Conditionally immortalised neural stem cells promote functional recovery and brain plasticity after transient focal cerebral ischaemia in mice

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Abstract Cell therapy has enormous potential to restore neurological function after stroke. The present study investigated effects of conditionally immortalised neural stem cells (ciNSCs), the Maudsley hippocampal murine neural stem cell line clone 36 (MHP36), on sensorimotor and histological outcome in mice subjected to transient middle cerebral artery occlusion (MCAO). Adult male C57BL/6 mice underwent MCAO by intraluminal thread or sham surgery and MHP36 cells or vehicle were implanted into ipsilateral cortex and caudate 2 days later. Functional recovery was assessed for 28 days using cylinder and ladder rung tests and tissue analysed for plasticity, differentiation and infarct size. MHP36-implanted animals showed accelerated and augmented functional recovery and an increase in neurons (MAP-2), synaptic plasticity (synaptophysin) and axonal projections (GAP-43) but no difference in astrocytes (GFAP), oligodendrocytes (CNPase), microglia (IBA-1) or lesion volumes when compared to vehicle group. This is the first study showing a potential functional benefit of the ciNSCs, MHP36, after focal MCAO in mice, which is probably mediated by promoting neuronal differentiation, synaptic plasticity and axonal projections and opens up opportunities for future exploitation of genetically altered mice for dissection of mechanisms of stem cell based therapy.

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Abbreviations: MHP36, Maudsley hippocampal murine neural stem cell line clone 36; MCAO, transient middle cerebral artery occlusion; CBF, cerebral blood flow; NAC, N-acetyl-l-cysteine; MAP-2, microtubule-associated protein 2; GFAP, glial fibrillary acidic protein; CNPase, 2′,3′-cyclic-nucleotide 3′-phosphodiesterase; IBA-1, ionised calcium binding adaptor molecule 1
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Introduction

Stroke has remained a leading cause of death and neurological disability worldwide in the past decades. According to the World Health Organization, 15 million people suffer stroke worldwide each year. Of these, 5 million die and another 5 million are permanently disabled (Mackay and Mensah, 2004). In the European Union, each year about 1.1 million people suffer a new stroke and currently 6 million subjects live, having survived a stroke (Mäkinen et al., 2006).
As calculated cost per patient, stroke is the third most costly neurological disorder (Makinen et al., 2006; Truelsen et al., 2005). There are currently no effective treatments to enhance functional recovery following stroke.

One of the most exciting frontiers in neuroscience is the potential use of stem cells for treating neurodegenerative disorders. Cell-based therapies are of particular interest in the central nervous system, because the mature brain has little capacity for self-repair. The plasticity exhibited by stem cells raises the possibility that they can be used to restore function compromised by brain insults, such as stroke (Makinen et al., 2006; Lindvall and Kokaia, 2004; Savitz et al., 2002). Initial studies using foetal tissue transplantation have been successful in ameliorating neurodegenerative deficits in animal experiments (Isacson et al., 1984) and this has been translated with some success in humans (Lindvall and Kokaia, 2005). However, further use of foetal tissue in treating human disease is problematic due to ethical considerations and a limited supply of tissue. In order to combat these problems, a variety of stem cell lines have been investigated for their potential in improving recovery in animal models of neurodegenerative disease. These include human bone marrow stromal cells (Chen et al., 2001), human neuroteratocarcinoma cells (Saporta et al., 1999) and conditionally immortalised neural stem cells (ciNSCs) (Sinden et al., 1997).

The ciNSC line, Maudsley hippocampal stem cell line clone 36 (MHP36), is derived from the embryonic stage 14 (E14) hippocampal proliferative zone of the temperature sensitive (tsA58) transgenic mouse, which constitutively expresses the tumour antigen (Tag) under the control of interferon-$\gamma$ (IFN-$\gamma$). MHP36 cells proliferate at low temperatures (33 °C–39 °C), however, the oncoprotein is inactive, the cells cease division and demonstrate the capacity to differentiate which is an important property of the cell lines which reduces the chance of producing tumours (Savin et al., 2002; Sinden et al., 1997). MHP36 grafts are well suited to repair the indiscriminate cell loss that occurs with middle cerebral artery occlusion because previously, they have been shown to have the capacity to differentiate into site appropriate phenotypes in response to host signals (Hodges et al., 2000; Gray et al., 2000; Wong et al., 2005; Modo et al., 2002a) and migrate to and engraf areas of damage in the host brain (Gray et al., 1999; Veizovic et al., 2001). Furthermore, they have been shown to reduce damage in several models of neurological impairment, including global ischaemia in mice (Wong et al., 2005), transient middle cerebral artery occlusion (MCAO) (Hodges et al., 2000b) and lesions to cholinergic forebrain projections (Gray et al., 2000) in rats but mechanisms involved still need to be clarified.

Previous MCAO studies of MHP36 cells have used rats and assessed functional recovery using behavioural tasks (e.g. neurological severity score, rotational bias, spontaneous activity, bilateral asymmetry test, water maze). Improvement within six weeks after administration has been demonstrated using the bilateral asymmetry test (Modo et al., 2002a), however recovery is incomplete. This is the first study that investigates whether MHP36 cells promote functional recovery after MCAO in mice utilising robust behavioural tests known to assess spontaneous forelimb use (Baskin et al., 2003; Fleming et al., 2004; Schallert et al., 2000; Li et al., 2004; Starkey et al., 2005) and foot faults (Farr et al., 2006; Metz and Whishaw, 2002; Riek-Burchardt et al., 2004). This in the future would allow a more mechanistic insight into how to maximise the function of stem cells by exploiting transgenic mice after stroke.

Results

Ischaemic deficits, neurological deficits and infarct volumes were equivalent across groups

We firstly ensured that all MCAO groups underwent similar stroke insults. Analysis of CBF did not show any difference between animals that later received vehicle and MHP36 stem cells, and CBF was reduced by $>85$–90% in all animals during MCAO when compared to pre-occlusion baseline values. Acute neurological deficit, evaluated using Clark’s deficit score, was significantly greater in both the general and focal scores post-MCAO when compared to sham animals and not significantly different in MCAO animals that received vehicle and MHP36 stem cells (Figs. 2A and B). At 28 days post-MCAO

![Figure 1](image-url)  
**Figure 1**  
Experimental regime used for the behavioural study. Mice were first handled before testing for 3 days each. The following day pre-surgery functional behaviour testing occurred for both tasks. Mice underwent focal MCAO (or sham surgery) on day 0 and were assigned a neurological score post-MCAO/sham on days 1, 3 and 4 using the Clark’s deficit score (dotted lines). Further, animals were tested for behavioural function (“post-surgery behaviour”) using ladder and cylinder tests on day 2 (prior to injection) and on days 7, 14 and 28.
there was no significant difference in lesion volume between the MHP36 (13.08 ± 1.3 mm³; n = 8) and vehicle (13.2 ± 1.97 mm³, n = 8) grafted mice. The topography of the lesion throughout the MCA territory is shown in Fig. 3.

ciNSCs improved foot-fault function using the ladder rung test

There were no significant placement errors observed on the ladder test in sham-treated animals (Fig. 4). Prior to MCAO, mice performed mainly correct placements with all four limbs. There was a significant increase in foot placement errors of the contralateral forelimb in MCAO mice at 2 days post-MCAO (vehicle, 4.029 ± 0.19; MHP36, 4.077 ± 0.13, p < 0.001) when compared to pre-MCAO (vehicle, −0.021 ± 0.15; MHP36, 0.024 ± 0.12) in both injection groups. Furthermore, this foot placement error was observed at 14 days post-MCAO (0.46 ± 0.1) and pre-MCAO score (p < 0.05). However, in the MHP36 group the scores between 28 days (−0.3 ± 0.16) and pre-MCAO baseline were no longer significant (ns) (Fig. 5). Furthermore, at 28 days the asymmetric score between MCAO+ vehicle and MCAO+ MHP36 animals showed a significant improvement in the MCAO+ MHP36 group (p < 0.001; Fig. 5).

ciNSCs improved asymmetric score using cylinder test

Spontaneous forelimb (cylinder) task results are shown in Fig. 5. In this task, the mean laterality score of the injured group, which measures preference for the forelimb unaffected by (ipsilateral to) the injury, peaked at 2 days post-MCAO time point. There were no significant asymmetries noted in the cylinder test in sham-treated animals (Fig. 5). There was a significant increase in left forelimb (ipsilateral) usage at 2 days post-MCAO (vehicle, 0.810 ± 0.19; MHP36, 0.767 ± 0.26, p < 0.001) when compared to pre-MCAO (vehicle, −0.021 ± 0.15; MHP36, 0.024 ± 0.12) in both injection groups. In the vehicle group there was a significant deficit between 28 days post-MCAO score (−0.3 ± 0.16) and pre-MCAO baseline were no longer significant (ns) (Fig. 5). Furthermore, at 28 days the asymmetric score between MCAO+ vehicle and MCAO+ MHP36 animals showed a significant improvement in the MCAO+ MHP36 group (p < 0.001; Fig. 5).

ciNSCs promoted integration, synaptogenesis and axonal projections

At 28 days post-MCAO staining of brain sections with neuronal marker MAP-2, revealed an increase in expression in the MHP36 group of animals when compared to the vehicle group (Figs. 6A and B) (Bregma: −0.38 ± 0.1 mm). Mean intensity of MAP-2 expression as well as the number of MAP-2 positive neurons was significantly increased in MHP36 versus vehicle animals (Fig. 6C). GFAP-positive astrocytes, in the peri-lesion region which included the cortex and caudate, showed a non-significant trend to decrease in the MHP36 group of animals when compared to vehicle (Figs. 6D, E and F) whereas CNPase-positive oligodendrocytes showed a non-significant trend to increase in the MHP36 group of animals versus vehicle (Figs. 6G, H and I) and there was no significant difference in IBA-1 positive activated-microglia cells in MHP36 versus vehicle groups (Figs. 6J, K and L).

At 28 days post-MCAO anti-Synaptophysin (Syn) immuno- staining was apparent in MHP36 grafted animals (Figs. 7A–C) and co-localisation of PKH26 labelled MHP36 cells with Syn was observed in the cortex within the peri-lesion. The mean average intensity for Syn-positive staining and the number of Syn-positive cells were significantly increased after MHP36 compared to sham surgery animals. There was a highly significant deficit at 28 days post-MCAO in vehicle group (4.03 ± 0.19, p < 0.001) when compared to pre-MCAO score (vehicle, 5.77 ± 0.05) which was improved in the animals receiving the MHP36 cells (pre: 5.73 ± 0.04; 28 days: 4.95 ± 0.12, p < 0.001) (Fig. 4). Furthermore, there was a significant acceleration of recovery observed at 14 days post-MCAO (4.71 ± 0.18, p < 0.05) in animals receiving MHP36 cells only when compared to 2 days post MCAO score. In addition, at 28 days the foot fault scores observed between MCAO + vehicle (4.95 ± 0.12) and MCAO + MHP36 (4.029 ± 0.19) animals showed a significant trend towards improvement in the MCAO + MHP36 (p = 0.049).
Discussion

Cell transplantation therapy for stroke has tremendous potential; however certain limitations are delaying their success in humans. The key findings of the present study are that ciNSCs, MHP36, restore lost sensorimotor function after transient MCAO in mice as demonstrated in both the cylinder and ladder rung tests. Improved function is not due to reduced lesion size but possibly by increasing neuronal differentiation and synaptic plasticity.

In a previous study, MHP36 improved functional recovery after transient MCAO, but in that study grafting was done in rats, at 8 distinct contralateral sites and at 2 to 3 weeks after MCAO (Veizovic et al., 2001). In addition, MHP36 cells have been tested in mice after 2 vessel global ischaemia and found to reduce ischaemic damage with 50% of cells having neuronal phenotype though no functional recovery was assessed (Wong et al., 2005). We now extend these studies showing success of MHP36 cells after transient MCAO when grafted in mice in 2 ipsilateral sites at 2 days after MCAO. The success of MHP36 cells in mice after focal ischaemia in the present study extends the use of these ciNSCs to genetically altered mice for subsequent research into mechanistic insight of these cells after stroke.

Improving functional outcome after stroke is the ultimate goal of stroke treatment. Therefore the detection of functional deficits is essential for potential translational applications. Mice due to their small size and quick movements can be perceived as more challenging to handle and train than rats (Wahlsten et al., 2003). Test strategies for mice are hence frequently based on evaluation of simple motor behaviour in standard test batteries (Li et al., 2004; Cook et al., 2002; Lalonde et al., 2003) or neurological score such as Clark’s deficit score (Clark et al., 1997; De Simoni et al., 2003). While these tests evaluate gross behaviour, they lack resolution when rather specific chronic deficits need to be identified. Clark’s deficit score has previously

Figure 3  ciNSC graft did not reduce lesion size by 28 days post-MCAO. (A) Composite images of a median representative of the topography of lesion from animals that received either vehicle or MHP36 cells inscribed on the line diagram from the mouse atlas and super-imposed on each other. (B) Graphical representation of the topography of lesion over eight coronal levels (0.10, 0.16, 1.98, 2.86, 3.34, 3.94, 4.66 and 6.02 mm with respect to interaural distance).
shown a significant difference between treatments up till but not beyond 4 days post-surgery (De Simoni et al., 2003; Storini et al., 2005). Clark’s score was not designed in the present study to differentiate between the injection groups given acute time points (3 and 4 days post-MCAO), but merely to evaluate if neurological deficit after MCAO was equivalent across groups. Regarding the recovery of sensorimotor deficits, much less has been described in the mouse. In rats and marmosets, tests that have shown successful sensorimotor recovery after MCAO with MHP36 include bilateral asymmetry and amphetamine rotation (Modo et al., 2002a; Veizovic et al., 2001), cognitive function and simple and conditioned discrimination (Virley et al., 1999). Since unilateral brain damage in human and rodents results in deficits of symmetry, we employed a test in mice that detects asymmetries, namely the cylinder test. The advantage of the cylinder test is that it can detect even mild neurological impairments (Hua et al., 2002) and factors out confounding variables such as overall decrease in activity after surgical induction of stroke as additional trials can be performed. This is the first time the cylinder test has been used to assess the potential of MHP36 cells include bilateral asymmetry and amphetamine rotation (Modo et al., 2002a; Veizovic et al., 2001), cognitive function and simple and conditioned discrimination (Virley et al., 1999).

Since unilateral brain damage in human and rodents results in deficits of symmetry, we employed a test in mice that detects asymmetries, namely the cylinder test. The advantage of the cylinder test is that it can detect even mild neurological impairments (Hua et al., 2002) and factors out confounding variables such as overall decrease in activity after surgical induction of stroke as additional trials can be performed. This is the first time the cylinder test has been used to assess the potential of MHP36 cells. We observed symmetrical use of both paws in sham animals and a marked preference for use of the non-impaired (ipsilateral) paw after MCAO. Importantly, treatment with MHP36 reduced this asymmetry [by >70%]. Therefore, we have introduced a mouse model in behavioural assay to better study potential functional effects of stem cells, enabling future studies on potential functional effects in mouse mutants that target particular signalling/developmental pathways for dissecting molecular mechanisms of stem cells. Neuronal connections are continuously remodelled and suffer intense adaptive functional and structural reorganisation after lesions (Carmichael, 2003; Giraldi-Guimaraes et al., 2009; Rossi et al., 2007). This restorative reorganisation is one of the most important mechanisms underlying functional recovery as described below.

Since topography of our lesion includes cortical and subcortical areas, motor impairments of limb functioning and placing deficits were evaluated in the present study by foot fault test which has commonly been used as an efficient and sensitive test strategy for chronic assessment of skilled fore- and hind-limb stepping in mice (Farr et al., 2006). This is the first study to show a significant improvement in stepping, at 28 days post-MCAO in mice receiving MHP36 cells. The improved functional recovery observed in the present work might be explained in part by a positive effect of the MHP36 cells in the structural plasticity induced by ischaemia. Wong et al. showed an increase in APO-E expression in rats with focal ischaemia relative to controls which was further

Figure 4 ciNSC grafts augmented recovery from foot faults after 28 days post-MCAO using ladder rung test. Stepping errors in vehicle and MHP36 cell treated sham and MCAO mice at pre-surgery (0 days) and at 2, 7, 14 and 28 days post-surgery (mean±S.E.M., n=8, **p<0.01, ***p<0.001 versus pre-surgery, ∆p<0.05 versus 2 days, **p<0.001 MCAO versus sham at 7 and 14 days post-surgery, ∆∆p<0.01 MCAO+vehicle versus MCAO+MHP36, repeated measure ANOVA). A score of 6 indicated correct placements.

Figure 5 ciNSC grafts reversed spontaneous forelimb use asymmetry by 28 days post-MCAO. Asymmetric score assessed by cylinder test in vehicle and MHP36 cell treated sham and MCAO mice at pre-surgery (0 days) and at 2, 7, 14 and 28 days post-surgery (mean±S.E.M., n=8, *p<0.05, ***p<0.001 (versus pre-surgery), ∆∆∆p<0.001 (MCAO+vehicle versus MCAO+MHP36), ns = not significant versus pre-surgery for all groups except MCAO+ vehicle, repeated measure ANOVA).

Figure 6 Histological assessment of differentiation at 28 days post-MCAO. Peri-lesion region of interest in the mice injected with (A) vehicle and (B) MHP36 cells stained for MAP-2 (neurons). (C) Significant difference was observed in the mean intensity (represented on the left side of Y-axis, solid red and blue bars) and number of MAP-2 positive neurons (right side of Y-axis, checks red and blue bars) when compared to vehicle group. Staining for (D, E) GFAP (astrocytes), (G, H) CNPase (oligodendrocytes (oligo)) and (J, K) IBA-1 (microglia) in animals receiving vehicle and MHP36 cells respectively. No significant difference was observed in the mean intensity (represented on left side of Y-axis, solid red and blue bars) and the number of GFAP, CNPase or IBA-1 positive astrocytes, oligodendrocytes or microglia respectively (right side of Y-axis, checks red and blue bars) when compared to vehicle group (F, I, L) (*p<0.05; ns = not-significant, scale bars for A, B and D, E=100 μm; for G, H=20 μm and for J, K=10 μm).
elevated in MHP36 grafted rats. This has been interpreted as an indirect evidence for remodelling of circuits (Wong et al., 2005). Thus to analyse this hypothesis, we studied the expression of synaptophysin and GAP-43 protein related to structural plasticity and axonal projections in the periphery of the lesion and in the contralateral (homologous) cortex. Our results showed a significant effect of the MHP36 treatment in the expression of synaptophysin and GAP-43 compared to vehicle suggesting that the observed recovery might be attributed to significant plastic structural changes.
involving synapse formation and axonal projections. The increased synaptogenesis in the ipsilateral cortex compared to contralateral cortex may also at least in part explain the asymmetric bias for impaired limb in cylinder test in MHP36 mice. In agreement, Shen et al. in 2006 showed an increase of the synaptophysin expression in the periphery of the ischaemic lesion in rats treated with bone-marrow stromal cells one day after MCAO (Shen et al., 2006). In terms of the mechanisms involved in increased synaptogenesis there are at least two possibilities: (1) that grafted MHP36 cells migrate to the area of damage and reconstitute local circuits that are sufficient to promote synaptogenesis and sustain some functions and (2) that grafts augment spontaneous reorganisation within the host environment sufficient to undertake, or compensate for, some lost function. We found co-localisation of Syn/PKH26 and GAP-43/PKH26 which on one hand may merely indicate the presence of these epitopes on grafted cells but on the other hand may indicate that the former of the two possibilities above occurs in our studies. In any case evidence from imaging studies suggests that both possibilities are reasonable (Cramer et al., 1997).

Whether transplanted cells reduce death of host cells is an important observation as enhanced recovery of function could result from neuroprotection (Shen et al., 2010). The lesion volume in the present study was not significantly reduced by MHP36 cell which was in agreement with Modo et al. where they showed no difference in lesion when transplantation was 2–3 weeks post-MCAO and lesion assessed at 14 weeks (Modo et al., 2002a). In other studies, MHP36 cells have been shown to reduce lesion size in rats (Veizovic et al., 2001; Modo et al., 2009). This was observed 11 months after transplantation of MHP36 grafts and may be due to the reduction of secondary degeneration and atrophy (Schallert et al., 1982), as reduction was not observed 3 months after transplantation, which already exerted positive effects on bilateral asymmetry. Other mechanisms of reconstituting local circuits by MHP36 could include filling out of cavities (Bible et al., 2009). However, in the present study it would not be possible for injections of 1 μl (25,000 cells) to fill out cavities averaging ~2–3 mm³, particularly since only a third of the cells migrate to the lesion side (Veizovic et al., 2001).

In terms of whether MHP36 grafts augment spontaneous reorganisation within the host environment, this could be induced by replacing lost cells and integration of MHP36 cells into host circuitry. We found a significant effect of MHP36 on induced by replacing lost cells and integration of MHP36 cells reorganisation within the host environment, this could be observed increased auto-fluorescence of the host brain after transplantation due to its chemical property.

Limitations for neural stem/progenitor cells include in vitro expansion for transplantation as well as loss of capacity to differentiate when expanded in vitro which limit their ability to form functional grafts (Svendsen et al., 1996) with lack of generation of specific cell types such as dopaminergic neurons (Bjorklund et al., 2002; Roy et al., 2006; Takagi et al., 2005) or motor neurons (Placoba et al., 2004). Still, neural stem cells have many advantages over other cells. Limitations of other cells include ethical concerns, limited availability (Wechsler, 2004) and potential tumour formation using embryonic stem cells (Erd et al., 2003); insufficient yield with endothelial progenitor cells (Rouhl et al., 2008) and mesenchymal cells (Rubic et al., 2005; Tolar et al., 2007); and the possibility for onco-genesis due to insertional mutagenesis that is inherent to stable genomic integration using retroviral or lentiviral vectors has been identified as a limitation using induced pluripotent stem cells (iPS) (Yamashita et al., 2011). Conditionally immortalised neural cell lines circumvent these limitations since they are capable of being maintained in vitro indefinitely and these cell lines are multipotent in nature, thus capable of repairing many different types of brain damage (Gray et al., 1999) and differentiating into different types of cells; neurones, astrocytes and oligodendrocytes (Mellode et al., 2004).

In the present study, we chose MHP36 cell line specifically for their capacity not to proliferate in vivo and since human equivalent neural stem cell line (conditionally immortalised using myCER) are currently in phase one clinical trial in the UK (Mack, 2011). It is important to recognise that these cells have transformed in nature, due to long-term culturing, even though they are potentially restricted by temperature and that they have a distinct molecular expression profile with high growth factor production (Ml et al., 2005). However our findings complement other stem cell work in mice. For example, stem cells have been shown to improve functional recovery in mice using other immortalised neural stem cells (Lee et al., 2007a) and using stem cells from other sources such as bone marrow non-haemotopoietic cells (Li et al., 2000) and, in agreement with our results, have been shown to be effective possibly by increasing neuronal differentiation (Lee et al., 2007b; Li and Chopp, 2009) and synaptogenesis (Li and Chopp, 2009).
Previous studies have implanted stem cells at various time points from 24 h to 2–3 weeks (Modo et al., 2002a,b; Ikeda et al., 2005) post-MCAO. This is the first time MHP36 have been implanted at 48 h post-MCAO in mice. The 48 h time point is late enough to avoid stem cell effects when the lesion is still evolving and to allow full anaesthetic recovery between surgeries and early enough to promote brain plasticity resulting in recovery of function (e.g. Ikeda et al., 2005).

Conclusions

In conclusion, the results suggest that MHP36 grafts ipsilateral to lesion exert a positive functional effect observed using the cylinder and ladder test. Increased MAP-2, synaptophysin and GAP-43 expression in animals receiving MHP36 cells suggests that complex processes of neuronal differentiation and synaptogenesis are activated,
whether by autocrine or paracrine effect are yet to be elucidated. Therefore the use of transgenic mice could help tease out the mechanisms clearly in the future.

**Methods**

**Subjects**

Forty-two males, 12–14 weeks old, C57BL/6 mice (Charles River, UK), weighing between 25 and 30 g, were used. The animals were housed in a controlled environment with a 12:12 h light cycle beginning at 06:00 and temperature maintained at 22 °C. The mice were allowed ad libitum access to food and water. The experiment was conducted complying with the UK Animals (Scientific Procedures) Act, 1986 and associated guidelines for the use of experimental animals.

**Surgery and measurement of cerebral blood flow**

Following completion of baseline testing, focal ischaemia was induced within the left hemisphere by transient (45 min) MCAO as described previously (McColl et al., 2007). The following occlusion time was used after a pilot study was carried out in order to acquire cortical as well as striatal damage. Briefly, mice were anaesthetised with 3% isoflurane (BIMEDA-MTC Animal Health Incorporated) mixed with 1% oxygen and maintained with 1.5 ± 0.25% isoflurane. The core body temperature was regulated at 37 ± 0.5 °C and a 7–0 silicone monofilament (Doccol, Ltd.) was introduced into the external carotid artery and advanced along the internal carotid artery (ICA) until occluding the origin of the MCA. Animals were maintained on 1.25% isoflurane during the occlusion time. After 45 min, the filament was withdrawn to establish reperfusion. Sham-operated mice underwent the same procedure except the filament was not advanced along the ICA.

In each animal, laser Doppler flowmetry (Moor Instruments) was used to monitor cerebral blood flow (CBF) continuously, before and during MCAO as well as during reperfusion. Briefly, a small incision of the skin overlying the temporalis muscle was made and a 0.7 mm, flexible, laser Doppler probe (model P10; Moor Instruments) was positioned on the superior portion of the temporal bone and secured with glue. This position corresponded to the MCA territory. Animals were included only when CBF was reduced by ≥ 85% during ischaemia, and successful reperfusion was subsequently achieved. No animal was excluded on these criteria.

**Cell maintenance and transplantation**

MHP36 cells were cultured from frozen stock and maintained in an undifferentiated state at 33 °C (Passage numbers 53–65) (Sinden et al., 1997). Before grafting, cells were labelled with the membrane bound fluorescent marker PKH26 (Sigma). Labelled cells were suspended in 1 mM N-acetyl-l-cysteine (NAC) in Hank's balanced salt solution without Ca²⁺ or Mg²⁺ at a concentration of 25,000 cells/μl. Cells were aspirated to form a single cell suspension and checked for incorporation of the PKH26 label prior to grafting. Viability was assessed using trypan blue exclusion in a haemocytometer. Pre-graft viability averaged 90% and post-graft viability averaged 78%. Two days post-MCAO or sham surgery, mice were randomly selected to receive a unilateral cortical and striatal graft of either MHP36 stem cells (n = 8) or vehicle (NAC solution) (n = 8). Mice were anaesthetised with isoflurane (3% induction, 1.5% maintenance) in a mixture of oxygen and mounted on a stereotaxic frame. Using Bregma as a reference point, a 2-μl Hamilton syringe was moved to the following coordinates (Medial/Lateral + 2 mm, Anterior/ Posterior – 0.26 mm). A burr hole was made, the dura was removed and the syringe was descended to a depth of −1.5 and 3 mm from the surface of the brain. Cell suspension or vehicle (0.5 μl per site of injection; cortex and caudate) was injected over 2 min, and the syringe was left in place for another 2 min. Treatment for immunosupression was not carried out in the present study because Modo et al. (2002b) showed that survival of MHP36 grafts was not affected by immunosupression.

**Experimental design**

The mice were handled extensively and habituated to the testing location and apparatus prior to training and testing (Fig. 1). Baseline (pre-surgery) testing was performed by placing the animals for two crossings on the ladder rung task with a set rung arrangement pattern (irregular). Mice underwent MCAO and received either the vehicle or MHP36 cells 2 days post-surgery. Behavioural testing and video recording of both ladder-rung and cylinder test occurred immediately after transplantation (2 days post-MCAO) and on 7, 14 and 28 days post-surgery. For all analyses, the experimenter was blinded to which treatment mice received (Fig. 1).

**Clark’s deficit score (CDS)**

One, three and four days after the induction of ischaemia, each mouse was rated on two neurologic function scales unique to the mouse (Clark et al., 1997) to establish severity of deficit. Scores for both scales range from 0 (healthy) to 28 and represent the sum of the results of all categories for each scale. The general deficit scale evaluates hair (0–4), ears (0–2), eyes (0–4), posture (0–4), spontaneous activity (0–4), and epileptic behaviour (0–12), whereas the focal deficit scale evaluates body symmetry (0–4), gait (0–4), climbing on a surface held at 45° (0–4), circling behaviour (0–4), front limb symmetry (0–4), compulsory circling (0–4) and whisker response to a light touch (0–4) (De Simoni et al., 2003). Animals that received a score ≥ 21 on the focal deficit scale were excluded from the study. Out of the 42 animals that underwent MCAO, 10 were excluded on this criteria.

**Ladder rung task apparatus and analysis**

The ladder rung task was adapted from the ladder rung walking task used previously in rats (Metz and Whishaw, 2002). The ladder rung apparatus was composed of two Plexiglas walls (69.5 cm × 15 cm). Each wall contained 121 holes 0.20 cm diameter, spaced 0.5 cm apart, and located 1 cm apart from the bottom edge of the wall (Farr et al., 2006). The holes could be filled with 8 cm long metal bars, diameter 0.10 cm in any pattern. The walls were spaced 5 cm apart to allow for passage of a mouse but prevent it from
turning around. The entire apparatus was placed atop two standard mouse housing cages, 17 cm above the ground. Animals were tested in an irregular pattern in which the distance of the rungs varied, ranging from 0.5 to 2.5 cm. Each post-MCAO test session included two crossings on the ladder. The animals’ performance was video-recorded from the side, with the camera positioned at a slight ventral angle to allow all four limbs being recorded at the same time. All video recordings were analysed frame-by-frame. Each of the four limbs was scored for placement errors with each step being scored. However, the first two initiation steps and last two final steps were omitted when an animal paused. Each step was scored according to the quality of limb placement based on the scale adapted from Metz and Whishaw (2002).

**Cylinder task apparatus and analysis**

The cylinder or spontaneous forelimb test modified for mouse (Baskin et al., 2003), involves the use of a 10 cm diameter transparent cylinder. Each animal was placed in the cylinder, and its spontaneous activity to rear up on its hind limbs and explore the vertical surface with its forelimbs was observed. Animals used either a single forelimb or both forelimbs for an exploration. The number of both, right only, or left only explorations was counted in a two-minute recording interval. One pre-injury measurement was taken to control for limb preference. The asymmetric score (Schallert et al., 2000) was computed as follows:

\[
\frac{\# \text{ of right only} - \# \text{ of left only}}{\# \text{ of right only} + \# \text{ of left only} + \# \text{ of both}}.
\]

Normal uninjured performance, for an animal with no preference for right or left forelimb, is at or near zero. Animals showed a tendency, in the measurements at 2 days injury, to explore the cylinder less frequently and to spend a larger proportion of time engaged in grooming activities. Additional two minute trials were performed until at least 10 rearing observations were made.

**Video recording and analysis equipment**

The mice were filmed with a high speed Panasonic digital camcorder (30 frames/s; shutter speed of 1/1000). The digital videotapes were analyzed using a HP Pavilion DV2000 laptop. Single frames were imported from the digital video records using Windows media player on a Windows operating system.

**Histology and lesion measurement**

Twenty-eight days post-MCAO, mice were perfused through the heart with 0.9% physiological saline followed by 4% paraformaldehyde. The brains were removed and cryo-protected in a 30% sucrose solution. The tissue was placed in a 2800 Frigocut E cryostat (Reichert–Jung) and sectioned in 20 μm thick sections. Eight coronal levels were selected from the mouse atlas (1.6, 1.1, 0.14, −0.38, −1.0, −1.9, −3.0, −4.0 mm with respect to Bregma, based on Osborne et al. (1987), modified for mouse). Distinct neuroanatomical landmarks defined which sections were chosen for the 8 pre-selected coronal levels which were then stained with haematoxylin and eosin. For volumetric assessment of ischaemic damage, the observer was unaware of treatment (injection). Areas of ischaemic damage were delineated onto scaled diagrams representing the eight coronal levels and then measured by means of an MCID image-analysis system. Approximation of the total volume (mm³) of ischaemic damage was achieved by integration of areas (mm²) with the distance between each coronal level (mm). The end points for integration were 2.9 mm (rostral limit) and −4.9 mm (caudal limit) with respect to Bregma.

Serial 20 μm coronal sections were processed for immunostaining with chicken anti-microtubule-associated protein (MAP-2, 1:500, Chemicon, UK), mouse anti-gliarial fibrillary acidic protein (GFAP, 1:200, Sigma, UK), mouse anti-CNPass (2′, 3′-cyclic-nucleotide 3′-phosphodiesterase) (1:200, Chemicon, UK) and goat anti-IBA-1 (ionised calcium binding adaptor molecule 1, 1:500, Abcam Plc., UK) to identify various populations of neural cells. Mouse anti-synaptophysin (Syn, 1:200, Abcam Plc., UK) was used to investigate synaptogenesis and anti-rabbit GAP-43 (1:200, Abcam Plc., UK) was used as a marker for axonal projections. Sections were incubated overnight at 4 °C and followed with application of secondary antibody, raised against chicken IgG bound to Alexafluor 488 (Chemicon, UK), mouse IgG bound to fluorescein isothiocyanate (FITC) (Sigma-Aldrich, UK) at a dilution of 1:100 in PBS for 1 h. After washing in PBS, cover slips were mounted with Vectashield and DAPI (Vector Labs) and viewed and photographed using a Nikon EclipseE600 Oil Immersion microscope connected to a photometrics (CoolSnapFx) digital camera managed by Meta-Morph software. A negative control was included in every run which included no primary antibody incubation.

Three animal brains were randomly stained for the different markers to avoid bias selection. During image acquisition, the threshold and gain on the confocal laser microscope were set using the control for each run of staining. This helped to subtract the background fluorescence. Cells positive for each marker were quantified by counting the number of positive cells and directly compared to vehicle group. In addition, the average intensity in the region of interest (ROI) per area of field of view for each marker was also measured to allow semi-quantitative comparison between MHP36 and vehicle treated animals using Image J software. The obtained values used for graphical representation are a ratio of intensity and area. ROI for differentiation consisted of the ipsilateral striatum and somatosensory cortex within each brain. ROI for synaptic plasticity consisted of the ipsilateral striatum and somatosensory cortex and contralateral striatum and somatosensory cortex within each brain (Bregma 0.3 ± 0.1 mm). Two images were taken in each ROI for quantification. No comparisons were made between the cortex and striatum. The images were acquired using a Leica Epi-fluorescence microscope (Leica Microsystems, Wetzlar, Germany) with 200×, 400× and 100× oil-immersion objectives connected to Metamorph-Pro software.

**Statistical analysis**

Statistical comparisons in all behavioural tests were made using repeated measures ANOVA. Two way ANOVA with a post hoc Bonferroni’s test to correct for multiple comparisons (three factors; surgery, time and injection) was used unless otherwise stated.
In histological and molecular assessments, unpaired t-test was performed unless otherwise stated. A p-value of less than 0.05 was chosen as the significance level for all statistical analyses. All data are presented as mean ± standard error of the mean (S.E.M.).

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