A Custom Made Electrode Construct and Reliable Implantation Method That Allows for Long-Term Bilateral Deep Brain Stimulation in Mice

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Objectives: The underlying mechanisms behind the therapeutic and side effects of deep brain stimulation (DBS) need further investigation. The utilization of transgenic mouse lines is a suitable approach to better understand the cellular and network effects of DBS. However, not many bilateral DBS studies have been conducted in mice. This might be due to a lack of commercially available bilateral DBS constructs.

Materials and Methods: We developed an approach to perform repetitive long-term DBS in freely moving mice. In this study, we implanted an in-house custom-made DBS construct containing two bipolar concentric electrodes to target the subthalamic nucleus (STN) bilaterally. Subsequently, we stimulated half of the animals with clinically relevant parameters three to five times a week with a duration of 20 min for ten weeks. Several behavioral tests were conducted of which the open field test (OFT) is shown to validate the reliability of this electrode construct and implantation method. Furthermore, we performed fiber photometry measurements to show the acute effect of STN-DBS on serotonin network activity in the dorsal raphe nucleus.

Results: Repetitive DBS and long-term behavioral testing were performed without complications. STN-DBS resulted in an increase of the distance traveled in the OFT and a reduction of calcium levels in serotonergic neurons of the dorsal raphe nucleus. None of the mice had lost their electrodes and postmortem evaluation of the tissue showed accurate targeting of the STN without excessive gliosis.

Conclusion: The DBS electrode construct and implantation method described can be used for long-term DBS studies to further investigate the mechanisms underlying DBS.

Keywords: DBS, deep brain stimulation, electrodes, implantation, method, mice

Conflict of Interest: The authors have no conflicts of interest to declare.

INTRODUCTION

Deep brain stimulation (DBS) is a dynamic area of translational neuroscience and used to treat motor symptoms in patients with Parkinson’s disease (PD) (1), other movement disorders (2,3), and nonmotor symptoms for instance in patients with obsessive-compulsive disorder (4,5). Recently, DBS is being explored as a potential therapy in other neurological and psychiatric disorders, including Alzheimer’s disease (6), depression (7,8), and addiction (9). Despite the beneficial effects of DBS in movement disorders, these studies show variable clinical outcomes and side-effect profiles. Therefore, it is important to gain more knowledge about its underlying mechanisms of action. DBS has been used in different neural targets using a variety of stimulation parameters, but its effect on a molecular, biochemical, and neural network level remains poorly understood.

A good way to study these underlying mechanisms would be by using transgenic animal models. Most transgenic animal models are created by using mice embryos, because mice are technically the easiest species for generation of transgenic lines (10). By using transgenic mice, the effects of DBS can be studied in different disease models. Furthermore, Cre transgenic mouse lines in combination with state of the art techniques, such as Optogenetics and Designer Receptors Exclusively Activated by Designer Drugs, give the opportunity to manipulate genes or cell types during DBS. However, not many bilateral DBS studies have been performed in freely moving mice. In the few bilateral DBS studies that have been done before, either a monopolar electrode...
or a bipolar twisted electrode was implanted per hemisphere (11–14). Both types of electrodes are not ideal since the current spread is too big in monopolar stimulation and twisted electrodes might bend, untwist, and distort brain tissue leading to reduced accuracy of the implantation and more brain damage. So far, a bilateral DBS construct with high-quality concentric bipolar electrodes is not commercially available.

Therefore, we developed a custom-made electrode construct and implantation method to be able to perform long-term bilateral DBS experiments. Using this technique, we were able to perform DBS and behavioral testing on a daily basis over the course of three months without losing any electrodes. In the present study, we show a systematic approach to perform DBS experiments in mice to stimulate further research into the mechanisms underlying the therapeutic and side effects of DBS.

MATERIALS AND METHODS

Overall Study Design

A proportion of patients with PD will develop adverse neuropsychiatric symptoms following subthalamic nucleus (STN)-DBS treatment, such as depression and anxiety (15,16). The overall experimental approach of this study was to investigate the therapeutic and side effects of STN-DBS in ePet-cre mice. Therefore, a behavioral test battery was performed in all mice to identify changes in locomotion, mood, and anxiety (timeline is shown in Fig. 1). To investigate possible changes in serotonin network activity that might be related to mood problems during STN-DBS, fiber photometry was used. To specifically record from serotonergic neurons in the dorsal raphe nucleus, ePet-cre mice (JAX stock #012712) were used in combination with a cre-dependent GCaMP6s virus. To perform such a long-term DBS experiment, it was necessary to establish a good method for implanting a high-quality DBS construct in mice. Therefore, this paper focusses on the developed DBS-construct and implantation method used for this study. Only data of the open field test (OFT) and fiber photometry measurements of one example animal are shown to validate the reliability of the DBS-construct.

Animals

Experiments were performed on ePet-cre male mice (JAX stock #012712). Of the 53 mice that were implanted, 49 completed the whole study of three months. Mice were socially housed under controlled conditions (21 ± 2°C and 40-60% humidity) in a reversed 12 hours day/night cycle (lights on, 7 pm) until they had received surgery. Mice were given ad libitum access to food and water. At the time of surgery, mice were six months of age. All tests and DBS took place under red light conditions during the active phase of the animals. Experiments were conducted according to the directive 2010/63/EU for animal experiments and in agreement with the Animal Experiments and Ethics Committee of the Maastricht University, Maastricht, The Netherlands.

DBS Electrode Construct

Several stimulation paradigms and electrode types are possible with experimental DBS. Stimulation with bipolar electrodes is the most appropriate for targeting the small brain structures in rodents. The concentrated current around the tip of bipolar electrodes will influence a smaller tissue area compared to monopolar stimulation in which electrons will diffuse from the negative pole through the way of least resistance. For this reason, we used a custom-made DBS construct with a bipolar concentric electrode for each hemisphere, which was manufactured by the engineering department (IDEE) of Maastricht University (Fig. 2).

The bipolar electrodes used for this DBS construct are commercially available and clinically used for electromyography recordings in human subjects (TE/D125-101, Technomed, Beek, The Netherlands). The bipolar electrode has an outer diameter of 300 μm and a tip diameter of approximately 50 μm. Apart from the 75 μm exposed tip, the whole electrode is insulated. The gold-coated electrode has a stainless steel outer part, which functions as a positive contact, and a platinum-iridium inner part, which functions as the negative contact. The distance between the positive and negative contact is 50 μm. The desired length of the bipolar electrodes was fixed in a custom-designed print plate with an adjusted interelectrode distance. The inner wire and outer surface of the needles were soldered to the print plate. Thereafter, connecting pins were soldered on the print plate. Melted plastic was used to cover and insulate the connections. The edges of the construct were trimmed, making it as small as possible. A little Plexiglas u shape was added onto the construct for a perfect fit with the skull of the mouse. A dental adhesive and light-cure flowable composite was used to create a stable and strong attachment between the Plexiglas u shape and the small skull of the mouse. A matching DBS construct holder was fabricated for easy and accurate stereotaxic implantation (shown in Fig. 3C).

Figure 1. Experimental timeline. Timeline showing the sequence of events and behavioral testing. The arrow shows the application of DBS (and sham stimulation in the control group) for three to five times a week for a duration of 20 min over the course of ten weeks. DBS was given during behavioral testing or in the homecage. [Color figure can be viewed at wileyonlinelibrary.com]
The electrode construct can be modified according to different targets and experimental designs. Both unilateral and bilateral stimulation are possible using this construct. The electrode length can be adapted to target any brain region of interest. For bilateral stimulation, the interelectrode distance can be modified as well. Furthermore, the addition of a recording electrode for electroencephalography (EEG) purposes is also possible (17). Another important advantage of this DBS construct is that only one connector is needed for connecting the mouse to the external device(s), regardless of number of implanted electrodes in the brain or on the skull, which makes connecting the animal easy and therefore less stressful. The total weight of this construct is 2 gm.

In this study, the STN was targeted bilaterally. Therefore, a construct was made with two bipolar electrodes having an inter-electrode distance of 3 mm and a length of 5.5 mm.

Stereotaxic Surgery

Buprenorphine 0.1 mg/kg was subcutaneously injected half an hour prior to surgery as an analgesic. Inhalational anesthesia was induced and maintained with isoflurane (Abbott Laboratories, Maidenhead, UK) at 4% and 1.5-3%, respectively. After adequate induction of the anesthesia, the mouse was placed in a small animal stereotaxic frame (Stoelting, Dublin, Ireland) and fixed by ear bars with zygoma ear cups (Kopf, Los Angeles, CA, USA) and a mouse gas anesthesia head holder (Stoelting). To maintain body temperature at 37°C throughout the whole procedure, the mouse was placed on a thermoregulator pad. An ocular lubricant was applied to prevent drying of the eyes. A subcutaneous injection of Lidocaine 1% (Streuli Pharma, Uznach, Switzerland) at the incision side was given for local anesthesia. After careful removal of hair and disinfection of the skin with betadine followed by 70% ethanol, a midline incision was made to identify the cranial sutures (e.g., bregma, lambda).

After leveling the skull, burr holes were made at the location of the brain region of interest by using a dental drill. The bipolar electrodes were targeted in the STN (AP $-2.00$ mm, ML $\pm 1.50$ mm, DV $-4.55$ mm) and the GCaMP6s virus and fiber (400 μm) were targeted in the DRN for fiber photometry measurements (AP $-4.7$, ML $-0.00$, DV $-2.9$ at a $32^\circ$ angle from the left) (18). For fiber photometry, 500 nL of AAV5.Syn.Flex.GCaMP6s.WPRE.SV40 was injected in the DRN using the Nanoject II (Drummond Scientific). Subsequently, the fiber and DBS construct were placed in their holders. The fiber and one of the bipolar electrodes were calibrated with bregma and positioned to the correct AP and ML coordinates. Before lowering them to their right DV coordinates, the skull was completely dried and OptiBond All-In-One (Kerr, Los Angeles, CA, USA) was used (Fig. 3a). This dental adhesive has etching, priming, and bonding properties, which prepare the skull for proper attachment to the flow able composite (Diadent, Almere, The Netherlands). The dental adhesive is air dried and light cured with UV light. Subsequently, composite was added in the middle of the area where the DBS construct would be placed on the skull before, lowering it to the correct DV coordinate (Fig. 3b). The composite under the construct is light cured from all sides (Fig. 3c). Multiple small layers of composite (2-3 mm) were added around the construct and light cured until there was a thick enascence. Next, the fiber was lowered until it reached the DV coordinate of the DRN and attached to the skull with the same composite. Afterward, the construct and fiber holder were removed and the skin of the mice sutured if necessary. After surgery, all mice were given a one-week recovery period prior to behavioral testing. During this period, their welfare was closely monitored and analgesic and anti-inflammatory agents were administered.

The electrode construct and dental composite add extra weight on top of the animal’s head. Besides, attaching and detaching the wires of the external stimulator may induce stress and alter behavior. Therefore, sham stimulation was performed as well. These animals were implanted with the same electrode construct.

Stimulation Protocol

To apply DBS, the electrode construct was connected to the stimulator by external wires and a swivel. The swivel allows the animal to move freely without the risk of cables being tangled up. A 3800 MultiStim 8-channel connected to Model 3820 Stimulus Isolation Units (A-M Systems, Carlsborg, WA, USA) was used for DBS. Animals of the DBS group were stimulated three to five times a week for 20 min over the course of ten weeks, either during behavioral tests or in their home cage. The sham animals were coupled to the external stimulator, but did not receive DBS.

Frequency, pulse width, current intensity, and pulse type are parameters that can be altered in DBS. Most animal studies use DBS parameters that are also used for treatment purposes in the clinic, which is a high-frequency stimulation (above ~100 Hz) and a pulse width between 60 and 90 μs (19-21). However, the current intensities used in animal studies are more variable. A very high current intensity can induce tissue damage and/or adverse behavioral side effects (22). Therefore, the maximum current intensity that can be used safely in neural tissue should be determined for the electrode used by charge density calculations (charge per phase divided by surface area of the electrode). The recommended charge density limit for DBS in clinical practice, which is also used in animal models, is 30 μC/cm² based on the Shannon model of neuronal damage (23-25). In addition, dose
response tests were performed with variable current intensities to determine which intensity elicits the desired behavioral response, but does not induce adverse behavioral side effects. In this study, monophasic stimulation at a frequency of 130 Hz, pulse width 60 μs, and a current intensity of 80 μA was used. The surface of the electrode is 0.021 mm², so the chosen parameters result in a charge density of 22.9 μC/cm², which is well below the limit of 30 μC/cm². Moreover, these parameters did not induce adverse behavioral effects.

Open Field Test
To test the effect of STN-DBS on locomotion, several behavioral tests were performed (Fig.1). In this manuscript, only the OFT is presented to show the validity and reliability of the electrode construct and implantation method. We performed the OFT two times in the same cohort of animals. The first time STN-DBS was only given prior to the OFT for a duration of 10 minutes and the second time STN-DBS was given during the OFT. The OFT arena consisted of a gray Plexiglas base (50 × 50 cm) and was divided into four equal arenas (25 × 25 cm each) separated by 25-cm-high black Plexiglas walls. A camera was placed above the center of the open field. Four mice were tracked simultaneously with a computerized system (Ethovision Color Pro, Noldus, The Netherlands) for 20 min. The floor of the open field was cleaned with ethanol after each session to prevent transmission of olfactory cues. Total distance moved was recorded for each animal.

Fiber Photometry
To investigate possible changes in serotonin network activity during STN-DBS, fiber photometry was used. Mice were connected to a Doric Lenses Inc. photometry setup by a 400 μm 0.48 NA patchcord. A 465 nm LED was coupled to a fluorescence mini-cube and a 1 × 1 fiber optic rotary joint to deliver light through the patchcord into the implanted optical fiber in the DRN. Light was delivered at an intensity of 40 microwatt at the tip of the patchcord. The emitted light that was collected from the brain was passed through the mini-cube and measured with a Newport 2151 Femtowatt Photoreceiver Module. To link the calcium-dependent fluorescent signal to DBS on or off epochs, the 3800 MultiStim 8-channel was directly connected to an analog input channel of the photometry console.

For the photometry experiment, mice could move freely in their home cage while they were coupled to both the patchcord and DBS cable. During the photometry measurements, mice were allowed to habituate for 5 min before DBS was given.
intermittently (2 min on – 3 min off) for ten trials. This means that one photometry measurement lasted for 55 min.

Electrode Localization

After the completion of experiments, verification of the electrode position was verified. At the end of the experiment, mice were deeply anesthetized with pentobarbital and transcardially perfused with tyrode buffer, followed by ice-cold 4% paraformaldehyde fixative in 0.1 M phosphate buffer. The electrode constructs were removed and the brains extracted from the crania. Brains were postfixed in 4% paraformaldehyde overnight and submerged in sucrose for cryoprotection (24 hours in 10% sucrose, thereafter 24 hours in 20% sucrose at 5°C). Coronal brain sections (20 μm) were cut on a cryostat, stained by hematoxylin and eosin (HE), and examined by an independent researcher. Animals with one or both electrodes misplaced were excluded from data analysis.

Data Analysis and Statistical Testing

Statistical analysis was performed using IBM SPSS Statistics for Windows (version 25.0, released 2017, IBM Corp., Armonk, NY, USA). Normality of OFT data was tested using the Kolmogorov-Smirnov test and homogeneity of variance was tested using the Levene’s test. The parametric data of the OFT were analyzed with the Student’s t-test and provided as mean ± standard error of the mean (SEM). A p-value ≤0.05 was considered significant.

Fiber photometry data were extracted, processed, and analyzed using custom MATLAB (Mathworks) scripts. The first two and a half minute of the data during the habituation period was discarded to remove the initial fast bleaching of the fluorescent signal. Next, the original sample rate of a 100 Hz was downsampled to 1 Hz and low-pass filtered using decimation. A two-term exponential model was fitted and subtracted from the decimated data to account for slow bleaching artifacts. Then, a single baseline fluorescence value (F₀) was calculated by averaging the fluorescent signals during the 60-sec time period pre-DBS. Subsequently, the normalized change in fluorescence (dF/F) was calculated as F − F₀/F₀. Data are presented as a heatmap of all single trials and an average plot with SEM.

A permutation test was used to analyze the statistical significance of the DBS-related fluorescent change. To compare the values of dF/F at each time point with the DBS-related fluorescent change, 10,000 permutations were used. An α-level of ≤0.05 was considered significant.

RESULTS

Histology

The surgical procedure was well tolerated by the operated animals. No complications such as infection, head/neck muscle injury, or intracranial ischemia were observed during or after surgery. Moreover, no behavioral abnormalities that could have been linked to internal brain damage were observed. At autopsy, macroscopic inspection of the scalp/skull did not indicate any sign of internal damage. Besides, there was no weight loss in both groups over the course of the study (data were presented during the reviewing process, but not shown here). After surgery, mice

Figure 4. a. Weight postsurgery. After an initial loss of 10% of their bodyweight, mice started to gain weight again from day four postsurgery onward. b. Electrode localization STN. The tip of the electrode is localized in the STN (black). The electrode shaft is schematically drawn to scale. 3 V, third ventricle; LV, lateral ventricle; cp, cerebral peduncle. [Color figure can be viewed at wileyonlinelibrary.com]

Figure 5. Open field test (OFT). Results of the OFT of the DBS group (n = 13) and sham control group (n = 12) are shown when DBS is given during the test (left) or shortly before the test (right). A significant increase in distance moved was seen when DBS is given during the test, while no differences between groups were found when DBS was given only shortly prior to the OFT in the same cohort of animals (**p < 0.01, independent samples t-test).
recovered fast. Initially, mice lost 10% of their weight during the first three days postsurgery (Fig. 4a). From day four onward, mice started gaining weight again and showed no signs of reduced welfare anymore. No electrodes were lost during this study. The location of the implanted electrodes was checked by histology. In 44 out of 49 mice, both electrodes were targeted correctly resulting in a targeting accuracy of approximately 90%. No excessive gliosis was found around the tip of the electrode (Fig. 4b).

Effect of STN-DBS on Locomotion

DBS settings for mice were selected based on our previous rat studies, but started off with a reduced current intensity of 100 μA (26–28). However, this current intensity was still too high and induced behavioral side effects. Therefore, stimulation was applied at a frequency of 130 Hz, pulse width 60 μs, and a current intensity of 80 μA. These settings did not induce abnormal behaviors during and in the absence of DBS such as seizures or turning. STN-DBS resulted in an increase of the distance traveled when given during the OFT (p < 0.01), but not when only given shortly before the test (Fig. 5).

Effect of STN-DBS on In Vivo Activity of 5-HT Neurons in the DRN Using the Genetically Coded Calcium Sensor GCaMP6s

Fiber photometry was used to find out if GCaMP6s fluorescence in 5-HT neurons of the DRN changes in response to STN-DBS. We found that upon the start of STN-DBS, the GCaMP6s signal decreases over time. As soon as STN-DBS stops, the fluorescent signal slowly goes back to baseline (Fig. 6). A permutation test revealed that this calcium related decrease in fluorescence was significant (p < 0.05). When STN-DBS was stopped, it took approximately 90 sec before the fluorescent signal recovered back to baseline.

DISCUSSION

By using a custom-made electrode construct containing two small bipolar concentric electrodes, we were able to target the STN of mice with high accuracy and perform DBS experiments over the course of three months. The combination of a dental adhesive and light-cured flowable composite ensures a strong bond between the electrode construct and the skull of mice, making it possible to perform DBS experiments for a long time. We performed these long-term DBS experiments without any problems regarding infections, loss of electrodes, or abnormal behavior. High-frequency DBS of the STN resulted in an increased distance traveled in the OFT, showing that DBS affects the basal ganglia network resulting in hyperactivity. Furthermore, STN-DBS also affected the serotonin network of the DRN by lowering the activity of 5-HT neurons, which might explain the occurrence or worsening of neuropsychiatric symptoms experienced by a proportion of PD patients following STN-DBS treatment (15,16).

The OFT is a widely used behavioral task to study general locomotor activity during DBS on and off conditions (29,30). The “distance moved” is a well-characterized readout parameter of the OFT that is affected in rodent models of PD which is (partly) restored by STN-DBS (31,32). Since STN-DBS improves locomotion in rodent models of PD, it is not surprising that it increases distance moved in healthy mice as well. The increase of activity in the OFT might be explained by activation of the motor cortex because of STN-DBS (31,33).

Our fiber photometry experiments showed that the calcium level in the serotonin network of the DRN was significantly lower during STN-DBS. This is in line with previous research in which STN-DBS inhibits the firing rate of 5-HT neurons of the DRN in anesthetized rats (28,34). Interestingly, there is no direct connection between the STN and DRN. Therefore, polysynaptic connections between the STN and DRN are likely to be involved. Future experiments are necessary to unravel which brain regions might play a role. Possible candidates might be the substantia nigra pars reticulata or the globus pallidus externa, because both brain regions project to serotonergic neurons of the DRN and receive direct inputs from STN (35,36).

Based on this study, it is possible to perform bilateral DBS experiments in mice for a long period of time. Therefore, mechanisms underlying DBS can be further investigated by using transgenic mouse lines. By using transgenic mouse models in combination with state of the art techniques (e.g., optogenetics, etc.), it might be possible to elucidate the roles of individual brain regions in PD.
fiber photometry, Designer Receptors Exclusively Activated by Designer Drug), molecular, cellular, and network manipulations can be applied and recorded to better understand the impact of DBS on the brain (37–39). This would not be possible in other animal species. Furthermore, several genetic mouse models for a variety of diseases are available nowadays, which can be used for preclinical testing of DBS (40).

A few studies have already been performed where bilateral stimulation was applied in mice. Either a monopolar electrode or a bipolar twisted electrode was implanted per hemisphere (11–14). The disadvantage of monopolar electrodes is that the current travels from the monopolar electrode through the brain to a ground electrode that is often attached to the skull or neck muscle (11,13), which means that the current spread is relatively big. Therefore, bipolar electrodes are preferred when targeting the small brain structures in mice. Based on our own experience, we know that twisted bipolar electrodes carry the potential to untwist inside the brain. In addition, twisting the electrodes can damage the insulation layer creating a short circuit. Furthermore, the rough shape of these electrodes might distort brain tissue during implantation. Moreover, the relative location of the anode and cathode is not consistent as well as the distance between them, as these twisted electrodes are cut arbitrarily. Another disadvantage is that the electrodes used in the aforementioned studies are not available as a bilateral construct. Therefore, one electrode has to be implanted per hemisphere and two connectors need to be attached to the skull. This can be difficult in brain regions where you need a small interelectrode distance. For these reasons, we developed together with the engineering department (IDEE) of Maastricht University the described bilateral electrode construct that can be adapted to the researcher’s wishes in terms of electrode length and interelectrode distance (Fig. 2). Furthermore, this construct provides a platform to incorporate additional stimulating, recording, and EEG electrodes, which can be connected to the other pins (17,41). Therefore, only one connector is needed to connect the construct to external devices. This results in a shorter handling time and therefore less stress for the animal, which is of course beneficial for the animal’s welfare and behavioral testing.

A limitation of our study is the use of monophasic stimulation. Monophasic stimulation can cause electrochemical damage and is therefore not often used in a clinical setting (42). In the majority of patients, charged balanced stimulation is used. Most external stimulators for rodents are not able to stimulate in a charged balanced manner. For this reason, most preclinical studies have been done using monophasic stimulation. Even though monophasic stimulation can induce tissue damage, in this study and previous studies of our group we have not seen this after histological examination (26,28). Moreover, the primary aim of this article was to introduce a reliable and reproducible electrode implantation method in mice. The DBS construct that we have described here can be used for all types of stimulation waveforms.

CONCLUSION

The implantation procedure and electrode-construct described here can be used to perform long-lasting studies in mice to unravel the mechanisms underlying DBS. We showed that it is feasible to perform a long-term bilateral DBS study in mice without complications. Combining this method of bilateral DBS in mice with genetic mouse models and state of the art techniques can contribute to gaining knowledge about the mechanisms of DBS and eventually clinical treatments of brain disorders.

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Authorship Statement

Drs. Sylvana Pol, Dr. Ali Jahanshahi, and Professor Yasin Temel designed the study. Drs. Sylvana Pol conducted the study, including data collection, and data analysis. Drs. Sylvana Pol prepared the manuscript draft with important intellectual input from Dr. Ali Jahanshahi and Professor Yasin Temel. All authors approved the final manuscript.

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REFERENCES


35. Pollak Dorocic I, Furth D, Xuan Y et al. A whole-brain atlas of inputs to serotonergic neurons of the dorsal and median raphe nuclei. *Neuron* 2014;83:663–678.


