetized using isoflurane and euthanized by cervical dislocation.

Experimental design

Four groups (N = 8) were created: balloon injury group, balloon injury and 14 Hz SNM group, balloon injury and 2 Hz SNM group, and sham balloon injury group. A sham SNM group was deemed unnecessary because the authors have previously shown that there is no effect of sham SNM on cortical EPs.[8] Balloon injury model Simultaneous inflation of two balloons in the pelvic cavity has been shown to be a valid model of nerve stretch and compression during the 2nd stage of labor

inducing pudendal neuropathy.10 In a supine position, a midline laparotomy was performed in the caudal 1/3 of the abdomen extending approximately 3 cm. The bladder and uterus were visualized and the bladder, if full, was emptied manually. The retro-uterine space was then carefully widened. Two silicone paediatric Foley catheters (Coloplast A/S, Humlebaek, Denmark) were inserted deep into the retro-uterine space. The abdominal wall was closed and both balloons were inflated in the pelvic cavity with 1.5 mL of deionised water. The correct placement of the balloons was confirmed when inflation caused a visible convexity in the pelvic area and the legs lifted slightly. After 60 min inflation, the balloons were deflated, but left in place to avoid any movement of the animal. In the sham group, the balloons were inserted, but not inflated.

Evoked potential recordings

Anal EPs were recorded over the primary somatosensory cortex as described previously.[8] Briefly,

the rat was placed in a stereotactic frame and a small craniotomy, circa 4 x 4 mm, was performed over the right hemisphere at the position of the anal canal representation (anteroposterior coordinate: ±0.6 mm, mediolateral coordinate: +2 mm measured from bregma). Evoked potentials were recorded with a 32-channel multi-electrode array (flexMEA, Multi Channel Systems, Reutlingen, Germany). Due to the limited size of the array (1830 µm x 1830 µm) recordings were taken from the right hemisphere only. Recordings were processed through a 10-fold miniature pre-amplifier (MPA32I; Multi Channel Systems, Reutlingen, Germany) and the MEA head stage, a combined amplifier, filter, and data acquisition system (USB-ME-FAI –System; Multi Channel Systems); MC_Rack 4.3.0 (Multi Channel Systems) was used to record data. Anal canal stimulation and the MEA head stage were both triggered by a programmable stimulator unit (Master 8; Grass Instruments Co., Slough, UK) to synchronize stimulation and recording. A sampling frequency of 10 kHz was used. For each trial, a sweep average of 400 EPs was created. Recordings were taken every 10 min.

Anal canal stimulation

A gold plated plug cathode (diameter: 2 mm) was placed in the anal canal and a silver wire anode (diameter: 500 µm) introduced subcutaneously lateral to the external anal sphincter (EAS) on the left side. Stimulation was applied at 10 V amplitude, 1 Hz frequency, and 1 ms pulse duration. A slight contraction of the EAS was visible during stimulation.

Acute SNM

For SNM a small midline incision was made over the sacrum and the first sacral foramen visualized. A SNM platinum iridium rodent lead was placed in the left S1 foramen to stimulate the first sacral

nerve root (Medtronic Inc., Minneapolis, MN, USA). S1 was chosen because the objective was to explore sensory function.[7,8] Correct placement of the electrode was confirmed by observation of a tail twitch. Stimulation was applied at motor threshold (3.5–9 V), 1 ms pulse duration and either 2 or 14 Hz for 10 min. Application of SNM was performed at a higher stimulation amplitude than is used in patients (motor threshold vs below or at the sensory threshold), because this allowed to confirm the right placement of the lead and stimulation greater or equal to 0.5 times the motor threshold up to the motor threshold has previously been shown to cause the same level of potentiation.[8] Recording protocol Anal EPs (duration 6 min) were recorded every 10 min during the study epoch, i.e., 4 min interval between recordings. Evoked potential data were thus recorded twice at baseline, six times during the 60 min balloon inflation and seven times after balloon deflation. A recording was not performed at 80 min to allow for the 10 min acute SNM administration (total protocol: 150 min).

Immunohistological experiments

Tissue harvesting Tissue for PSA-NCAM immunocytochemistry was harvested in four animals per group and three additional (unstimulated) animals for the performance of control experiments. The location of the recording was marked with an injection of 2% pontamine sky blue (10 lL; BDH Laboratory Supplies, Poole, UK). On completion of the experiment, rats were euthanized and the whole brain quickly harvested. The brain, covered in optimum cutting temperature compound (VWR International, Leuven, Belgium), was snap frozen in liquid nitrogen cooled isopentane (Fisher Scientific, Leicestershire, UK). Samples were stored at -80 °C until further processing. Tissue processing All tissue was sectioned using a cryostat (Acc Leica Cryostat CM30505, Wetzlar, Germany) at 12 µm thickness and sections were placed on polylysine-coated glass slides (VWR International). Systematic uniform random sampling was applied over the area of interest (-0.4 to 1.6 mm anteroposterior from bregma). Eight coronal slices were taken 228 µm aside from each brain after randomization of the starting point.

PSA-NCAM immunocytochemistry

Fixed sections were incubated for 20 h at 4 °C with anti-PSA-NCAM primary antibody (mouse monoclonal IgM anti-PSA-NCAM; Millipore, Cork, Ireland) diluted 1 : 1000 in antibody diluent (50 mL PBS, 0.5 mL goat serum and 0.5 g bovine serum albumin; Sigma-Aldrich, Arklow, Ireland). Following 3 h dark incubation with 1 : 100 secondary antibody (FITC-conjugated goat anti-mouse IgM; Sigma-Aldrich), sections were counterstained for 2–3 s with 40 ng/mL propidium iodide (nuclear counterstain; Sigma-Aldrich) in PBS and mounted in fluorescence enhancing medium (Vectashield®; Vector Laboratories, Peterborough, UK). Appropriate positive and negative controls were performed. Hippocampus slices were stained for positive control. Omission of the primary antibody

and its replacement by a mouse IgM isotype control (Mouse IgM (MOPC-104E) purified immunoglobulin raised in mice; Sigma-Aldrich) were used as negative controls. Slides were examined using a fluorescence microscope (CM 3050 S; Leica, Hickville, NY, USA). The areas of interest -2 mm lateral from Bregma in the right and left hemisphere were imaged, saving eight random images per hemisphere for the calculation of the numeric density of PSA-NCAM positive cells and three additional images, that in combination covered all cortical layers, for the measurements of cell location. The set of sequential slices was stained with Cresyl violet (Sigma-Aldrich) and light microscopy images for the measurements of the cortical layers were taken.

Numeric density of PSA-NCAM positive cells

A stereological counting frame (250 x 250 µm), with inclusion and exclusion boundaries, was placed over each image to facilitate counting PSA-NCAM positive cells. To simplify the analysis of a large number of images, images were combined in a video file, overlaid with the counting frame and imprinted with the frame number using VirtualDub (virtualdub.org). PSA-NCAM positive cells were defined as those with at least 50% green staining. The observer was blinded to the group and hemisphere identity of the image. Position of PSA-NCAM positive cells A montage of three images was created to span the whole thickness of the cortex and the distance of each PSA-NCAM positive cell from the surface was noted. Data were expressed in a density map. j-means cluster analysis was used to sort the PSA-NCAM positive cells into clusters. For creation of density maps and cluster analysis, SPSS version 21 (IBM, New York, NY, USA) was used. The thickness of the cortical layers in the area of interest was measured to allow localization of the PSA-NCAM positive cells.

Statistical analysis

In electrophysiological experiments, the channel showing the maximal amplitude was selected for detailed analysis. Using Spike2 (C.E.D., Cambridge, UK), amplitudes and latencies were measured. For further analysis, the percentage change was calculated in relation to baseline values. A two-way repeated-measure ANOVA with Bonferroni post test was used to analyze the independent effect of SNM within data modelled over repeat time points.

For immunohistochemistry, each brain had a stimulated and unstimulated hemisphere, as SNM was applied on the left only and therefore served as its own control. The frequency of PSA-NCAM positive cells was Poisson distributed and counts from both hemispheres were compared by a Poisson test. Poisson distribution was confirmed using a Chi-squared test in Excel (Microsoft®, Redmond, WA, USA). Poisson tests were performed using R Studio (Boston, MA, USA). In a second step, the numerical density of PSA-NCAM positive cells was calculated. To control for possible

double counting of spherical objects in thin slices, the Abercrombie correction factor was applied. This was deemed appropriate because the objects of interest (nuclei) are approximately spherical and the section thickness is constant for a left to right hemisphere comparison.

Data are presented as mean ± SEM. Analyses were performed using proprietary software (GraphPad Prism 5; GraphPad Software Ind., La Jolla, CA, USA), with statistical significance defined at the $p < 0.05$ level.

RESULTS

Electrophysiological experiments

Cortical EPs were recorded successfully in all animals. The anesthetic level was stable during each experiment. Blood pressure after completion of surgeries, after 1 h of balloon inflation and at the end of the experiment was 96 mmHg ± 2 mmHg, 89 mmHg ± 2 mmHg and 88 mmHg ± 2 mmHg, respectively. Values were within physiological limits. Evoked potentials consisted of one upward deflection followed by a less marked downward deflection. At baseline, the first deflection lasted from 10.3 ms ± 0.4 ms to 161 ms ± 8.3 ms with its maximum at 40.1 ms ± 1.8 ms with an amplitude of 52.1 µV ± 4.4 µV. The second deflection had its minimum at 289 ms ± 12 ms and 30.1 µV ± 2.3 µV. The area under the first deflection was 3890 µVms ± 350 µVms and the slope was 0.23 ± 0.03 (example trace in Fig. S1). Evoked potential amplitudes in the sham group were unchanged; the coefficient of variation over the 150 min was 7% ± 1%. Evoked potential amplitudes declined gradually during 60 min balloon inflation. In the recovery period (after balloon deflation and 2 Hz/14 Hz SNM), EP amplitudes recovered partially in the balloon injury group, to baseline in the 14 Hz SNM group and above baseline in the 2 Hz SNM group (Table 1, Fig. 1A–C). Statistical analysis showed important differences between the groups. Evoked potential amplitudes decreased at the same rate in all three groups with balloon injury. In the recovery period after balloon deflation, amplitudes in the 14 Hz SNM group were significantly higher than in the balloon injury group and at the same level than in the sham group, while amplitudes after 2 Hz SNM were significantly higher than after 14 Hz SNM, in the sham and control group (two-way repeated-measure ANOVA and Bonferroni post test: Group factor $F(3, 364) = 31.14$, $p < 0.0001$; Time factor $F(13, 364) = 43.47$, $p < 0.0001$; Interaction: $F(39, 364) = 8.19$, $p < 0.0001$). Onset latencies were longer during balloon inflation and shortened again after injury deflation. They were unchanged in the sham group (Fig. 1D); the coefficient of variation over the 150 min was 12.6% ± 1.6%. In comparison to the sham group, onset latencies were significantly longer at the end of balloon inflation in all groups with balloon injury (two-way repeated-measure ANOVA and Bonferroni post test: Group factor $F(3, 364) = 5.51$, $p = 0.004$; Time factor $F(13, 364) = 10.68$, $p < 0.0001$; Interaction: $F(39, 364) = 2.21$, $p < 0.0001$.

Immunohistological experiments

Control experiments showed PSA-NCAM positive cells in the innermost part of the granule cell layer of the dentate gyrus and no green staining was observed after either omission of the primary antibody or after application of an isotype control (Fig. S2). The frequency of PSA-NCAM positive cells counted per field of view (250 lm 9 250 lm) was Poisson distributed (Chi-squared test, $p > 0.05$). The mean number of cells/250 lm 9 250 lm square is given in Table 2A. The number was increased in the right (stimulated) hemisphere of both SNM groups (Poisson test, $p < 0.001$), but not in the sham or balloon injury group ($p = 0.43$, $p = 0.74$, respectively). Using the mean diameter of the nuclei, the Abercrombie correction factor was applied (Supplemental formula 1). The resulting numerical density of PSA-NCAM positive cells (Table 2B) was likewise increased in the 14 Hz and 2 Hz SNM groups (paired Student's t-test, $p = 0.01$, $p = 0.02$ respectively), but not in the sham or balloon injury groups ($p = 0.53$, $p = 0.55$ respectively, Fig. 2). The distribution of PSA-NCAM positive cells over the cortical layers between stimulated and unstimulated cortices was different (Fig. 3 and Fig. S3). PSA-NCAM positive cells could be sorted into three clusters for both stimulated and unstimulated cortices. The cluster centers were located 230 µm ± 13.4 µm, 793 µm ± 27.3 µm, and 1261 µm ± 26.1 µm from the surface, corresponding to layers II, IV and V. In unstimulated cortices, the cluster in layer V was most prominent. The number of cells per cluster increased in stimulated cortices for each cluster, with the largest increase in the layer II cluster (+271% and +124% in the 14 Hz and 2 Hz group, respectively). Changes in stimulated cortices after 14 Hz and 2 Hz stimulation were similar.

DISCUSSION

Acute SNM restored anal EPs diminished by pudendal nerve injury and, in the model used, 2 Hz SNM was more effective than 14 Hz SNM. The immunohistological study of PSA-NCAM confirmed the presence of synaptic plasticity in the somatosensory cortex involving layers II, IV, and V. There was no difference in synaptic plasticity between 14 Hz and 2 Hz SNM.

Electrophysiological experiments

Two main factors were studied, EP amplitude as the primary outcome and onset latency, which represents the main outcome measure in human EP studies.[11,12] Although EP amplitudes show substantial inter-animal variations, they can easily be studied in an acute setting using the baseline recording in each animal as the reference point. Reproducibility in any one animal was good. Evoked potential onset latency, on the other hand, is more difficult to study in rodents than in humans due to the very short distance between stimulation and recording site.

The balloon injury model of FI has been shown to cause pudendal nerve damage leading to transient muscle atrophy,[13] axonal degeneration of sensory fibers[10] and also a long-term reduction in somatosensory cortical activation.[14] In a behavioral model, rats displayed signs of fecal continence by showing place preference for depositing fecal pellets.[15] Balloon injury has been shown to cause FI, as measured by an incontinence index (non-latrine defecation rate/total defecation rate) in a subpopulation (1/3) of rats for 3 weeks following injury.[16] Therefore, it may adequately represent conditions of neuropathic FI. In this study, somatosensory EPs after balloon injury were consistently reduced with partial recovery after balloon deflation. The reason partial recovery was seen may be due to the fact that a few nerve fibers were transiently and reversibly injured.

From a translational point of view, reduced anal sensation plays a vital role in the pathobiology of FI[17,18] and an improvement of anal sensation after commencement of SNM has been described previously.[19] In addition, specific changes in sensory cortical activation following SNM have been demonstrated in patients suffering urinary or FI using positron emission tomography[20] and pudendal EPs recordings.[11,12] Overall, loss and regain of anal sensation may play a major role in FI and successful SNM. This is the first time that the reduction in sensory function and its subsequent restoration have been demonstrated in an animal model.

The effect of stimulation frequency was also investigated. The authors have previously shown 2 Hz to be the optimal stimulation frequency on SNM in an acute rodent model.[8] The current electrophysiological experiments support those results. Stimulation at 2 Hz SNM caused a 30% greater increase in EP amplitude.

Some limitations need to be considered. Firstly, results were derived from a rodent model. An animal model was utilized because it facilitated invasive physiological testing. Secondly, only a brief experimental period was examined in comparison to the chronic nature of SNM therapy. While initial plasticity leading to LTP happens within the first 2 hours and may be appropriately represented, nerve injury is present chronically in patients with FI before commencement of treatment and may therefore be more difficult to imitate. It remains to be determined if the dramatic improvement in the anal evoked EPS by SNM ultimately affects fecal continence. To further explore this key translational question, chronic experiments of implanted rats will be required in addition to a working model of chronic FI. The latter hurdle has been surmounted by our group recently[16] and we are actively exploring the former, i.e., the effects of longterm neuromodulation.

Immunohistological experiments

Expression of PSA-NCAM in the somatosensory cortex was equally increased for 14 Hz and 2 Hz after SNM. PSA-NCAM positive cells were clustered in layers II, IV, and V, and each cluster showed a substantial increase after SNM with the largest increase in the layer II cluster. Positive and negative control experiments were performed (Fig. S2). Plentiful PSA-NCAM staining could be observed in the innermost part of the granule cell layer of the dentate gyrus. Two negative control experiments were performed: deletion of the primary antibody and an isotype control. Statistical considerations (number of slices, number of fields of view) have been taken into account and the observer was blinded to group and hemisphere affiliation. PSA-NCAM expression did not change in the balloon injury control group. A study showed that PSA-NCAM expression was increased 24 h after loss of input by dorsal rhizotomy.[22] There are two possible explantation, why no change in PSA-NCAM was observed in this study: (i) the time was too short for significant changes to take place; and (ii) the loss of signal was incomplete. There was no significant relationship between changes in SEP amplitude and PSA-NCAM cell density (linear regression, $p = 0.82$ [14 Hz] and $p = 0.72$ [2 Hz]).

A previous study demonstrated an increase in the numerical density PSA-NCAM positive cells by 50% after acute SNM.[7] In this study, the degree of increase was larger (150% for 14 Hz and 125% for 2 Hz), which may reflect the longer recording protocol in this study which allows for more time for PSA-NCAM translocation to the cell surface. In contrast to electrophysiological findings, 14 Hz and 2 Hz SNM had equal effects on the neuroplasticity marker.

The study of the location of PSA-NCAM positive cells identified three areas of interest: layer II, IV, and V. Layer IV, which is most prominent in unstimulated cortices, is the primary receiving site for thalamocortical projections of somatosensory afferents.[23,24] Ascending cortical projections from layer IV mainly travel to layers II/III,23 but connections to all other cortical layers exist.[25,26] Neurons in layer V are well connected within all other layers, project to association areas,[27] and are integrated in downstream feedback loops.[28] Signals from layer II, which shows the greatest increase in neuroplasticity after SNM, are relayed to other intra-hemispherical areas,[29] of which the secondary somatosensory cortex and the motor cortex are the most important.[26,30] Sacral neuromodulation may therefore increase cortical activity in cortical association centers in the same hemisphere or cause translocation of the cortical representation of the anorectum.

Limitations of this study are that only the primary receiving area of the cortex was investigated; further transmission of these signals was not studied and, as only one plasticity parameter was investigated, the transmitters and cell types involved were not explored.

From a clinical perspective, a better understanding of the mechanism of action of SNM is essential in improving patient selection, optimize stimulation protocol, and reduce treatment failures. A recent

review found SNM successful in 63% of patients only in the short term, if data were analyzed on an intention-to-treat basis.[31] Possible causes for suboptimal treatment may be poor patient selection and suboptimal historically unchanged stimulation parameters.

CONCLUSION

This study gives further insights into the mechanism of action of SNM. For the first time, the restorative action of SNM on diminished anal sensation was shown and this observation was linked to the induction of LTP. The superiority of 2 Hz to 14 Hz SNM in previous rodent experiments was confirmed in electrophysiological experiments, although frequency had a similar action on neuroplasticity as measured by PSA-NCAM. This plasticity was mainly located in areas relaying signals to different cortical association areas of the same hemisphere.

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FUNDING : This paper represents independent research funded by Science Foundation Ireland (grant 11/RFP/3115). The authors acknowledge additional funding from the Royal College of Surgeons, the Bowel Disease Research Foundation and HEFCE. In addition, a research agreement with Medtronic Inc. to use customized rodent leads for SNM was in place for this study.

DISCLOSURE: The authors make the following disclosures. Charles H. Knowles has received remuneration for expert panels, speaker bureau and consultancy from Medtronic Inc., Uroplasty Ltd, and Shire. P. Ronan O'Connell has received remuneration for expert panels and consultancy from Medtronic Inc. and James F.X. Jones has been supported by Medtronic Inc. (2012-2014).

Table 1: Percentage change in evoked potential (EP) amplitudes at baseline, after 60 min balloon inflation and in the recovery period. Note the decrease in the groups with balloon injury and the different levels of recovery

Group	Baseline (10 min)	After 60 min balloon inflation (70 min)	Recovery period (100–150 min)
Sham group	-0.8% ± 2.0%	-0.8% ± 3.7%	7.2% ± 1.0%
Balloon injury group	-0.8% ± 2.2%	-71.1% ± 6.4%	-35.1% ± 2.0%
14 Hz SNM group	1.9% ± 1.2%	-67.8% ± 7.1%	-2.8% ± 3.6%
2 Hz SNM group	3.3% ± 2.5%	-67.1% ± 5.1%	33.5% ± 3.9%

Table 2: (A) Mean number of polysialylated neural cell adhesion molecule (PSA-NCAM) positive cells/250 µm x 250 µm square (data shown as mean ± SEM). A Poisson test was performed. (B) Numerical density of PSA-NCAM positive cells (data shown as mean ± SEM). A paired Student's t-test was performed.

Group	Sham group	Balloon group	14 Hz SNM group	2 Hz SNM group
(A) Cells/250 µm x 250 µm square				
Left hemisphere	0.88 ± 0.07	1.15 ± 0.08	1.02 ± 0.07	0.75 ± 0.06
Right hemisphere	0.95 ± 0.07	1.77 ± 0.07	2.46 ± 0.10	1.55 ± 0.09
p-value	p = 0.43	p = 0.74	p < 0.0001	p < 0.0001
(B) Cells/mm^3				
Left hemisphere	790/mm^3 ± 110/mm^3	930/mm^3 ± 130/mm^3	800/mm^3 ± 110/mm^3	440/mm^3 ± 180/mm^3
Right hemisphere	810/mm^3 ± 90/mm^3	950/mm^3 ± 160/mm^3	2000/mm^3 ± 220/mm^3	990/mm^3 ± 300/mm^3
p-value	p = 0.53	p = 0.55	p = 0.01	p = 0.02

Figure 1: (A) Development of maximal EP amplitude over time in the SNM restoration, balloon injury and Sham group (N = 8). Note the gradual decrease in EP amplitude during balloon inflation in the balloon injury and SNM restoration group. Evoked potential amplitude recovers to baseline after 14 Hz SNM and above the baseline after 2 Hz SNM; (B) Average traces (N = 8) from the balloon injury and 14 Hz SNM group. Traces from baseline (blue), after 1-hour balloon inflation (red) and during the recovery period (green) are shown. (C) Exemplary color maps from the balloon injury and 14 Hz SNM group. Three time points are shown: baseline, after one hour balloon inflation and recovery. (D) Development of EP onset latency over time in the SNM restoration, balloon injury, and Sham group (N = 8). Note the increase in onset latency during balloon inflation in the balloon injury and SNM restoration group.

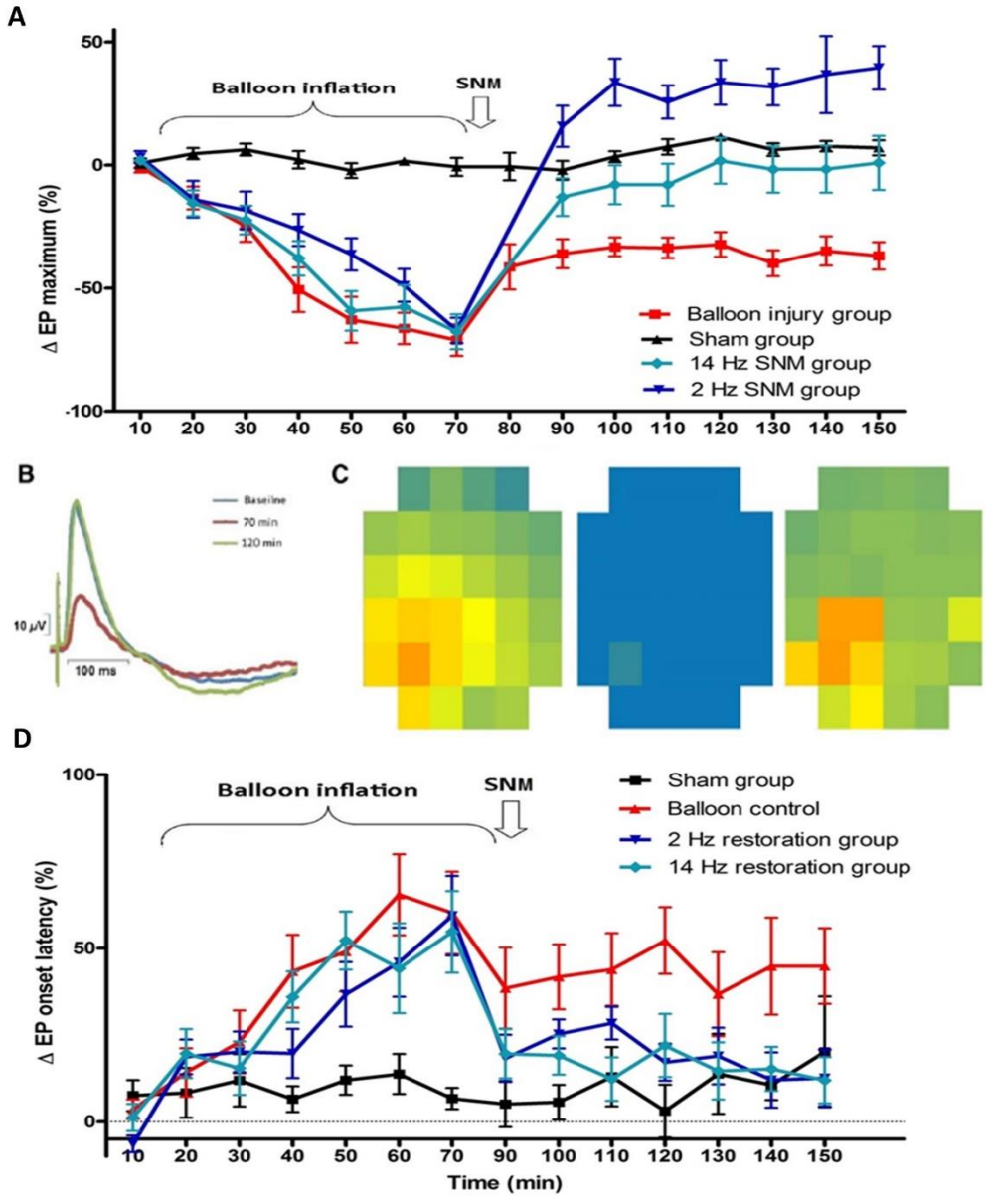

Figure 2: Corrected numerical density of PSA-NCAM positive cells. Note the increase in PSA-NCAM positive cells in the two SNM groups.

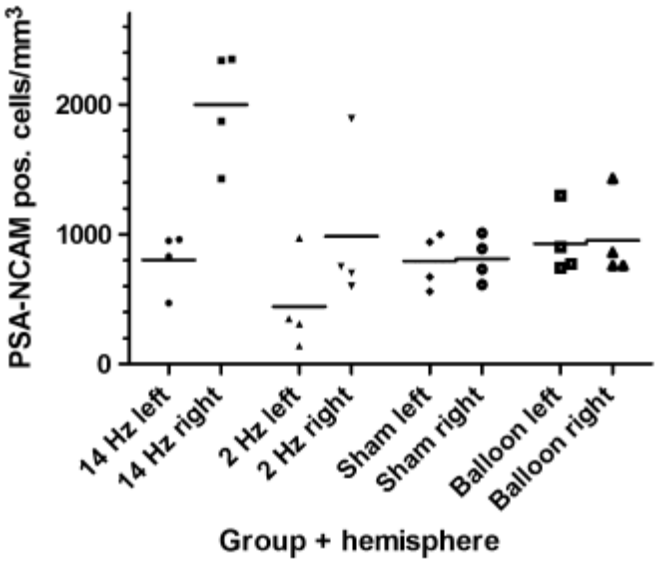

Figure 3: (A) Image of anti-PSA-NCAM immunohistocytochemistry spanning the whole thickness of the somatosensory cortex. Nuclei are stained red and PSA-NCAM green. (B) Examples of PSA-NCAM positive cells. Cells with green staining surrounding greater than 1/2 of the nucleus are counted as positive. (C) Nissl stain of the whole thickness of the somatosensory cortex overlaid by PSA-NCAM positive cell distribution exemplary for the 14 Hz SNM group. Note the increased frequency of PSA-NCAM positive cells in the stimulated right cortex in layer II, IV, and V.