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Atlanto-occipital catheterization of young rats for long-term drug delivery into the lumbar subarachnoid space combined with *in vivo* testing and electrophysiology *in situ*

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Highlights

- A method of lumbar catheterization of young rats (3-week-old) is described.
- The high catheterization success rate was demonstrated for young rats (about 80%).
- No adverse effects on peripheral sensitivity, animal locomotion or anxiety were found after spinal catheterization.
- Whole-cell recordings from sensory interneurons *in situ* are demonstrated following spinal treatment with genetic material *in vivo*.
- The technique is feasible and useful for studies both *in vivo* and *in situ.*

Abstract

BACKGROUND: Catheterization has been widely used in neuroscience and pain research for local drug delivery. Though different modifications were developed, the use of young animals for spinal catheterization remains limited because of a little success rate. A reliable technique is needed to catheterize young animals aimed for *in vivo* testing combined with spinal cord electrophysiology, often limited by animal age, to facilitate pain research.

NEW METHOD: We describe intrathecal catheterization of young rats (3-week-old) through atlanto-occipical approach for long-lasting drug delivery into the lumbar subarachnoid space. The technique represents a surgical approach of minimized invasiveness that requires PE-10 catheter and few equipment of standard laboratory use.

RESULTS: Behavioral assessments revealed that spinal catheterization does not change peripheral sensitivity of different modalities (thermal and mechanical) and gives no rise to locomotive deficit or anxiety-like behavior in young rats. The long-term administration of

genetic material (oligodeoxynucleotides given up to 4 days), examined both *in vivo* and *in situ*, produced no adverse effects on basal peripheral sensitivity, but changed the AMPA receptor-mediated currents in sensory interneurons of the spinal cord.

COMPARISON WITH EXISTING METHODS: Dissimilar to already described methods, the method is designed for the use of young rats for behavioral testing *in vivo* and/or spinal cord electrophysiology *in situ*.

CONCLUSIONS: A practical method for spinal catheterization of young animals designed for studies *in vivo* and *in situ* is proposed. The method is rapid and effective and should facilitate investigation of therapeutic effects on both systemic and subcellular levels, as an advantage over the existing methods.

Keywords

Spinal catheterization; intrathecal delivery; young animals; genetic material; oligodeoxynucleotides; local treatment; spinal cord electrophysiology; sensory interneurons; behavioral testing; peripheral sensitivity.

1. Introduction

Intrathecal catheterization has been widely used for local delivery of compounds of interest into the spinal cord and different methodological modifications were developed to prompt higher success rate in various species (Yamashita et al., 2003, Malkmus and Yaksh, 2004, Federici et al., 2012, Lambertini et al., 2015, Hou et al., 2016, Mazur et al., 2017). The main benefits of the technique is local delivery of a drug to avoid systemic blood circulation and reduce high doses required to achieve efficient drug concentrations associated with a risk of developing side effects (Humphreys et al., 2005, O'Donnell and Iohom, 2008). Among advantages, there is a capability for delivery of genetic materials, which could not cross the blood brain barrier due to a large molecular weight (hundred thousand Da). Catheterization also yields long-term delivery of a drug whenever repeated injections needed in much less invasive way as compared with technically challenging punctures into the subarachnoid area, associated with a risk of damage to the spinal cord.

Despite advantages, the procedure of spinal catheterization via atlanto-occipital membrane could cause postoperative mortality and neurological deficits that prompted up methodological excellence through modifying catheters (Pogatzki et al., 2000) or using needles for a catheter guidance in rodents (Storkson et al., 1996, Wu et al., 2004). A high catheterization success rate has been demonstrated for adult rats (LoPachin et al., 1981, Malkmus and Yaksh,

2004), whereas it remains considerably low for young and small animals, e.g., mice (Wu et al., 2004, Oladosu et al., 2016). The young animals, however, are of a particular interest for electrophysiological studies of the spinal cord where recordings become limited with aging of experimental animals (e.g., whole-cell recordings from vulnerable sensory interneurons in slices or the whole spinal cord demand by up to 3-4-week-old rats (Voitenko et al., 2004, Szucs et al., 2009). Though the emerging call, a technique for spinal catheterization of young animals has not yet been described whilst would benefit pain research with intracellular recordings from spinal interneurons following-up behavioral assessments *in vivo* after local treatment.

Here we provide the detailed description of intrathecal catheterization of young rats (3-week-old) through the atlanto-occipital membrane and figure out few technical improvements how to avoid typical complications that could arise upon and following the procedure when catheterizing the small-sized animals. The method does not require special equipment and can be readily implemented for routine use to facilitate studies of central pain processing.

2. Methods

2.1. Animals

Male Wistar rats (20 ± 3 day-old) were used in this study (Fig. 2A), whose body weight was 40 ± 10 g (n = 114 rats in total, including 18 control, non-catheterized animals). All animal procedures were approved by the local Animal Ethics Committee in Bogomoletz Institute of Physiology (Kyiv, Ukraine) and were in accordance with the European Commission Directive (86/609/EEC) and ethical guidelines of the International Association for the Study of Pain.

2.2.Materials and tools for intrathecal catheterization

For the procedure of intrathecal catheter implantation following tools and instruments are required:

- PE-10 polyethylene tube (ID 0.11", OD 0.24"),
- scales for laboratory animals,
- stereotaxis or head holder,
- shaver (cream) for removing fur,
- scalpel (blade size #11 or #15),
- 2 forceps (one with wide branches, another with tiny ones),
- slant tip tweezer,
- silk suture,
- scissors,
- cotton (or cotton-tipped applicators),
- ethanol and betadine (or other antiseptics of choice).

2.3. Pre-surgical preparation

Standard PE-10 polyethylene tubing (Instech Solomon, PA, USA; Fig. 1A) was used in the study. We cut PE-10 tubing to make a near 10 cm long catheter; tube cuts of blunt ends are more preferable than those of slant shapes to reduce a risk of stabbing tissue when inserting catheter (Fig. 1B). A knot was tied at approximately the middle of the tube and tightened up to have one arm of the catheter of slightly longer length (near 6 cm long that is an external part) and a shorter another one (~4 cm long – an internal part; Fig. 1C-D). In such length, the internal part of catheter would reach lumbar spinal segments, as confirmed in young rats (n = 96 catheterized animals) with the caudal end of the catheter located within Th_{13}-L_2 spinal segments. Before implantation, we used to stretch the internal arm of the catheter to make tube straight that enables managing its insertion in less compressive and more controllable manner. To check out that there is no leakage or any occlusion, catheters were flushed with saline before implantation.

2.4. Anesthesia

Animals were weighed and individual doses of anesthetics were prepared (Fig. 2). For anesthesia of young rats we used a mixture of ketamine (70 mg/kg) and xylazine (15 mg/kg) given intraperitoneally. Other anesthetics, either liquids or inhalators, are a subject of choice. The depth of anesthesia was confirmed with the loss of corneal reflex. At this point, it might be helpful to double verify that the length of the internal part of catheter fits an anesthetized animal, reaching 0,5 cm caudally the last ribs (measured from the atlanto-occipital membrane on the neck) and adjust the length, if needed (note that the internal arm has to be stretched up when fitting on animal's back).

2.5. Intrathecal catheterization

Anesthetized rat was placed in a stereotaxic frame and animal's head was securely fixed with the ear bars. For spinal catheterization the fixation is secured when animal's head is positioned symmetrically, allowing head movements only in up and down directions without any lateral move; the body trunk locates perpendicularly, in a right angle with animal's head. Fixation in other way may increase a risk of damage to spinal cord upon implantation, resulting in neurological deficits post-operatively. To prevent animal's eyes to get dry, an ophthalmic ointment was applied. Skin on the animal's head back was shaved out (3 x 2 cm area) and swiped with ethanol. A 1-cm longitudinal incision was made through skin below the nape where the occipital bone terminates, caudal to the neck (Fig. 3A-B). The occipital crest muscles were gently retracted out to expose the atlanto-occipital membrane. For muscle retraction we use sharp forceps, not cutting muscles with a scalpel. Once the atlanto-occipital membrane had been exposed, surrounding tissues were kept using the wide-branch forceps in left hand (Fig. 3C)

while small incision was made in the membrane (1-2 mm long) with a scalpel in right hand. We prefer to make incision in very caudal part of the membrane since inserting catheter at that area diminishes a risk of damage to the spinal cord. In young animals, the atlanto-occipital membrane is thin and gentle, therefore no hook is needed for opening dura, in opposite to adult animals (Malkmus and Yaksh, 2004). Opening of the atlanto-occipital membrane is confirmed with a cerebro-spinal fluid coming up immediately through incised membrane. Then, the catheter was placed into the subarachnoid space at the rostral level of the spinal cord. The procedure of intrathecal insertion requires catheter to be kept in a straight position, parallel to the spinal cord, as close to the dorsal side as possible (Fig. 3D). A proper insertion into the thecal space is fairly validated by a cerebrospinal fluid flow through tube following-up insertion. A sudden resistance might be felt at any point – no forced pressure should be applied there to avoid any damage to the spinal cord. In a case of resistance, the catheter needs to be taken up to a previous position and further inserted down in a slightly different angle. Catheter should be inserted until a knot reached the neck muscles (Fig. 3E). Afterwards, suture ligatures were applied to tighten muscles, but catheter knot shall remain right above the muscles to prevent any damage to the atlanto-occipital membrane. Finally, the external part of the catheter was tunneled throughout skin to exit on animal's head (between ears) using sharp forceps or a needle (20G or others) to puncture skin. Incised skin is closed (2-3 suture ligatures usually suffice; Fig. 3F) and treated with betadine (Egis Pharmaceutical PLC, Hungary).

The surgery typically takes 10-15 min for each animal. The surgical approach is bloodless – appearance of blood at any step indicates tissue damage upon operation. Animals were kept postoperatively on a warm surface (28-30 ^{0}C) until fully recovered from anesthesia. Catheterized animals were housed separately and monitored for their behavior and health conditions before being taken into experiments. A period for full animal recovery, including complete wound healing, typically takes 3 days. From the day 1 post-surgery, catheter has to be flushed with sterile saline once a day, daily, to avoid possible occlusion.

2.6. *The Hargreaves plantar test for measuring the thermal peripheral sensitivity*

The Hargreaves technique was used to measure peripheral sensitivity of rats to the thermal stimulus (heat) as described recently (Kopach et al., 2016). Briefly, after an animal habituated to a Plexiglas chamber, a radiant heat was applied to the middle of the plantar surface of one hind paw (Ugo Basile Model 7370 Plantar Test). The light beam was automatically turned off when animal lifted its paw. The time between starting the stimulus and animal lifted its paw – the withdrawal latency – was measured, which reflects the thermal nociceptive threshold. Trial was repeated 3–5 times with an interval between measurements 3 to 5 minutes and values were

averaged. We examined catheterized animals for their peripheral sensitivity on the day 5 post-surgery. The age-matched naïve animals, quasi-randomly sampled, were used as control. Animals were eliminated after termination of experiments with overdose of anesthetics.

2.7. Assessment of mechanical peripheral sensitivity with von Frey's monofilaments

The method of von Frey monofilaments was used to measure mechanical peripheral sensitivity in rats, as described earlier (Kopach et al., 2016). Briefly, after an animal was habituated to an experimental chamber located on an elevated mesh screen, von Frey monofilaments of different intensity of stimulus (Bioseb) were applied to each hind paw. Applications were repeated 10 times for each hind paw with an interval between stimulations for at least 2 min for each tested filament. The percentage of responses was calculated for every trial and averaged; it was defined as the paw withdrawal threshold. Catheterized animals were examined for peripheral mechanical sensitivity on the day 5 post-surgery. The age-matched naïve animals, quasi-randomly sampled, were used as control.

2.8 The open-field test for animal locomotion and anxiety

For the assessment of general activity and locomotion of young animals after spinal catheterization the open-field test was performed, as described in details recently (Kopach et al., 2016). Briefly, an animal was placed in the open-field arena, a 75 x 75 x 40 cm wooden box with a digital camera (Logitech C270) attached above to record animal relocations within the box. The total distance explored by an animal for the defined period of time (5 minutes) was calculated.

Anxiety is a commonly used readout of side effects, which could rise following treatment. Anxiety in rodents is typically characterized by suppressed exploratory activity of animals those avoid entering the arena center (open area) whilst keep within the box corners or travel close to the walls (Kopach et al., 2016). We measured both parameters of anxiety-like behavior – the number of crossing the arena center and the time spent within the central area. Catheterized animals were examined for their locomotive activity on the day 5 post-surgery. The aged-matched naïve animals were used as control.

Tests were performed within the same daily period for different experimental groups to avoid (or minimize) an influence on behavior assessment with daily activity of laboratory animals.

2.9. Dye injection

To validate the delivery of a drug into the subarachnoid space in lumbar segments through implanted catheter we used morphological dye Alexa Fluor 594 (Molecular Probes, USA). Catheterized animals were given a single injection of Alexa (10 μl, 100 μM), flushed with saline (10 μl). The spinal cord was examined for the Alexa-mediated fluorescence shortly after injection of the dye (~15 min post).

2.10. Spinal administration of genetic material

For long lasting spinal treatment with genetic material the antisense (AS) oligodeoxynucleotides (ODN) specific to PKC subtype α were used with the following sequence, 5'-GACATCCCTTTCCCCCTCGG-3'. Missense (MS) oligodeoxynucleotides with the sequence of 5'-CGTCCTCAGTCGTCCCTCAC-3' were used as control. Genetic material was intrathecally delivered through implanted catheter with a 25-gauge needle connected to a 25 μl Hamilton syringe. Animals received intrathecal injections of AS ODN or MS ODN (10 μg/10 μl) once a day for 3 days. The reduced expression of PKCα protein at the lumbar enlargement segment following such a treatment has been confirmed in our previous studies (Kopach et al., 2013).

Animals were assessed for their peripheral sensitivity before initiating a treatment with genetic material and then every day following (treatment of a 3 day-duration), with measurements performed once a day, daily.

2.11. Spinal cord slice preparation

Spinal cord slices were prepared from catheterized animals those received spinal treatment with genetic material (see above section 2.10) according to methodology described in details previously (Kopach et al., 2013, Kopach et al., 2015). Briefly, the spinal cord was quickly dissected out and placed in an ice-cold dissection solution that contained (in mM) 250 sucrose, 2 KCl, 1.2 NaH_2PO_4, 0.5 $CaCl_2$, 7 $MgCl_2$, 26 $NaHCO_3$, 11 glucose, oxygenated with 95% O_2 and 5% CO_2. Transverse slices (350-μm thick) were cut with a HA 752 vibratome (Campden Instruments, Loughborough, UK). Slices were maintained at room temperature in a physiological Krebs bicarbonate solution that contained (in mM) 125 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 2 $CaCl_2$, 1 $MgCl_2$, 26 $NaHCO_3$, 10 glucose, oxygenated with 95% O_2 and 5% CO_2 (pH 7.4).

2.12. Electrophysiology in situ

Whole-cell recordings were made from lamina II DH interneurons using an Axopatch 200B amplifier controlled with pClamp 9.2 software (Molecular Devices, USA). Neurons were visualized with an infrared optics using a ×60.09 water-immersion objective on an Olympus BX50WI upright microscope (Olympus, Japan). Patch pipettes (resistance of 5 MΩ) were filled with an internal solution containing (in mM) 130 Cs-methylsulfonate, 10 NaCl, 10 EGTA, 2 $CaCl_2$, 10 HEPES, 5 QX-314, 0.1 spermine tetrahydrochloride, 2 Mg-ATP, and 0.1 Na-GTP (pH 7.2). The membrane resistance was constantly monitored by applying a short hyperpolarizing pulse (–5 mV).

The AMPA receptor (AMPAR)-mediated currents were induced by a selective receptor agonist, AMPA, bath applied in the continuous presence of TTX (0.5 μM), cadmium chloride (100 μM), APV (50 μM), bicuculline methiodide (10 μM), and strychnine hydrochloride (2 μM). To prevent a fast desensitization of AMPARs, the agonist was applied in the continuous presence of cyclothiazide (20 μM). Typically, one neuron was studied *per* slice. For quantification of changes in the current-voltage relationship of the AMPAR-mediated currents the rectification index (RI) was calculated as the peak current amplitude at +30 mV to the peak current amplitude recorded at –50 mV, as we described in details earlier (Kopach et al., 2013).

2.13. Statistics; power analysis

The data sets were probed for normality using the Shapiro-Wilk test. Since the data sets have been normally distributed, the results are presented as mean ± standard error of the mean (SEM) with n referring to the number of animals / cells tested. Student's t-test was used to determine statistical difference between experimental groups using OriginPro software and Excel package. A *p* value of less than 0.05 was considered as statistically significant.

Power analysis was performed to determine the sample size required to detect an effect of spinal catheterization on peripheral sensitivity and/or animal locomotion, if any, in young animals. The analysis determined that in order to detect 10% changes in either thermal threshold value (peripheral sensitivity) or total distance value (animal locomotion), the group size required to be 21 and 30, respectively, to give a degree of confidence 90%.

Results

The group of catheterized animals in this study consisted of 53 rats, including 3 animals those did not recover from surgery/anesthesia (~6%) and 7 rats those developed neurological deficits post-operatively (~13%). Neurological deficits were forelimb paresis, chromodacryorhea ('bloody' eyes) or tremor, which animals displayed post-surgery. Examination of those animals revealed compression, in some cases lesion in the spinal cord, produced by catheter. Therefore,

the effectiveness of intrathecal catheterization of young rats counted ~ 81% (43 catheterized animals without any deficit out of 53). None animal withdrew the catheter after implantation.

3.1. The lumbar catheter location for intrathecal delivery

For the assessment of lumbar placement of catheter in young rats and local delivery of a drug into the subarachnoid space we used morphological dye Alexa Fluor 594 (see Methods for details). A robust Alexa-mediated fluorescence was observed within the lumbar spinal cord, the area where implanted catheter ended, when examining the spinal cord shortly after dye injection (n = 3 rats; Fig. 4B).

Necroscopic analysis confirmed the catheter location within the lumbar thecal space (n = 50 catheterized animals for post-euthanasia analysis). The catheter was predominantly positioned within the lateral spinal cord that counted up ~ 62% (31 out of 50 animals), with less occurring placement at the dorsal area that estimated ~24% (12 out of 50 animals).

3.2. Catheterization changes neither thermal nor mechanical peripheral sensitivity of young rats

To examine if spinal catheterization could give rise to adverse effects and impair the peripheral sensitivity of young animals, we tested out basal peripheral sensitivity of different modalities – thermal and mechanical. The Hargreaves plantar test demonstrated no differences in the thermal nociceptive sensitivity of animal's hind paw between catheterized animals and control group. The average thermal threshold was 14.6 ± 0.3 s (n = 21) for catheterized animals and 15.0 ± 0.3 s (n = 18; $p > 0.3$) for their age-matched control on the day 5 post-surgery (Fig. 4C).

The method of von Frey monofilaments demonstrated no changes in peripheral sensitivity to mechanical stimulation between catheterized animals and control group. The average mechanical threshold was 2.8 ± 0.4 g (n = 24) for catheterized animals and 3.0 ± 0.4 (n = 18) for their age-matched control ($p > 0.8$; Fig. 4D). Thus, spinal catheterization does not change basal peripheral sensitivity of different modalities in young rats.

3.3 Catheterization gives no rise to locomotive deficit and animal anxiety

Next, we performed the open-field test to examine if spinal catheterization gives a rise to locomotive deficit and/or anxiety in young animals. No changes in general activity of young animals were observed after catheterization. There was no difference in animal locomotion between catheterized and control animals (Fig. 5A). The total distance of animal's movement was 25 ± 1 m (n = 30) for catheterized rats and 26 ± 1 m (n = 18, $p > 0.3$) for control group (Fig. 5B). There were also no signs of anxiety in young animals after catheterization. Catheterized rats

actively explored novel surroundings and freely entered the centre of arena (Fig. 5A, see Video recording). There were no significant changes in any tested parameters for the anxiety-like behavior – the number of animal crossing the arena center ($p > 0.5$; Fig. 5Ci) and the time spent within the central area ($p > 0.1$; Fig. 5Cii) – after catheterization.

3.4. Long term spinal administration of genetic material does not change basal peripheral sensitivity

Given that spinal catheterization of young animals produces no changes in their basal peripheral sensitivity and gives no rise to common side effects (e.g., locomotive deficit and/or anxiety), we finally examined whether intrathecal drug administration through implanted catheter would affect peripheral sensitivity of catheterized animals following treatment with genetic material for a prolonged period of time (3-day-duration treatment, see Methods for details). Intrathecal administration of AS ODN or MS ODN (daily injections for 3 days) did not change basal peripheral sensitivity of young animals for the whole period. There were no changes in the thermal threshold in both experimental groups at every time-point tested, as compared with "0" time (before initiating a treatment) or between groups (n = 14 rats treated with AS ODN and n = 7 rats treated with MS ODN, $p > 0.01$; Fig. 6A).

3.5. Genetic inhibition of spinal PKCα changes the AMPAR-mediated currents in sensory interneurons

Finally, a capability of catheterized animals after prolonged spinal treatment *in vivo* for the reliable spinal cord preparation aimed for electrophysiological studies *in situ* was validated with acute spinal cord slices prepared from catheterized animals following long-lasting genetic treatment with AS ODN or MS ODN. We enabled the acute spinal cord slice preparation for the intracellular recordings made from lamina II DH interneurons in whole-cell configuration (see Methods for details). Consistent to our previous findings (Kopach et al., 2013), electrophysiological parameters of the DH interneurons did not differ between AS ODN- and MS ODN-treated groups for the resting membrane potential, input resistance, capacitance, and series resistance of patched interneurons (data nor shown). However, we found changes in the AMPA-induced currents in lamina II interneurons after spinal administration of AS ODN (genetic inhibition of PKCα). The AMPA-induced currents demonstrated a linear current-voltage relationship in the AS ODN-treated group, whereas the opposite inward rectification was observed at positive potentials in the MS ODN-treated group (Fig. 6Bi). The rectification index of the AMPAR-mediated currents (see Methods for details) was 0.94 ± 0.11 (n = 9 cells) for the

AS ODN-treated group, but 0.51 ± 0.08 (n = 7 cells; $p < 0.01$) for the MS ODN-treated group (Fig. 6Bii). The difference indicates changes in the proportion between Ca^{2+}-permeable and Ca^{2+}-impermeable AMPARs in DH interneurons by local genetic inhibition (knocking down) of spinal PKCα. These results are consistent with our previous findings of the role of PKCα in AMPARs trafficking in DH interneurons, demonstrated for both synaptic (Park et al., 2009) and extrasynaptic pools of AMPARs (Kopach et al., 2013).

4. Discussion

The present study described in details the technique of spinal catheterization of young rats (3-week-old) aimed for combined use of the animals for both *in vivo* (testing the therapeutic effects of a drug) and *in situ* studies (electrophysiology of the spinal cord), as advantage over the existing methods. The technique represents a surgical approach of minimized invasiveness, which is simple and prompt, bloodless and could be performed without specialized equipment (for instance, monitoring the cardiorespiratory parameters that could be implemented, if necessary). The effectiveness of the described methodology is high (more than 80% catheterization success rate). This rate is similar to that reported for mice (Oladosu et al., 2016), although it remains lower comparing with the modified methods for catheterization of adult rats where success rate may raise up to 95-98% (Malkmus and Yaksh, 2004). Such discrepancy is due to a little thecal space in young animals that complicates catheter placement by challenging the procedure with highly increased risk of spinal cord damage in the small-sized animals. In order to boost catheterization success rate, the use of smaller gauge catheter might be helpful, as suggested (Pogatzki et al., 2000). Nevertheless, we routinely use standard PE-10 tubing for catheterization of young rats, achieving a success rate about 80%. To obtain this rate, we elaborated few technical improvements for the atlanto-occipital catheterization, those have been particularly essential for implanting catheter into young animals, as described in the study. In the atlanto-occipital approach, it is crucial positioning of the animal's head in right angle to body trunk with further catheter insertion in a very straight direction, and towards the dorsal side. The insertion should follow without any sharp pressure applied over the entire procedure.

Behavioral assessments *in vivo* using different tests for measuring peripheral sensitivity of different modalities (thermal, mechanical) and locomotive activity (locomotion, anxiety-like behavior) confirmed that spinal catheterization gives no rise to development of any deficit in young animals. Neither thermal nor mechanical peripheral sensitivity changed after spinal catheterization. The open-field test, a robust assay for the assessment of general activity and anxiety (as a readout of side effects that could develop following treatment), demonstrated no changes in locomotion and exploratory behavior of young animals after catheterization. Thus, no evidence for locomotive deficit and/or anxiety status has been found in catheterized rats.

Furthermore, the fluorescence detection within the lumbar spinal cord confirmed localized delivery of a compound of interest through implanted catheter into the thecal space. Altogether, our results prove that the described technique is reliable for testing fine therapeutic effects of localized drug treatment *in vivo*.

Our whole-cell recordings from sensory interneurons in acute spinal cord slices prepared from catheterized animals after prolonged spinal treatment *in vivo* have also confirmed the use of the animals for reliable spinal cord preparation and following-up electrophysiological studies *in situ*. Interneurons of the superficial dorsal horn (lamina I-II) represent a highly vulnerable population for *in situ* preparation, often limited by aging of experimental animals that compromised obtaining of intracellular recordings from elder animals (Voitenko et al., 2004, Szucs et al., 2009). Therefore, the demonstrated capability of acute spinal cord preparations from catheterized animals to obtain whole-cell recordings from sensory interneurons provides substantial advantages of the described technique over the existing methods since enables investigations on subcellular level after long-term spinal treatment *in vivo*. The changes observed in sensory interneurons *in situ* (the AMPAR-mediated currents) following treatment with genetic material *in vivo* (knocking down of spinal PKCα) are consistent with our previous findings of the role of spinal PKCα in AMPAR trafficking in DH interneurons in persistent inflammatory pain conditions (Kopach et al., 2013). Together this confirms the use of catheterized animals for localized treatment (e.g., gene silencing of spinal PKCα (Kopach et al., 2013) to investigate the precise intracellular mechanisms of pain processing in central pathways.

Summarizing, our data demonstrate that the described technique of spinal catheterization of young animals provides the reliable use of animals for combined testing of therapeutic effects *in vivo* and consistent electrophysiological studies *in situ* to yield benefits of exploring effects on both systemic and subcellular levels to facilitate pain research and beyond.

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Fig. 1. Photographs of PE-10 tubing used in the study (A) and a fabricated catheter of 10-cm long cut (B) with a knot made approximately at the middle (C), tightened then out (D).

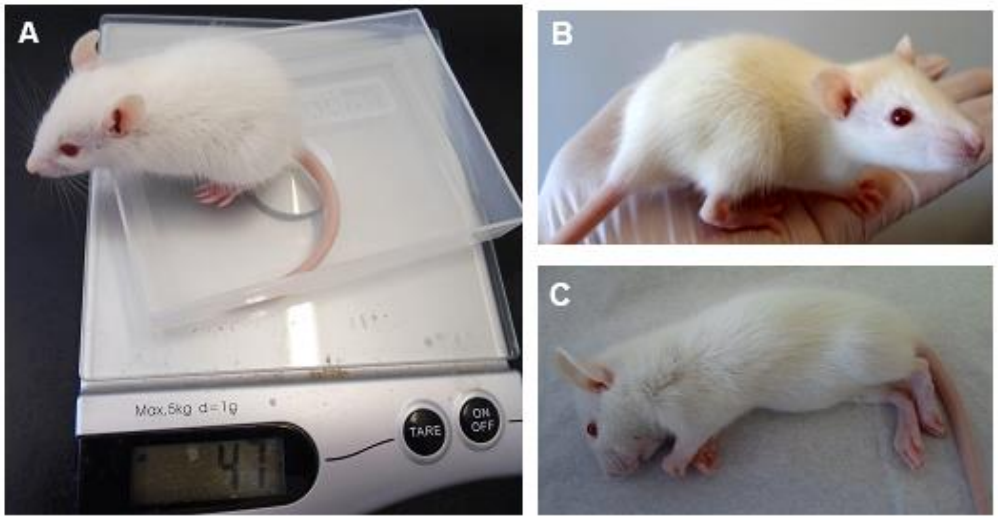

Fig. 2. Photographs of a 19 day-old rat with weight of 47 g (A) before (B) and after intraperitoneal injection of a mixture of ketamine and xylazine (C).

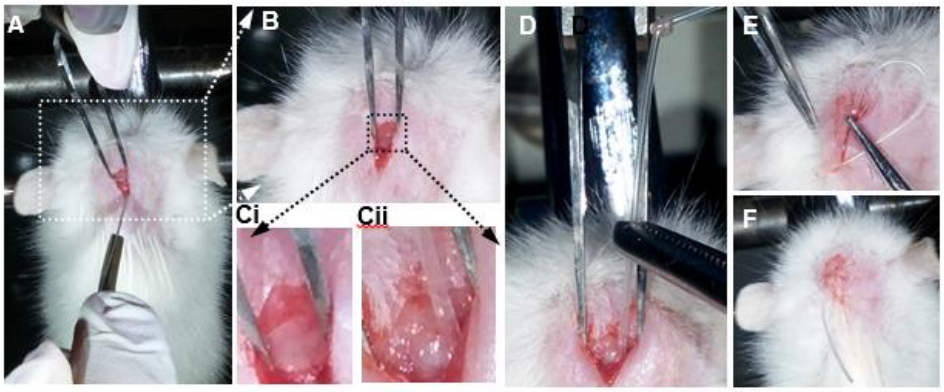

Fig. 3. Photographs depicting the procedure of intrathecal catheterization of a 19 day-old rat. After an animal's head was fixed, one incision in skin was made as shown in A, and muscles were retracted to expose the atlanto-occipital membrane, as demonstrated (B-Ci). A small cut was made in the caudal part of the membrane, where catheter was inserted intrathecally down (Cii-D) until a knot reached muscles (E). Catheter was anchored beneath skin, leading its external part through skin to exit out (F).

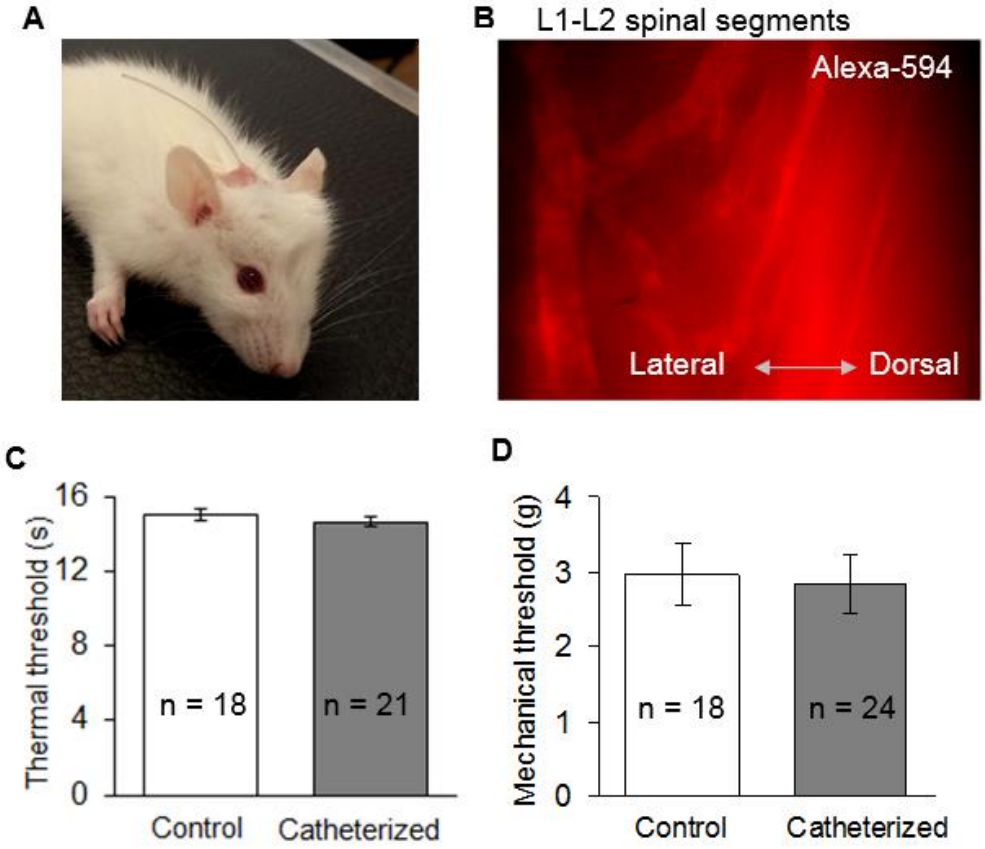

Fig. 4. Spinal catheterization changes neither thermal nor mechanical basal sensitivities of young animals.

(A) Photograph of a 19 day-old young rat shortly after intrathecal catheterization. **(B)** Epifluorescence imaging of the lumbar spinal cord after dye injection (Alexa-594, 100 µM / 10 µl). **(C)** Measuring of the thermal nociceptive threshold revealed no changes in the peripheral thermal sensitivity between catheterized animals (n = 21 rats) and the age-matched control (n = 18 rats). **(C)** The plantar measurements of the mechanical threshold with von Frey monofilaments showed no difference in the peripheral mechanical sensitivity between catheterized animals (n = 24 rats) and control (n = 18 rats). Data are expressed as mean ± SEM.

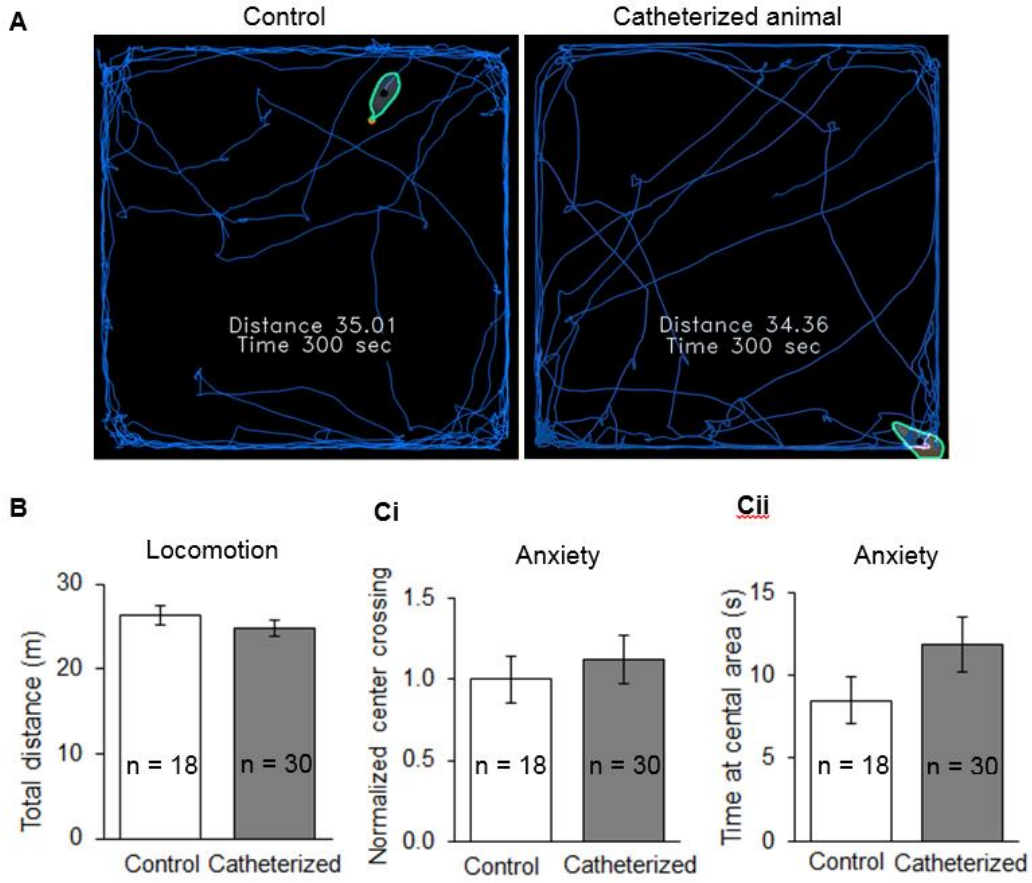

Fig. 5. Spinal catheterization gives no rise to locomotion deficit or anxiety-like behaviour of young rats.

(A) The open-field test analysis snapshots taken from a control animal (left) and a catheterized rat (right) demonstrate no difference in locomotor activity between animals. Sketches of the animal movement trajectory and the total distance indicated are for 5 min-duration recordings. **(B)** Average distance explored by animals during the open-field test demonstrates no difference between catheterized animals (n = 30 rats) and their age-matched control (n = 18 rats). Data are expressed as mean ± SEM. **(C)** The parameters for anxiety-like behaviour, the crossing of arena centre (Ci) and the time spent within the central area (Cii), are similar between catheterized animals (n = 30 rats) and control (n = 18 rats), indicating no anxiety after catheterization. Data are normalized to control for Cii and present as mean ± SEM for Cii.

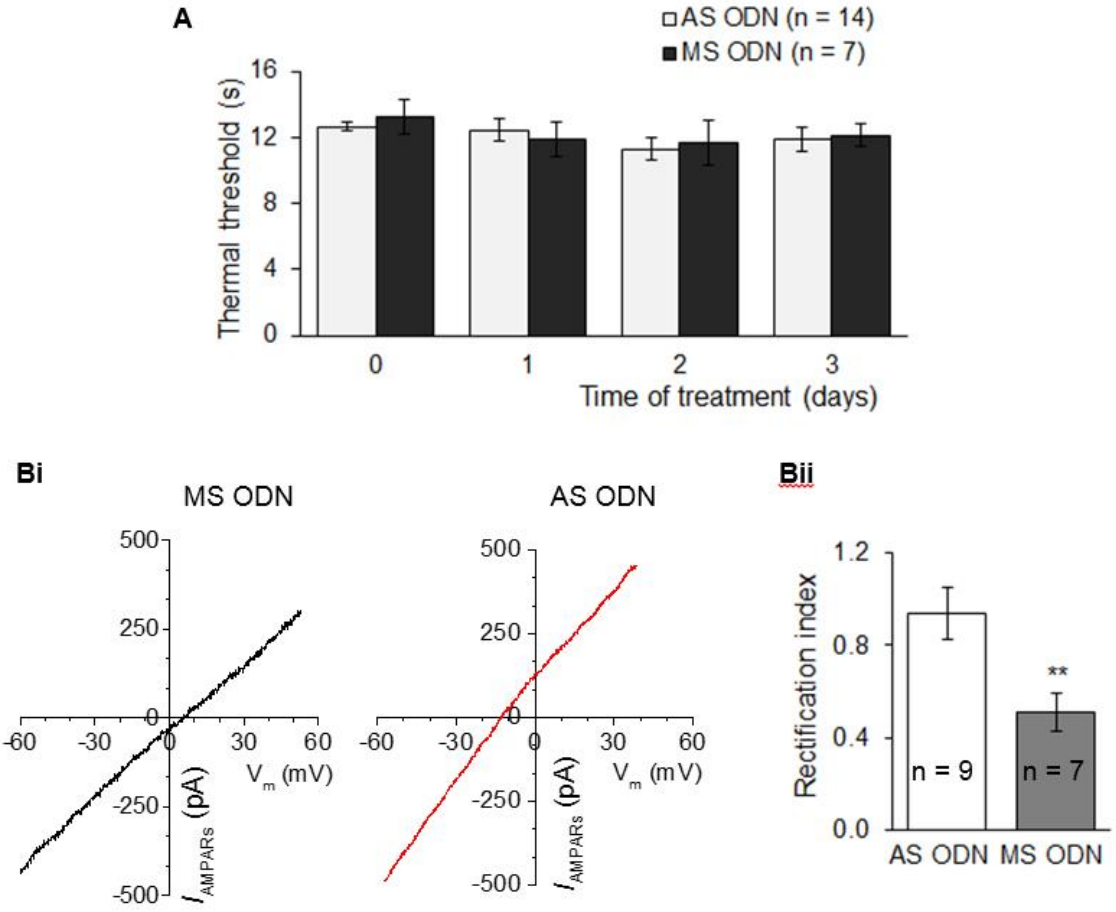

Fig. 6. Long-lasting intrathecal administration of genetic material does not influence basal peripheral sensitivity *in vivo*, but changes the AMPA-induced currents in sensory interneurons.

(A) The thermal nociceptive threshold in catheterized animals remains at similar level before and following spinal treatment with antisense (AS) or missense (MS) oligodeoxynucleotides (ODN) specific to PKCα. Data are expressed as mean ± SEM, n = 14 rats treated with AS ODN and n = 7 rats for MS ODN. **(Bi)** Whole-cell recordings from the lamina II dorsal horn interneurons in spinal cord slices *in situ* revealed the difference in the current-voltage relationships of the AMPAR-mediated currents between experimental groups given spinal treatment with MS ODN (left) and AS ODN (right). **(Bii)** The difference in current-voltage relationships was expressed by quantifying the rectification index of the currents at different membrane potentials (+30 mV to –50 mV). Data are expressed as mean ± SEM, n = 9 neurons from AS ODN-treated group and n = 7 neurons from MS ODN-treated groups. ** $P < 0.01$ (unpaired *t*-test).