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14. ABSTRACT Amyotrophic lateral sclerosis (ALS) is an incurable fatal paralytic disease that affects adults. ALS is characterized by the degeneration of the "motor neurons" innervating the muscles. Our proposal investigated the neuroprotective effect and therapeutic value of a drug: the translocator protein (TSPO) ligand PK11195 (PK), which we found to be very promising in cell models of ALS. These models take into consideration that in ALS, motor neurons are killed by neighboring cells called astrocytes, which transform from supportive to neurotoxic cells. Over these two years of the award, we were able to meet our main objectives. ***First, in our cell study,*** we found that PK acts directly on motor neurons to prevent their death, not via the astrocytes. Then, we evidenced that PK protects not only the motor neuron cell bodies, but also their long axons. We also found that TSPO is not expressed in the same cell compartments in motor neurons and astrocytes, suggesting cell-specific roles. Finally, we demonstrated that PK is not neuroprotective by inhibiting TSPO. In contrast, an increase in TSPO content may protect motor neurons against ALS. ***Second, in our preclinical study in ALS mice,*** we have determined that the best route to administer PK is continuous delivery via osmotic mini-pumps placed under the skin of the animals. We tested two doses of PK, well tolerated by the mice; both allow PK to reach desirable brain and spinal cord levels. Overall we found that both doses of PK significantly improve grip strength, motor performance, and muscle innervation of ALS mice in the early phase of the disease. However, PK does not delay the onset of paralysis and does not appear to protect motor neuron survival or extend mouse lifespan at later disease stages. In diseased ALS mouse spinal cord, we found that TSPO expression was primarily co-localized with inflammatory reactive astrocytes. Our future studies will have to determine whether over time PK's neuroprotective effect, which acts via an increase of motor neuron defense against astrocytes is not overruled by the continuous and possibly exacerbated detrimental effect of astrocytes. This supports the view that only the combination of therapies targeting neuroprotection and lessening of glial cell neurotoxicity will hold some promise for clinical efficacy in ALS.

15. SUBJECT TERMS
Amyotrophic lateral sclerosis, preclinical study, motor neuron death, translocator protein ligand, astrocyte toxicity, SOD1 mice

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1. Introduction:

Amyotrophic lateral sclerosis (ALS) is a fatal adult-onset paralytic disease (Hirano 1996). There is, at present, no effective treatment for this devastating disorder. Most cases of ALS occur spontaneously (sporadic ALS), although sometimes it can be inherited. Mutations in the antioxidant enzyme superoxide dismutase-1 (SOD1) are a cause of inherited ALS, and animals that express mutated SOD1 mimic the hallmarks of ALS, including the loss of specific brain cells, the motor neurons (Gurney et al. 1994). The search for agents that can slow or even stop ALS has always been paramount. However, this quest has been hampered by a lack both of screenable disease models with clear relevance to the human condition and existing drugs that prevent motor neuron loss. Previously, our group and others made the following groundbreaking observations: motor neurons can be taken from mouse brains or spinal cords and, when these motor neurons are placed in co-culture with non-neuronal supportive brain cells called astrocytes from animals that express mutant SOD1, the motor neurons die in a strikingly similar fashion to how they die in ALS (Nagai et al. 2007; Di Giorgio et al. 2007). Recently, even more fascinatingly, we demonstrated that astrocytes directly taken from sporadic ALS patient fresh autopsied tissues are killing motor neurons, recapitulating *in vitro* the most common form of this human disease (Re et al. 2014). By using back-to-back these mouse and human ALS models, we showed that the demise of motor neurons can be prevented in both by the addition of a small molecule named PK11195 (PK) which binds to the translocator protein (TSPO). This striking prevention of motor neuron degeneration in our models of both familial and sporadic ALS strongly suggest that PK could be a drug candidate for the vast majority of ALS forms, but also its proven efficacy on both mouse and human cells predicts for a more reliable clinical application to humans compared to previous targets identified solely with mouse cells. ***Together with our promising preliminary data, the fact that PK freely permeates the blood-brain barrier and is already approved for imaging in humans to follow brain inflammation makes it an ideal candidate for preclinical testing for ALS.*** The objective of our research for this Therapeutic Idea Award was twofold. **First, in specific aim 1**, we proposed to **elucidate: i) the cellular target(s) of PK in our motor neuron-astrocyte co-culture models; ii) the subcellular distribution and expression of TSPO in motor neurons and astrocytes in control and in ALS conditions; iii) the protective effect of PK on motor neuron axons as compared with their cell bodies; iv) whether PK is protective by antagonizing TSPO; v) the mechanism(s) underlying PK's protective effect**. **Second**, our study proposed to rigorously **assess the therapeutic value of PK in the best preclinical animal model of ALS available so far.** For this, the **first phase of SA-2** would determine the best route, regimen, and dose(s) of PK for administration to ALS mice to reach brain and spinal cord levels allowing PK to effectively bind on TSPO, also making sure the mice satisfactorily tolerate the drug treatment. Then, based on the optimal conditions of PK administration, the **second phase of specific aim 2** would determine whether PK can mitigate the expression and progression of the paralysis in the transgenic mutant SOD1 mouse model of ALS using a comprehensive set of motor tests, and morphological investigations to assess the protection of motor neurons and the preservation of their axons.

2. Keywords:

Amyotrophic lateral sclerosis (ALS)
Motor neuron death
Preclinical study
Mutant SOD1 mice
Astrocytes
Translocator protein (TSPO) ligand
Neuroprotective drugs
In vitro models of neurodegeneration
Axon preservation
Neuromuscular junctions

3. **Accomplishments**:

What were the major goals of the project?

Specific Aim 1: Define the cellular targets of PK11195 and its mechanisms of neuroprotection, *in vitro*.

Major Task 1. Clarify whether the protective effect of PK on motor neurons co-cultured with SOD1 astrocytes is due to its activity within motor neurons, astrocytes, or both. **Milestone(s)**: Elucidation of the cellular target of PK, 3rd month of the Award. **100% completion**

Major Task 2. Evaluation by immunoelectron microscopy in which subcellular compartment TSPO is expressed in control vs. SOD1 astrocytes and in motor neurons exposed to either control or SOD1 astrocyte conditioned media. **Milestone(s)**: Elucidation of the sub cellular expression of TSPO, by the end of the 5th month of the Award. **100% completion**

Major Task 3. Measure whether PK protects only motor neuron cell bodies or their neurites as well. **Milestone(s)**: Elucidation of PK effect on motor neuron dendrites and axons, by the end of the 6th month of the Award. **100% completion**

Major Task 4. Investigate whether PK is protective by antagonizing TSPO. **Milestone(s)**: Elucidation of the agonist or antagonist effect of PK for TSPO, by the end of the 9th month of the Award. **100% completion**

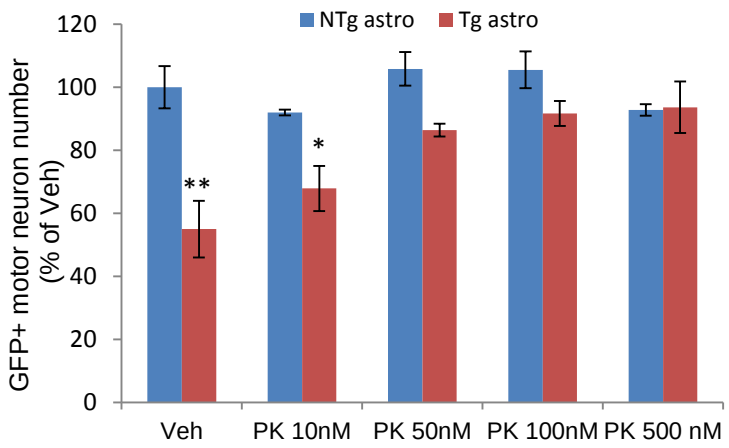
Major Task 5. Investigate the mechanism underlying PK protective effect. **Milestone(s)**: Elucidation of the molecular mechanism underlying PK neuroprotective effect, 24th month of the Award. **50% completion**

Specific Aim 2: Preclinical assessment of PK11195 efficacy in ALS mice.

Major Task 1. Pharmacokinetic, optimal route of administration, chronic tolerability, and dose study. **Milestone(s)**: Determination of the optimal dose and route of administration of PK to reach neuroprotective brain concentrations, by the end of the 12th month of the Award. **100% completion**

Major Task 2. Preclinical study. Clinical and neuropathological expression of the disease will be compared between 15-20 transgenic mutant SOD1^{G93A} mice, per arm, treated with the compound at two different doses and 15-20 transgenic SOD1^{G93A} mice treated with vehicle. **Milestone(s)**: The clinical efficacy of PK in ALS mice will be verified al all the levels: motor improvement, paralysis onset, mouse lifespan, neuroprotection, protection of the axon myelination, protection of muscle innervation, and modulation of the gliosis, by the end of the 24th month of the Award. **80% completion after the 24th month of the award**

Fig. 1. PK11195 is dose-dependently neuroprotective



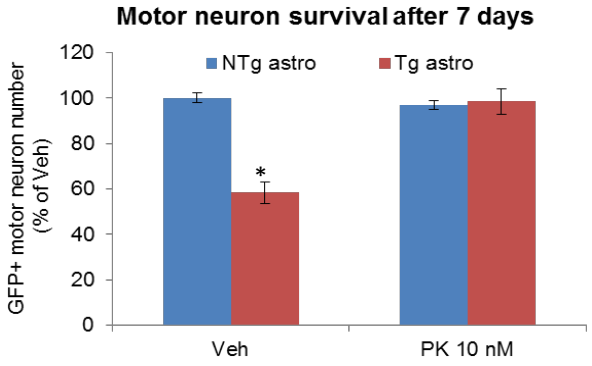
What was accomplished under these goals?

Specific Aim 1: Define the cellular targets of PK11195 and its mechanisms of neuroprotection, *in vitro*.

To limit variability between experiments and complete this two year study with a unique batch of PK, we have ordered a new batch of highly-purified R- enantiomer form (active form) of PK11195 (PK) from ABX advanced biomedical compounds Inc. (Germany), in a quantity sufficient to cover all the experiments that we have planned (=1.5 g).

Accordingly, before starting any task of the project, we have performed a dose-response experiment with this new lot of PK in our rodent co-culture model of ALS. For this, primary motor neurons co-cultured on the top of control (non-transgenic; NTg) or ALS (Tg; mutant SOD1^{G93A}) confluent astrocyte layers were treated twice over 7 days (at day 1 and day 4, as determined to be necessary in our pilot study) with vehicle (Veh; DMSO) or PK at doses ranging from 10 to 500 nM, for which PK was demonstrated to be a selective ligand of TSPO (Le Fur et al. 1983). As previously reported (Nagai et al. 2007; Re et al. 2014), to facilitate identification, motor neurons were produced from transgenic mice expressing green fluorescent protein (GFP) under the motor neuron specific HB9 promoter. Motor neuron survival was evaluated as the number of GFP^{+} motor neurons, exhibiting a healthy morphology and at least one neurite twice the length of their soma, in the entire cell culture well, after 7 days of co-culture. As expected, we found tha PK is dose-dependently protective for primary motor neuron against mutated SOD1^{G93A} transgenic astrocyte (Tg astro) toxicit (**Fig. 1**; ** p≤0.001; * p≤0.05; p values are from post Two-wa ANOVA Newman-Keuls test; n=4 independent experiments) Though, this new lot of PK shows a maximal protective effect a ~50-100 nm whereas the previous lot was already showing maximal neuroprotective effect at 10 nM (**Fig. 2**; * p≤0.001; pos Two-way ANOVA Newman-Keuls test; n=3 independer experiments). We will take this important new factor int consideration for the preparation our preclinical study. ***We nov know that we will have to reach levels of PK approximating 10 nM in the brain and the spinal cord of the animals to ensure maximal efficacy of the drug.***

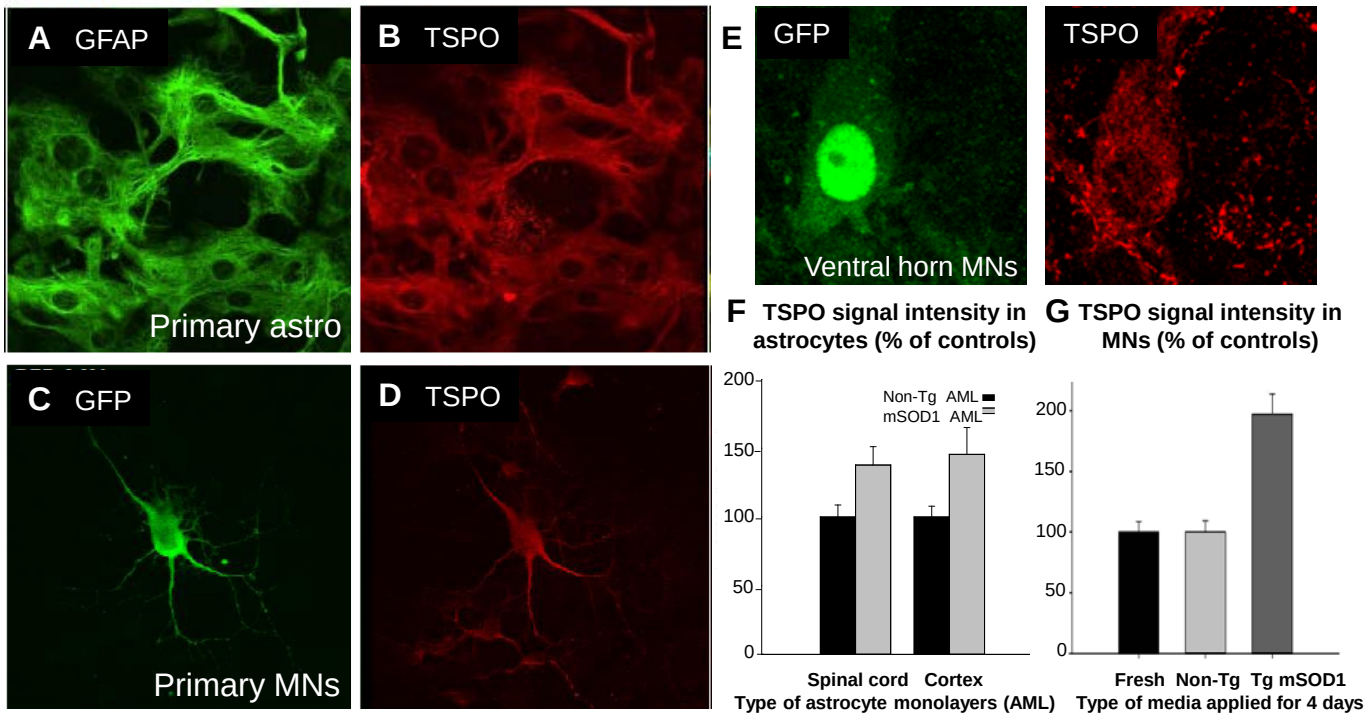
Fig. 2. Efficacy of the previous batch of PK.



Major Task 1. Elucidation of the cellular target of PK.

Previously, we found in our co-culture models of ALS that TSPO is not only expressed by astrocytes (**Fig. 3 A-B**) but also by motor neurons (**Fig. 3 C-D**). We have further confirmed that TSPO expression in motor neurons is not an *in vitro* artifact, as it is clearly detected *in vivo* in ventral horn spinal motor neurons of mice expressing GFP under the motor neuron-specific promoter *HB9* (**Fig. 3E**). In addition, our pilot data show that SOD1 astrocytes express the TSPO protein at higher levels than control astrocytes from NTg spinal or cortical monolayers,

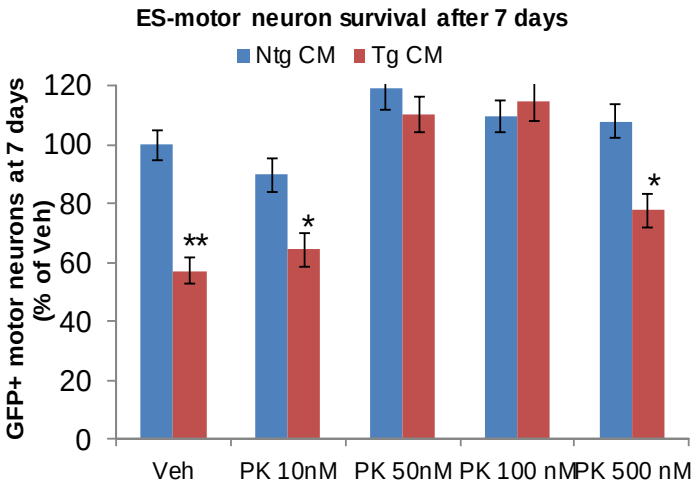
Fig. 3. TSPO is expressed by both astrocytes and motor neurons and its expression is increased in ALS experimental conditions.



indicating that these ALS astrocytes may be in a higher state of reactivity (**Fig. 3F**). Even more strikingly, motor neurons exposed for 4 days to Tg mSOD1 astrocyte conditioned medium dramatically up-regulate their TSPO expression as compared with motor neurons exposed to control astrocyte conditioned medium (Non-Tg) or fresh motor neuron medium (Fresh; **Fig. 3 G**).

Elucidating the cellular target of PK is essential to discover the mechanism of PK's protective effect and build-up on this knowledge for future drug development of TSPO ligands that could be even more efficient than PK itself. To address this question, as before (Re et al. 2014; Ikiz et al. 2015), we cultured magnetic activated cell sorted (MACs)-purified embryonic-stem cell (ES)-derived motor neurons in media conditioned for 7 days with control (NTg CM) and ALS (Tg CM) astrocytes (**Fig.4**). These ES-motor neurons were treated twice with Veh and PK over 7 days and survival was assessed as in the experiments described for Fig. 1 and 2. Strikingly, we found that PK was still dose-dependently protective to motor neurons against ALS astrocyte conditioned media (Tg CM), even when astrocytes were not present in the cell culture wells (**Fig. 4**; ** p≤0.001; * p≤0.05; post Two-way ANOVA Newman-Keuls test; n=4 independent experiments). In agreement with the co-culture experiments we found that maximal neuroprotection was reached at 50-100 nM of PK. However, in this conditioned medium experiment, PK's neuroprotective effect was lost at 500 nM, suggesting that in absence of astrocytes PK could also have some detrimental effects above a certain concentration. Yet, a dosing of PK leading to brain and spinal cord concentrations of ~500 nM should not be a concern for our *in vivo* experiments where astrocytes and other cells will be present around motor neurons, as in the co-culture model where 500 nM of PK is still fully protective (**Fig. 1**). On the other hand, it is notorious that at concentrations ≥ 1 µM, TSPO ligands have off-target effects (Le Fur et al. 1983; Caballero et al. 2014). Therefore, we will seek to stay at nM levels of PK below that off-target threshold. ***Altogether, importantly, the work accomplished in Major Task 1 indicates that motor neurons are the cellular target of PK neuroprotective effect and confirms that 50-100 nM of PK is the optimal dose range we will seek to reach in mouse brain and spinal cord for our preclinical study.***

Fig. 4. Motor neurons are the target of PK neuroprotective effect.



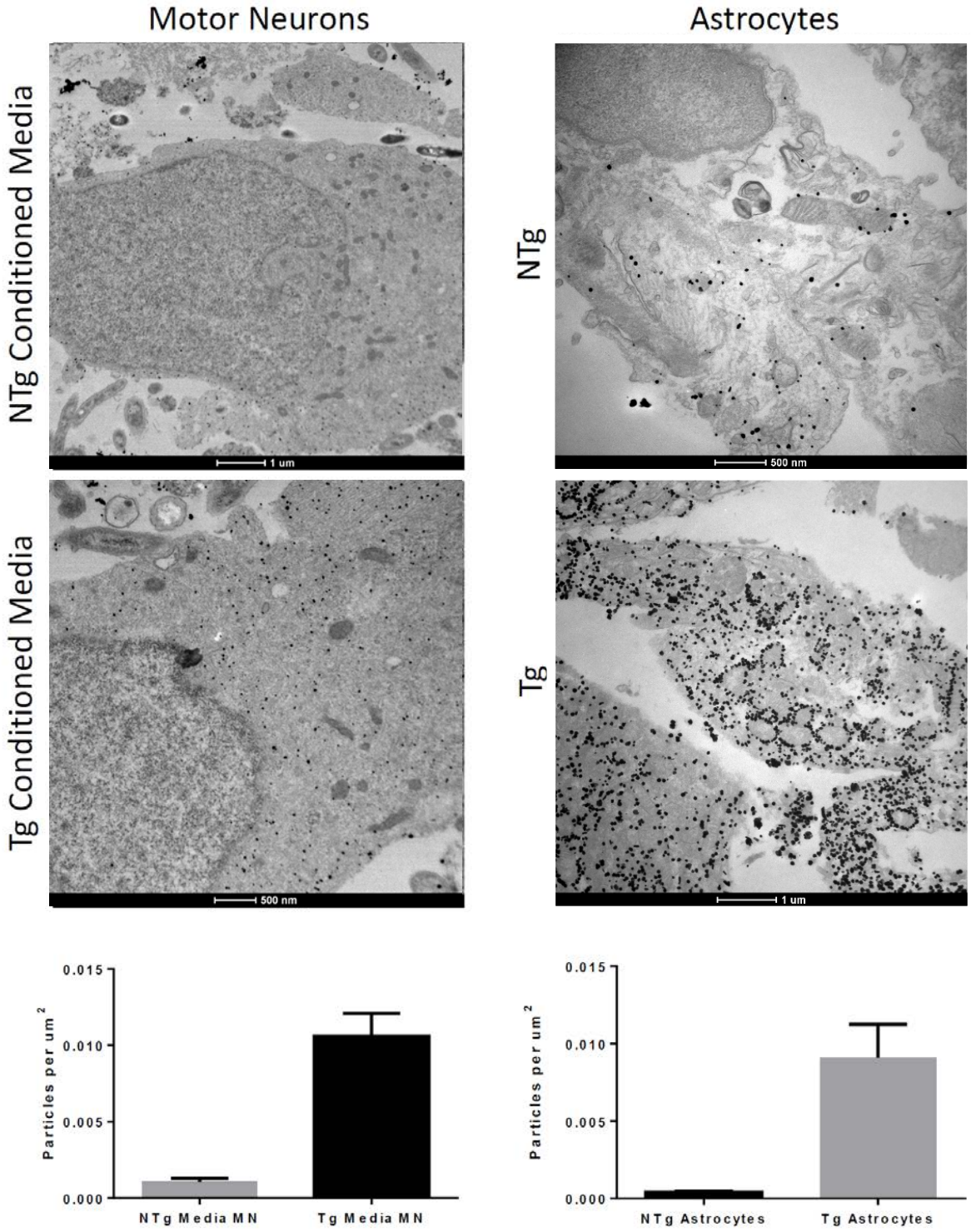
Major Task 2. Investigation of the subcellular distribution of TSPO in control vs. ALS astrocytes and in motor neurons exposed to either control or ALS astrocyte conditioned media.

To address this question, we carried out an immunoelectron microscopy study. Cells were fixed in a mixture of paraformaldehyde and glutaraldehyde. Membrane permeabilization was accomplished using a freeze-thaw method. Cells were immunostained with Rabbit anti-TSPO (validated with knockout animal cells; Abcam, MA, USA) and a goat anti-rabbit secondary antibody with a 0.5 nm colloidal gold particle attached. The gold particle was amplified using HQ Silver. Cells were then routinely processed for electron microscopy. For qualitative and quantitative analysis of TSPO expression and localization, 20 cells were randomly selected for imaging. To quantitate the expression of TSPO in cells, the amount of TSPO in a square µm was determined using the particle analysis command on ImageJ. Only cytoplasmic regions were selected for analysis.

As illustrated in **Fig. 5**, we confirmed here that motor neurons that were exposed to transgenic (Tg) astrocyte conditioned media for 3 days had a marked increase of TSPO expression ($p < 0.001$, T-test, Welch Correction). TSPO was even more significantly increased in Tg astrocytes ($p = 0.0021$, T-Test, Welch Correction). In motor neurons TSPO was mainly localized in the cytoplasm near the endoplasmic reticulum (ER), Golgi, vesicles, microfilaments (neurofilaments?), or near the plasma membrane, but it was only occasionally found to be associated with mitochondria. This subcellular distribution is in far contrast with what we previously found in microglial cells where TSPO is mainly associated with mitochondria (publication in preparation). In astrocytes,

TSPO is more often associated with mitochondria but it is also found in the cytoplasm near smooth ER, Golgi, close to vesicles, and sometimes in the nucleus. This differential distribution among motor neurons, astrocytes and microglia suggests that TSPO probably has different cell-specific functions. The level of TSPO expression was clearly up-regulated in motor neurons and astrocytes in the ALS experimental conditions. Regarding the sub-cellular distribution of TSPO as compared with control conditions, the only noticeable changes are a stronger association with mitochondria for astrocytes and less association with ER and more co-localization with vesicles for motor neurons.

Fig. 5. Immunoelectron microscopy of TSPO in motor neurons and in astrocytes.



Major Task 3. Evaluate whether PK protects only motor neuron cell bodies or their axons as well.

In this task, we proposed to investigate whether PK works beyond preventing the death of the motor neurons to also preserve the integrity of their long processes that travel the body to contract our muscles. This point is very important as among these neurites, the longest ones (axons) are also degenerating in ALS, causing the progressive paralysis of patients. Accordingly, the preservation of neurites is an equally important target for achieving real clinical motor benefit in ALS. To address this question, we used an automated imaging platform, the Flash Cytometer, originally developed by the biotech company Trophos (Marseille, France). The Flash Cytometer has a motorized X-Y stage and a proprietary auto-focus system automatically eliminating cell debris and aggregates from the imaging process. This apparatus can screen 3- to 4-times faster than other high-content screening microscopes such as the Cellomics and the Universal Imaging system (a whole 96-well plate can be scanned in less than 5 minutes). Another important feature here is that the captured image contains the entire 96-well dish, removing sampling bias. Illumination is by specific high-intensity LEDs, providing the possibility of tri-colored analysis in green, blue, and UV wavelengths. The Flash Cytometer has already been used in the Motor Neuron Center at Columbia University for high-throughput screens to measure survival and axonal outgrowth of GFP$^+$ ES-motor neurons, using the MetaMorph image analysis software. Accordingly, we have cultured purified GFP$^+$ ES-motor neurons in 96 well-plates containing either control (NT) or SOD1 ALS (Tg) astrocyte conditioned media (CM) for 7 days (**Fig. 6**). As for **Fig 1 and 4**, ES-motor neurons were treated twice over 7 days with Veh and PK at doses ranging from 10-500 nM. Pictures were taken with the Flash cytometer at day *in vitro* (DIV) 1 and 7, and neurite outgrowth was analyzed with MetaMorph. As illustrated in **Fig. 6** and previously reported (Nagai et al. 2007), Tg astrocyte CM (Veh condition) reduces motor neuron neurite and axon length as compared with NTg CM (* p≤0.001; from post Two-way ANOVA Newman-Keuls test; n=4 independent experiments). ***This effect is dose-dependently reversed by PK (fully at 100 nM) indicating that this drug can preserve both motor neuron soma and axons from mutant SOD1 astrocyte toxicity. In our preclinical study, we will determine whether this* in vitro *phenomenon can be predictive of PK effectiveness in preserving the integrity of skeletal muscle innervation, and whether or not it may have to be combined with other agents to provide overt clinical benefit in ALS.***

Fig. 6. Motor neurons are the target of PK neuroprotective effect.

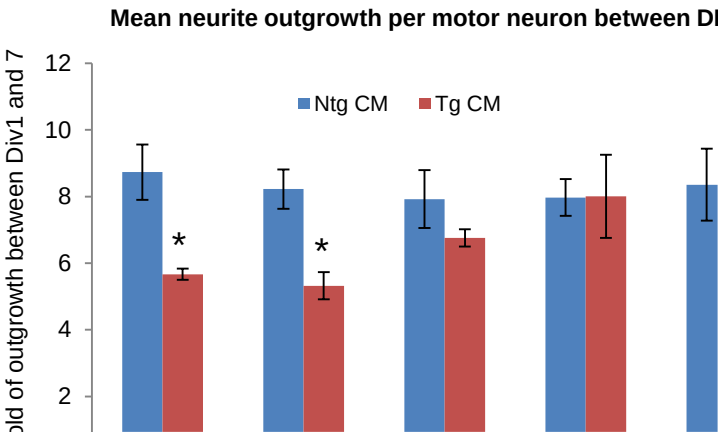
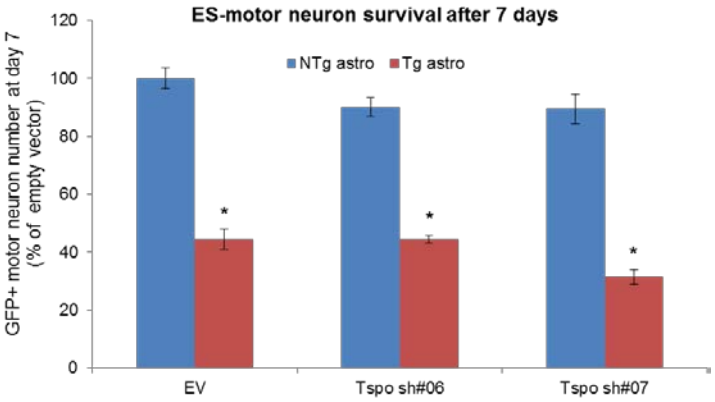


Fig. 7. Silencing TSPO in Motor Neurons does not emulate PK neuroprotection



Major Task 4. Investigate whether PK is protective by antagonizing TSPO.

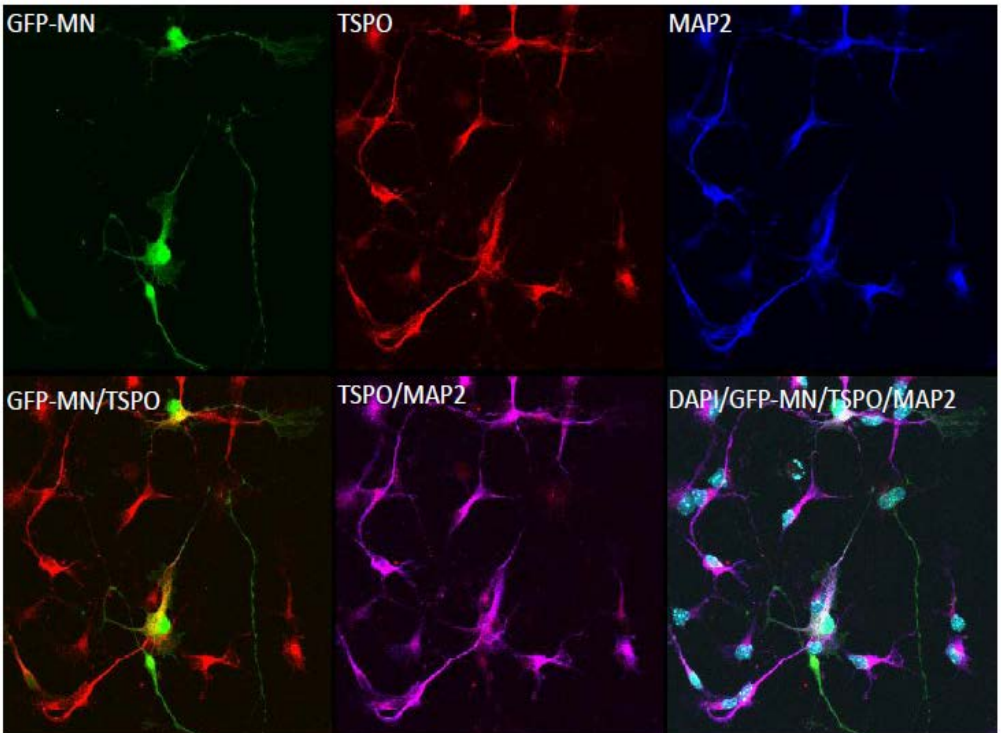
In Major task 1, we found that motor neurons are the cellular target of PK's effect (**Fig. 4**). Therefore, to try to get some molecular insights into the mechanisms underlying PK neuroprotection (antagonist or agonist of TSPO), we will first evaluate whether it is mimicked by the knockdown of TSPO in motor neurons, which would suggest it acts as a TSPO antagonist. Gene silencing is very difficult to successfully achieve in primary and ES-derived brain cells with regular transfection. Hence, we used lentivirus-delivered shRNAs. From Sigma Mission, we ordered three types of shRNA

directed against mouse tspo already packaged in lentiviral particles (tspo sh#06, #07 and #09), as well as control lentiviruses containing an empty vector (EV). In preliminary experiments (not shown), following the same protocol as described in (Re et al. 2014), we tested the silencing efficiency of these different viruses in purified ES-derived motor neurons. By quantitative real-time PCR, we found that both sh#06 and sh#07 efficiently knock down TSPO in motor neurons by ~70% when used at a multiplicity of infection (MOI) of 30 as compared with motor neurons infected with EV. Unfortunately, sh#09 did not work even at the highest MOI tested (=300). Therefore, we selected sh#06 and 07 and spinoculated freshly purified motor neurons in suspension before co-culturing them for 7 days with control (NTg) and mutant $SOD1^{G93A}$ (Tg) astrocyte layers (astro). We found that the silencing of TSPO in motor neurons, with two different shRNAs, did not emulate the neuroprotective effect of PK (**Fig. 7**; * p≤0.001; from post Two-way ANOVA Newman-Keuls test; n=4 independent experiments). ***This indicates that in our co-culture model of ALS, PK is not acting as an antagonist of TSPO. This also suggests that TSPO activation in motor neurons is involved in a neuroprotective pathway rather than in a cell death cascade. Another interesting note is that the silencing of TSPO shows a tendency to worsen the death rate of motor neurons, which plateaus at 50-60% in our* in vitro *models*** (Nagai et al. 2007; Re et al. 2014)***. It would be interesting to see if this phenomenon could reach statistical significance with a higher number of experiments or a more profound silencing of TSPO (over 70%). In view of these data, the increased expression of TSPO that we have measured in motor neurons, after 4 days of exposure to ALS astrocytes (Fig. 3G), before the death of motor neurons is massive*** (Nagai et al. 2007; Re et al. 2014; Ikiz et al. 2015)***, may represent a defense mechanism which is maximized by the application of PK.***

Major Task 5. Investigate the mechanism underlying PK protective effect.

The experiments that we initially planned for our investigation of the mechanism of PK's neuroprotective effect have been revisited in light of our progresses in SA-1 and of some recent breakthroughs in the TSPO research field. First, our finding in **Major Task 1** that PK's neuroprotective effect is entirely mediated by TSPO in the motor neurons has completely refocused our investigations toward the motor neuron compartment. Then, the insight we made in **Major Task 4** regarding the fact that TSPO silencing was not mimicking PK's neuroprotective effect has dismissed the possibility that TSPO is part of the cell death cascade leading to motor neuron loss in our *in vitro* model of ALS. In support of this as illustrated in **Fig. 8**, we also found that in our mouse spinal cord cultures, TSPO is not selectively expressed by GFP^+ motor neurons which are vulnerable to ALS astrocyte toxicity. Indeed, TSPO is also expressed by other neurons ($MAP2^+/GFP^-$) that we previously characterized to include mainly GABAergic interneurons, which are resistant to ALS astrocyte toxicity (Nagai et al. 2007; Re et al. 2014). Besides, our discovery that in motor neurons TSPO is not localized mainly at the mitochondria like it was described in

Fig. 8. Both ALS astrocyte-resistant and vulnerable neurons express TSPO.



microglial cells, suggest cell-specific roles for TSPO in the CNS. Finally, two recent studies reporting new animal models knockout for TSPO (Tu et al. 2014; Banati et al. 2014) have been groundbreaking for the field of TSPO research, as they have reported that TSPO knockout is not embryonic lethal, as previously thought, and that the complete absence of TSPO has no bearing on cholesterol transport or steroid synthesis, the two better defined roles of TSPO. Altogether, this leaves TSPO with an unknown (or uncertain) function that will be much more challenging to explore and will require investigation extending outside of the scope of this 2 year project.

To start to unravel what could be the role of TSPO in neurons, we have first extended our analysis of TSPO expression over time, in our cell model of ALS (**Fig. 9**). We found that at days 0 and 1, TSPO expression was very low in motor neurons cultured on either non-transgenic or transgenic astrocyte conditioned media. At day 4, TSPO expression is markedly increased; 4 fold higher in non-transgenic and 5 fold higher in Tg conditioned media. And this genotypic difference is even more pronounced at 7 days, when all vulnerable motor neurons have degenerated and only resistant motor neurons have contributed to the data acquisition. This indicates that resistant motor neurons probably have a higher capacity to increase their expression of TSPO in response to the toxic astrocyte signal. To further evaluate this hypothesis, we have evaluated TSPO expression in non-motor neurons i.e. MAP2+/GFP- neurons in the same culture wells (**Fig.10**). We found that other neurons were also progressively increasing TSPO expression as a function of their exposure time to the toxic astrocyte conditioned medium. ***It will be important to evaluate whether***, as suggested by the data from **Fig. 7**, ***a more profound silencing of TSPO in these resistant neurons and motor neurons can reverse their vulnerability phenotype. Besides being a therapeutic target, TSPO could be a marker of neuronal resistance to the ALS pathogenic process. It remains to be determined which protective pathway or defense mechanism involving TSPO is stimulated by PK in motor neurons exposed to ALS astrocytes.*** The final currently ongoing effort that we are carrying out to get some clue into TSPO protective function in motor neurons for this project is a confocal analysis of the co-localization of TSPO with different types of intracellular organelles and vesicles. This will complement our electron microscopy analysis by confirming, thanks to specific markers, what the exact subcellular localization of TSPO in motor neurons is. We already ordered all the necessary antibodies and produced and preserved by fixation the tissues to be analyzed. Our different hypotheses are that TSPO could be involved in: 1) misfolded protein degradation pathway for this we will test its presence in lysosome (LAMP1); 2) in lipid metabolism in the ER (PDI); and/or 3) in vesicles trafficking between cis-Golgi (GM130), plasma membrane (pan-cadherin and α1 sub-unit of Na+/K+ ATPase), early (EEA1) and late endosomes (rab7) for the production of extracellular vesicles to eliminate misfolded proteins. This final study will provide critical insights for future investigations for which we will apply to other sources of funding.

Fig. 9. TSPO expression in Motor Neurons increases over time

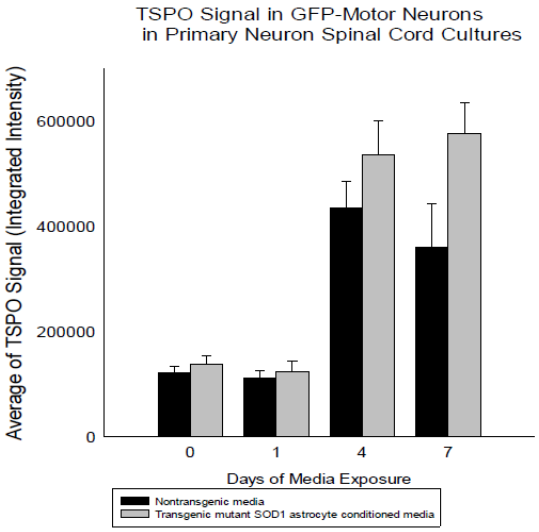
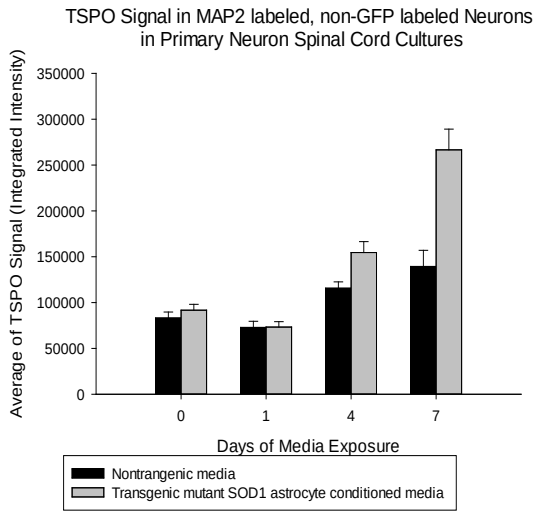


Fig. 10. TSPO protein is also increased in non-Motor Neurons



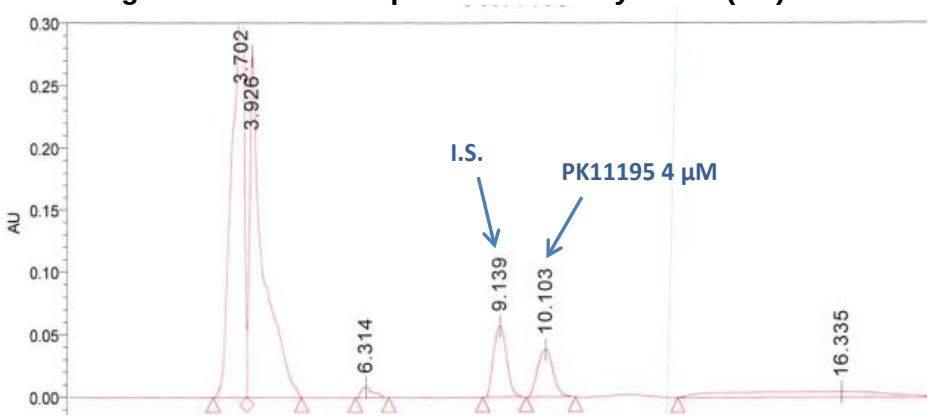
Specific Aim 2: Preclinical assessment of PK11195 efficacy in ALS mice.

Major Task 1. Pharmacokinetic, optimal route of administration, chronic tolerability, and dose study.

At the time the PI wrote this proposal, the laboratory of Serge Przedborski at the Motor Neuron Center of Columbia University was starting another preclinical study in ALS mice in collaboration with the Scripps Institute. This

previous preclinical study aimed at testing the therapeutic values of small inhibitors with c-Jun N-terminal-Kinase (JNK) inhibitors. These small inhibitors had a similar pharmacokinetic profile than the one predicted for PK: blood-brain-barrier penetrant, small lipophilic molecules. The preliminary pharmacokinetic data of the JNK study were showing that daily gavage (30 mg/kg/day) allowed an efficient delivery of the drugs in the brain and the spinal cord (400 nM) of wild-type mice from the same genetic background as the Tg SOD1^{G93A} mice. Accordingly, we proposed in the present preclinical study to assess in priority the same route of administration of the drug. However, before we started the present major task, more data available from the JNK study made us considerably revise our strategy. Indeed, in this previous study we found that after 100 days of daily gavage with 2 different JNK inhibitors (30 mg/kg/day), the brain concentrations of the inhibitors were~65% lower in Tg SOD1^{G93A} mice than in the NTg mice. We haven't clarified the origin of the difference in brain concentration between NTg and Tg SOD1^{G93A} mice, but ***we decide to evaluate more direct route of administration that would allow us to bypass any potential intestinal absorption problem in Tg SOD1^{G93A} mice such as intraperitoneal injection (IP) or implanted subcutaneous osmotic pumps.*** Of course, based on our need of chronic administration of PK for months in ALS mice, if PK was found to be stable at 37ºC for several weeks, the minipumps would be a much more optimal route of administration. However IP injections on alternate sides o the animals can be acceptable if they do not need to b performed more often than once a day.

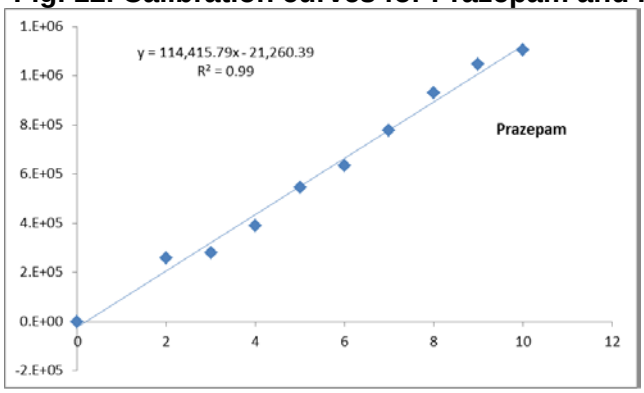
Fig. 11. PK and Prazepam detection by HPLC (UV).

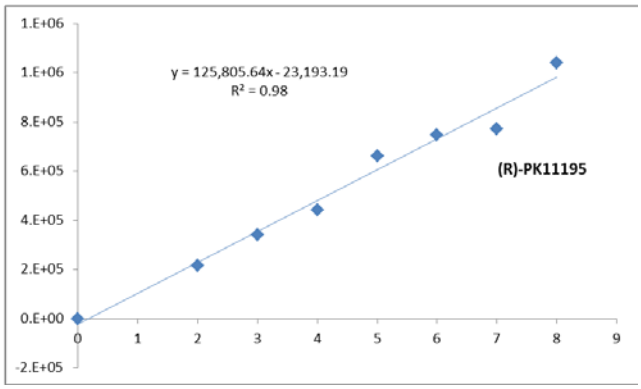


Fine-tuning of the HPLC detection method and dru extraction protocol from brain tissue

Before starting our pharmacokinetic study, we thoroughl validated our UV HPLC detection method for PK. Briefly, ou HPLC system consists of a Waters 515 pump, a Waters 71 plus autosampler injector, a Waters 996 photodiode arra detector set at 235 nm, Empower software and an analytica HPLC column Supelcosil LC-8 column (15 cm x 4.6 mm; µm) with Supelguard LC-8 (2 cm). The mobile phase consist of a mixture of 0.01 M K_2HPO_4 (pH 3.8), acetonitrile (25:75 and phosphoric acid with a flow rate of 1 mL min^{-1} in isocrati conditions. As internal standard (I.S.), we used the compoun "Prazepam", which is similar to (R)-PK11195, in size an chemical nature (small lipophilic compound), but it i chromatographically distinguishable from PK as previousl described (Wala et al. 2000). ***In our conditions, Prazepar (I.S.) and PK are separated by ~1 minute in their retentio times and are not masked by endogenous components c the brain matrix*** (**Fig. 11**). The internal standard is spiked i both calibration and actual samples after tissu homogenization to serve as an index of variability during th analytical determination and of possible drug losses durin sample preparation. Indeed, it is critical to have an internal control attesting to the efficiency of drug extraction from tissues within each sample. Then, the amount of the compound of interest is related to the amount of internal

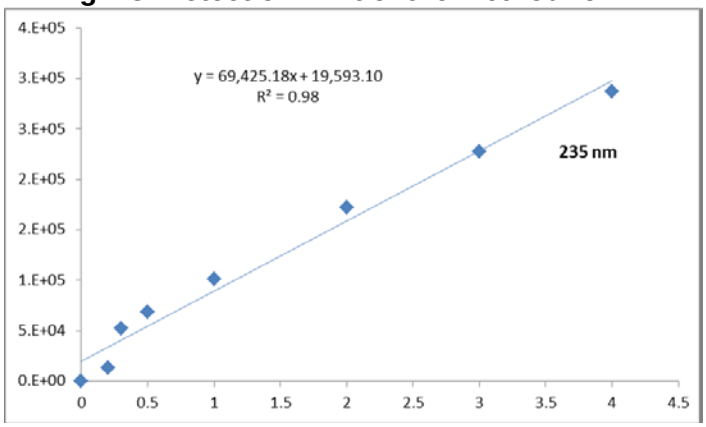
Fig. 12. Calibration curves for Prazepam and PK





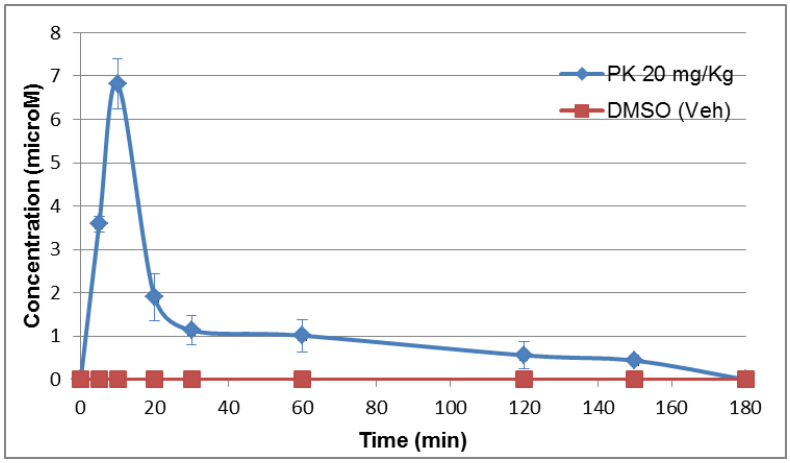
standard successfully recovered and measured. We have first run calibration curves with both prazepam and PK, and were able to obtain very high coefficient of correlation (R^2=0.99 and 0.98, respectively; **Fig. 12*). We found that the detection limit of our method for PK was of 30 nM*** (**Fig.13**). Below that, the peaks were not reproducibly measured. Knowing that we are seeking to ultimately detect ~100 nM levels of PK in brain and spinal cord, our objective was to find a method of extraction with minimal loss and offering possibility of sample concentration before analysis. We first evaluated a solid extraction method previously described to recover PK from brain tissue (Wala et al. 2000). For this, we first spiked 10 µM of PK and of prazepam in control brains homogenized with ice-cold PBS 1:4. Briefly, Bond Elut Certify columns (3 cm^3) were used for solid phase extraction. The columns were washed three times with methanol and then with water before being buffered with borax (pH 9.4; 300 µL). The columns were then washed with water. Brain homogenates (250 µL) were placed on the column and washed twice with water and then methanol. The columns were placed in a SP-10 vacuum system and eluted with methanol (600 µL). Eluates were dried under N_2 in an N-Evap analytical evaporator. Residues were then reconstituted in 100 µL methanol before injection in the HPLC system. Despite multiple attempts, this extraction procedure did not allow us to successfully recover detectable levels of either PK or prazepam from the original samples. Therefore, we tested a series of alternative methods of phase extraction. With a first method including methanol extraction, we were able to recover 10% of the starting sample before concentrating it by a factor of 2, so to measure 20% of an initial sample but we anticipated it would not be enough. Finally, we found that the Bligh & Dyer extraction method (BLIGH & DYER 1959) is particularly suitable for lipid extraction of tissue homogenates. Here, the tissue is homogenized with a mixture of chloroform and methanol which, when mixed with the water in the tissue, yields a monophasic solution. The resulting homogenate is then diluted with water and chloroform to produce a biphasic system, the chloroform layer of which contains the lipids and the methanol-water layer the non-lipids. After evaporation, the lipid extract is re-dissolved in a small volume of methanol, allowing its concentration. Thanks to this method we were able to recover on average ~70% of the initially spiked PK and prazepam (n=4 independent experiments), confirming that both compounds are extracted with an equal efficacy, and to concentrate the initial sample by a factor of 3.5 times (0.7 x 3.5=2.45 fold of concentration). Thanks to this extraction method we should be capable of detecting brain levels of PK which are 2.45 lower than our detection limit i.e. samples containing ~40 nM of PK.

Fig. 13. Detection limit of the method for PK



However, we found the Bligh & Dyer extraction method to be much more efficient to extract PK from brain than for spinal cord tissue (lower detection limit 200 nM), probably due to differences in lipid composition. Therefore, we continued investigating and found that dichloromethane (DCM) extraction method (Wang & Lemmer 1989) had a better and more consistent recovery for PK in both brain and spinal cord samples between experimental runs (~ 75%; n=5 independent experiments) as well as a lowest detection limit for spinal PK ~50 nM of PK. The brain or spinal cord samples (0.2-0.25 g wet/weight for hemi forebrain, 0.065-0.95 g wet/weight for spinal cord were gently homogenized with 0.9-

Fig. 14. Brain levels of PK over time after a single IP injection



2mm magnetic beads (Bullet Blender homogenizer) in 2 volumes (w/v) of 0.9% saline solution. Then the obtained homogenate was transferred to polypropylene copolymer (PPCO) tube and extracted with the following protocol: prazepam was spiked and then we added 1 ml of 1 M sodium hydroxide, 250 mg of sodium chloride, and 6 ml of DCM per 1 mL homogenate. The tubes were shaken for 15 min, and after centrifugation at 1500g for 15 min the aqueous phase was discarded. The remaining organic phase was transferred to a conical tube and evaporated (SpeedVac). The residue was redissolved in a small volume of methanol. The accuracy of the method was evaluated by spiking drug-free brain/spinal cord samples with prazepam, followed by the extraction procedure. The recovery was calculated by comparing peak-height ratios of these extracts with peak-height ratios of a standard solution injected directly.

Pharmacokinetic study

We did not find any previous pharmacokinetic studies of PK in mice after IP injection, but two published reports describe the levels of PK reached in various organs including brain after a single intravenous injection of PK in mice (Hashimoto et al. 1989) and rats (Wala et al. 2000). Based on these studies, we decided to evaluate first the levels of PK reached in the mouse brain at 5, 10, 20, 30 min, and 1, 2, 2.5, 3 hours after a single IP injection of 20 mg/Kg of PK dissolved in dimethyl sulfoxide (DMSO). Control mice were injected with the same volume of DMSO alone. Each experimental group was composed of 2 males and 2 females from the same background (B6SJL) as the $SOD1^{G93A}$ mice that used for the preclinical study. Note that we did not observe any difference in PK kinetic to reach the brain between males and females, therefore the results were pooled. For each time point, 4 mice were treated with PK and 4 with DMSO (Total=8 mice X 6 time points = 48 mice). As illustrated in **Fig. 14**, PK reaches the brain very efficiently (peak ~ 7 µM at 10 minutes) but then its levels quickly drop to ~1 µM in the next twenty minutes, before decreasing more slowly and reaching about ~ 500 nM at 2 hours and ~ 400 nM at 2.5 hours. At 3 hours, the levels of PK were undetectable in the brain. ***Based on these data, we can extrapolate that a single IP injection of PK per day could yield desired nM levels of PK specific for TSPO binding for ~ half hour per day (between 2 and 2.5 hours post injection). However for the first hour, the levels of PK reached will be far higher than the desirable levels and after 3 hours probably too low. It is difficult to predict whether such a short and non-specific exposure will allow us to fairly evaluate the therapeutic promise held by PK.***

Stability study

A valuable alternative to daily IP injections could be the continuous delivery of the drug thanks to implanted subcutaneous osmotic pumps. Usually mini-pumps adapted to subcutaneous implantation in mice have a delivery capacity of 4-6 weeks. Therefore, we evaluated whether PK was stable when stored at 37^0C, in a dark incubator saturated with humidity for several weeks. We tested 3 different vehicles compatible with osmotic pump delivery (30% b cyclodextran/0.07% HCl, PEG400 100%, and DMSO/PEG400 at 50/50). **Fig. 15a-c** illustrates the best and most reproducible results that we obtained for PK 25 µM were with the vehicle PEG400/DMSO (a) and two different doses for PEG400 100%, 15 µM (b) and 500 nM (c). ***In both vehicles PK was found to be perfectly stable over 4 or 6 weeks at 37 ºC. These data greatly support that PK dissolved in PEG400 can be efficiently and continuously delivered to ALS mice via osmotic minipumps for up to 4 weeks.***

Fig. 15a. PK Stability study at 37ºC in DMSO/PEG400

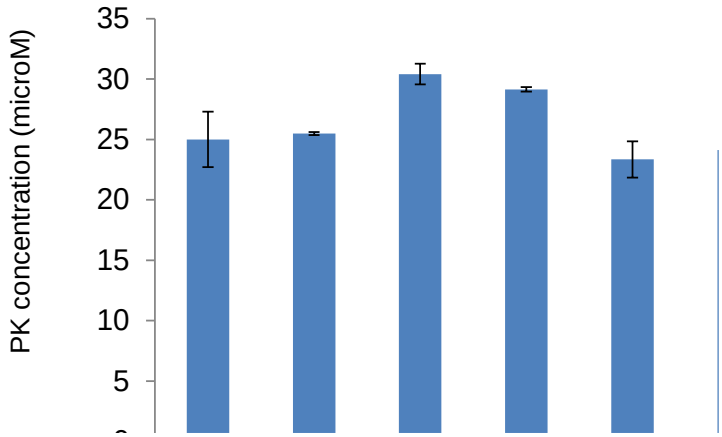


Fig. 15(b). PK Stability study at 37ºC in PEG400

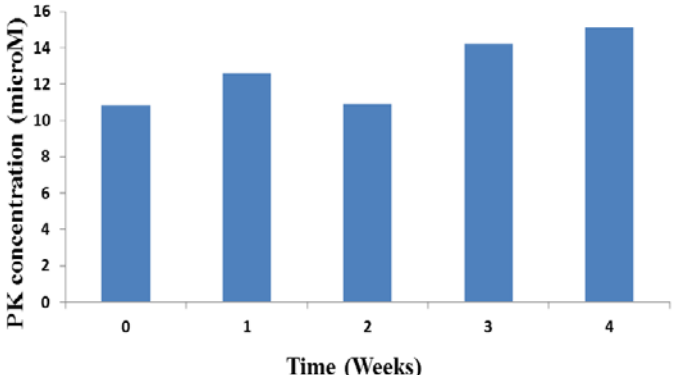
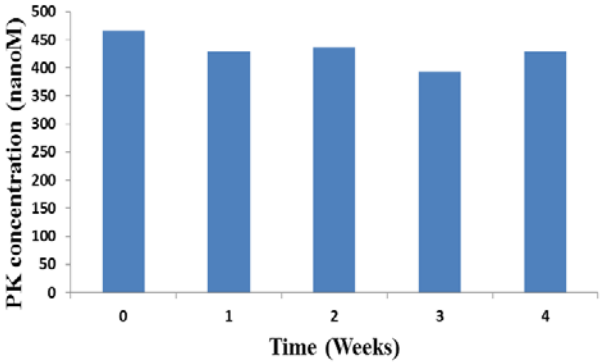


Fig. 15(c). PK Stability study at 37ºC in PEG400



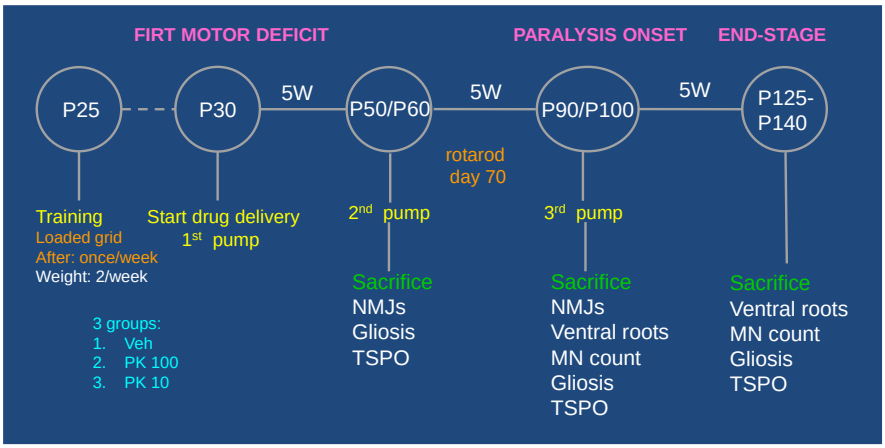
Dose and Tolerability studies

To address the issues of chronic tolerability and doses, we implanted 8 adult mice (P60), four males and four females with the maximal dose deliverable in a 4 week-minipump, based on the maximal solubility of PK in the vehicle selected, i.e. 25 mM in PEG400. The mini-pumps (ALZET Models 1004) held 100 µL and release the solution at a nominal rate of 0.11 µl/hr over 28 days. Mini-pumps were filled with solution sterilely and soaked in PBS at 37°C for 24 hrs prior to surgery according to data obtained from stability in saline. For the surgery, animals were anesthetized with ketamine/xylazine (80/8 mg/kg, ip) and after depth of anesthesia has been achieved, as assessed by hindlimb toe pinch, the upper back area of the mouse was shaved then cleaned x 3 with betadine and alcohol. An incision (0.5-1.5cm,) was made between the shoulder blades and a pocket is formed by spreading the subcutaneous connective tissue apart with a hemostat. The pump was positioned with the flow moderator facing caudally. Care was taken not to disrupt BAT tissue. The incision was closed with two wound clips followed with application of triple antibiotic to minimize the risk of infections. Autoclips were removed 10-14 days after surgery. 2 mice of each gender were sacrificed after 2 and 4 weeks to measure the levels of PK reached in the brain. Over the 4 weeks of the experiment, animals were monitored twice a week for weight, and behavioral signs of tolerability (grooming, cage exploration, social interaction with other mice). ***No adverse effects of the treatment were observed over this period. Similarly, autopsy did not reveal any obvious sign of toxicity in any organ. This indicates that the chronic delivery of PK is very well tolerated by the animals at the maximal soluble dose of PK,*** Following the Bligh & Dyer extraction method described above, we found that ***the average level of PK in the brain of mice implanted with osmotic mini-pumps for 2 weeks was of 271.78 nM +/- 13.42*** which are within the desired 100-500 nM range. However, the average level of PK in the brain **after 4 weeks was of 1.21 µM +/- 0.1** and the mean level reached **in the spinal cord was of 3.41 µM +/- 1.91**. Besides, when we repeated some animals, extracted this time ***with the more consistent and efficient DCM method***, we found that we were reaching **≥10 µM** in the spinal cord of some animals. These levels are too high for our purpose, knowing that above 1 µM PK is known to have some off target effects. To find some doses more appropriate for our preclinical study, we implanted several pumps containing lower doses of PK: 12, 2.5, 1.25, 0.625, 0.2 and 0.1 mM. We found that at 0.1 mM (100 µM) the average level of PK reached in the brain after 2 weeks was of 530 nM +/- 0.13, compatible with specific TSPO activation, but close to the limit. For the second dose, we decided to compare the levels of PK reached in the brain and spinal cord for pumps filled with 25 µM and 10 µM of PK (1/4 and 1/10 of 0.1 mM) and we found that the average levels of PK reached in the brain were 186.8 nM +/- 93 (n=3) and 101.118+/- 28.1 (n=3), respectively, both within the desired levels. The average levels at the spinal cord were of 769.17 nM +/- 32.1 and 316.73 nM +/- 0.18. Therefore, we selected 10 µM as the second dose ***for our preclinical assessment of PK therapeutic value in ALS mice, because these data perfectly meet our objective which was to reach between 100 and 500 nM max of PK continuously in both brain and spinal cord.***

Major Task 2. Preclinical study.

Experimental Time-Line and Study Design

As depicted on the opposite schematic, the 3 groups of SOD1 animals enrolled in the study (Veh, PK10 and PK100) first underwent training to the loaded grid test starting at ~P25-30, and were tested once a week thereafter. The rotarod test was started at P70 and performed weekly until end-stage. Mice were implanted with the first osmotic mini-pump delivering PK at ~P30-35. The pump was then renewed twice at P50-60 and P90-100. A first set of animals was sacrificed at P50-60, a second at P90-100 and a third at end-stage for morphological and pathological analyses. ***The 2 persons assessing motor performance and neuropathology were blinded to treatment.***

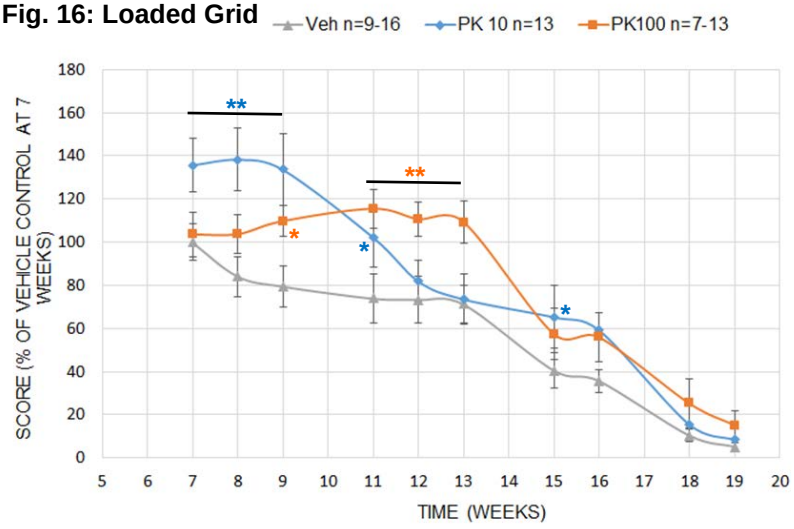


Behavioral testing

SOD1-G93A mice recapitulate many of the pathological features of ALS in humans such as progressive muscle weakness and atrophy, with eventual paralysis and death. We therefore evaluated the possible beneficial effects of the two doses of PK selected from our dose-response study (10 µM [PK10] and 100 µM [PK100]) on lessening the ALS-linked motor deficit in two cohorts of transgenic SOD1^{G93A} mice. We used two motor performance-related behavioral tasks, loaded grids and rotarod (Barneoud et al. 1997). All mice used were ***on the same B6SJL strain background***. ***To control for the effect of sex,*** a known confounder in ALS (Wang et al. 2011), females and males were equally balanced in all our groups. ***To control for the effect of litter,*** our study was powered based on the number of dams rather than pups, and we have ensured that littermates were distributed across different experimental arms. We have estimated sample size for each experiment outlined in this report by power analysis (SigmaStat for Windows version 2.0; Jandel Corporation, San Rafael, CA) using our own pilot or published values (means and SDs) (Wu et al. 2006). We originally enrolled 20 mice per group (10 males and 10 females) to cover all the different studies (behavior, morphological analysis and survival). As these different motor tasks require a learning period before animals reach a stable performance, all mice received pre-training before the tests. Also, we know by experience that a small percentage of mice (≤10%) that we call “non-performers”, may systematically fail the test on purpose (immediately drop the loaded grid or fell from the rotarod) while they do not have any physical motor impairment. “Non-performers” mice just refuse to provide a motor effort that they perceive unpleasant, as attested by the fact that some non-performers for loaded grid excel in rotarod and vice-versa. Non-performers are systematically excluded from the study. A small cohort of Ntg littermate “sentinels” (4 mice) was also tested in parallel, to gauge the extent of motor deficit in ALS mice, although we could not include another powered experimental group for budget reasons. In addition to the two motor tasks, we used a simple neurological scoring system (NeuroScore) focused on hind limb function, because neurological signs of disease in SOD1-G93A mice start with hind limb deficits before paralysis progresses to the rest of the body (Gordon et al. 2009). NeuroScore provides an unbiased assessment of onset of paresis (slight or partial paralysis), progression and severity of paralysis. When first symptoms of paresis were observed, access to food and water was facilitated by placing food pellets and hydrogel® on the floor of the animal cage. Testing was stopped and mice were euthanized when they reached “end-stage criterion”, corresponding to the maximum NeuroScore (NS=4), defined by “a rigid paralysis in the hind limbs”: when the mouse is allowed to walk, there is no forward motion and when the mouse is placed on its left and right side it is not able to right itself within 15 sec from either side. i.e. in absence of “righting reflex”. The following tests were used to assess performance of the three groups of SOD1 mutant mice: PK10, PK100 and vehicle (Veh).

Loaded grid: This test detects forelimb muscle strength impairment much earlier (P40-P50) than overt paralysis symptom onset (Barnéoud et al. 1997). The mouse was allowed to grip a loaded grid with several weights (10, 20, 30, or 40 g) and was then lifted by the tail. A behavioral score was calculated as follows: $\sum(t_w \times W)$, where t_w corresponds to the time for which the mouse was able to carry the loaded grid with weight W. Performance is based on the duration of time in seconds (sec) and allowed 30 s before dropping the weight with a 15 s inter-trial interval and 30 s inter-weight interval. Each mouse was tested three times with each weight (10, 20, 30, and 40 g in that order) for a 30-sec fixed duration once a week. Prior to the initiation of testing at 7 weeks, all mice received pre-training with the unloaded grid starting at 4 weeks of age. Each mouse was given two trials with a 10 g weight and while suspended by the tail was allowed unlimited time to hold the loaded grid by the forelimbs before dropping it, only adding 20 g, 30 g, and 40 g at 5 and 6 weeks (data not shown). **Figure 16** illustrates the loaded grid performance of SOD1 mutant mice PK10 and PK100 vs Veh from 7 weeks to end-stage. From 14 mice originally enrolled per group (16 for vehicles) in the loaded grid test study, we have about n=9-13 mice for the majority of the time points in the curves (some animals were excluded as non-performers, few others had adverse events such as wound from fight, and at the last time points, 18 and 19 weeks, several animals in each group already reached end-stage). Note that we did not observe any difference in loaded grid score between males and females, therefore the results were pooled. The score is represented as the percentage of vehicle control at 7 weeks. Results are expressed as mean ± SEM; the number of animals for each condition is indicated in the bars of the graph. For this time-course experiments differences among group means were analyzed by One-Way Repeated measures ANOVA. When ANOVA shows significant group differences, pair-wise comparisons between group means were tested by post-hoc Newman-Keuls to determine p values: * p<0.05; ** p<0.001. Note that at 7 weeks (P49) the deficit of SOD1 mice as compared to their NTg littermates is already significant ~26%. ***The group treated with PK100 shows an immediate striking beneficial effect on mouse grip strength from 7 weeks but this effect is of short duration as it loses its significance as compared with the Veh group by 12 weeks***. Thereafter, we observed a slight rebound in the beneficial effect of PK 100 at 15 weeks, which progressively disappears up to end-stage. ***The group treated with PK10 shows an initially lower, but more progressive and sustained beneficial effect***, which is significant as compared to the vehicle group from 9 to 15 weeks. ***For the vehicle group, as previously described the motor deficit progresses first slowly before to accelerate at 13 weeks. Altogether, we can summarize that PK initially offers a dose-dependent beneficial effect on the preservation of animal grip strength. However as disease progresses, the effect of the lower dose (PK10) is more sustained over time before to become indiscernible from PK100 at 15 weeks. The atypical biphasic profile of the benefits offered by PK100 is suggesting that at this dose, PK may have another molecular target than TSPO. This is supported by our HPLC data showing that PK100 over time leads to accumulation of PK superior to 1µM. It is possible that the stimulation by PK of these multiple targets lead to a combination of postive and negative effects on ALS disease progression. However, this dose-response experiment has the merit to demonstrate that we could probably not reach higher grip strength beneficial effect with further higher doses of PK.***

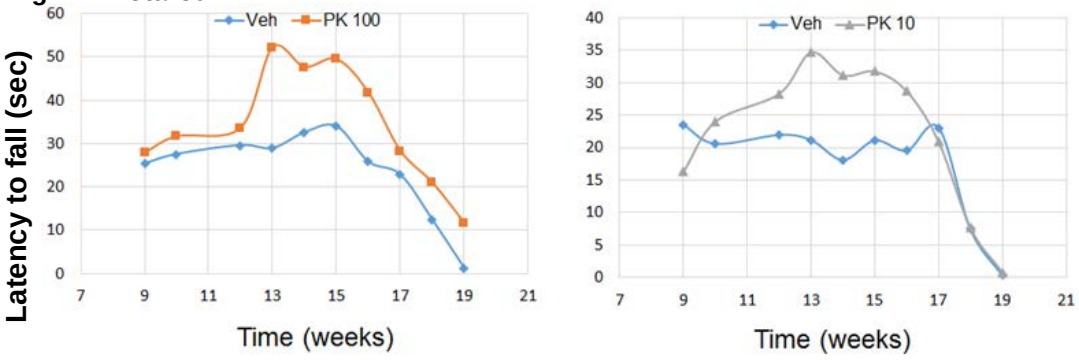
Fig. 16: Loaded Grid



Rotarod: The same three experimental groups were also tested to assess motor coordination, strength and balance. Animals were placed onto the apparatus, an accelerating rotarod where a rotating rod or drum functions as a treadmill for the rodent placed on top. This accelerating version devised by Jones and Roberts (Jones & Roberts 1968) overcomes the inconvenience of running separate tests at different speeds for the same animal. A constant acceleration of 2.5 rpm/s was applied; starting with a speed of 10 rpm to reach a maximum speed of 50 rpm (rotating cylinder was 3.5 cm in diameter). Four mice were tested simultaneously, with each mouse separated

by a 3 mm wide × 60 cm high opaque Plexiglas wall. The time for which and animal could remain on the rotating rod was measured. After a training phase of three trials, each animal was given two trials and the longest latency without falling was recorded. The animals were trained the week before the testing giving each mouse 3 trials a day for 3 days. **Figure 17** illustrates the latency before fall (seconds) of SOD1 mutant mice PK10 and PK100 vs SOD1 vehicle. Note that we did not observe any difference in rotarod score between males and females, therefore, again the results were pooled. Results are expressed as mean ± SEM; the number of animals for each time point is ~n=10-13. Please note that we are still considering different possibilities to combine the results of the two cohorts, as for unknown reason the Veh group of cohort 1 (run in parallel to PK100) were better performers (50% better performance over several weeks) than the Veh group of cohort 2 (run in parallel to PK10). Statistics were not performed yet, but for this time-course experiment differences among group means will be analyzed by One-Way Repeated measures ANOVA. When ANOVA shows significant group differences, pair-wise comparisons between group means will be tested by post-hoc Newman-Keuls. In agreement with the loaded grid test, we observed an initially dose-dependent beneficial effect of PK (80% improvement of motor performance for PK100> 64% improvement for PK10). But for this more general test of locomotor performance, the effect of PK100 was more consistent over time (not bi-phasic). For both doses the beneficial effect was more prolonged (up to 17 weeks) than for the more precisely related to muscle strength loaded grid test.

Fig. 17: Rotarod



Body weight: It has been proposed that one of the simplest parameters that could be used as a surrogate for defining "clinical onset" or "overt paralysis onset" (inappropriately called by some "disease onset") in transgenic SOD1G93A mice is **a ≥5-10% decrease in peak body weight**. Indeed, ALS is accompanied by a significant weight loss related in particular to muscle atrophy in both human and mice. Therefore, body weight was assessed twice a week for all the animals. **Figure 18** illustrates changes over time in weight of the three groups (PK10 n=8-13, PK100 n=8-13, and vehicle n=8-14). Data are shown as mean ± SEM. ***Peak body weight was reached at 15 weeks in the three groups. Thereafter, weight started to decrease gradually and we did not observe any improvement or delay in peak body weight decline as disease progresses in any of our treatment groups as compared with Veh.*** The only difference that we observed is a non-significant tendency toward a higher weight early in the study (7 to 10 weeks) for the mice enrolled in the PK10 group.

Fig. 18:

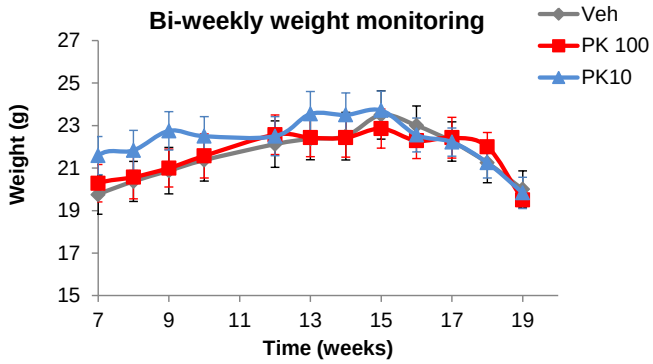
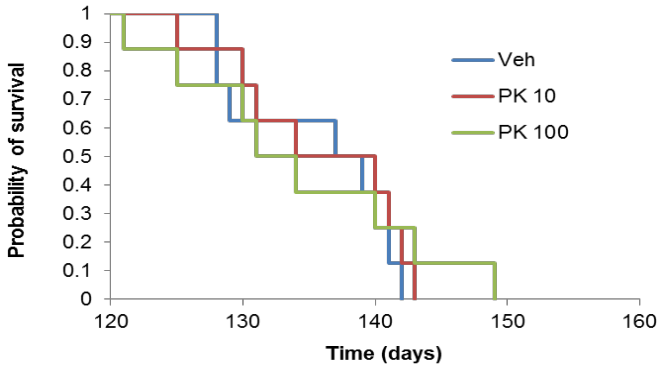


Fig. 19: Survival comparison between groups.



Survival study:

Some mice in each treatment group (n=8) were also followed until end-stage to assess the potential effect of PK in increasing disease duration and prolonging ALS mouse survival. As illustrated in **figure 19, when probability of survival over time was analyzed, no significant difference was observed among the**

different experimental groups. Kaplan–Meier survival curves shows that the groups treated with PK 10 and PK 100 survive for an average of 134.75 ± 2.36 days (n =8) and 133.13 ± 3.32 days (n = 8) respectively. Mice from the Veh group survive on average, 134.50 ± 2.16 days (n = 8). In contrast with other studies using less severe transgenic SOD1 mouse models, we did not observe any difference in average survival between males and females, therefore the results were pooled.
Altogether, our results demonstrate that PK10 and PK100 both have a significant but transient beneficial effect on ALS mouse muscle strength and motor performance before overt onset paralysis. However, PK even at the highest dose tested does not significantly delay the clinical onset of paralysis (Figure 18 and Neuroscore not shown) or prolong mutant SOD1 ALS mouse lifespan **(Figure 19)**.

Histopathological analysis

One of the earliest pathologic events observed in diseased SOD1G93A mice is the denervation of muscles i.e. the progressive disappearance of the neuromuscular junctions (NMJ) i.e. the unique synaptic sites where spinal motor neurons connect with myofibers to form a functional motor unit and induce voluntary muscle contraction. The loss of NMJs is followed by an active degeneration of the nerves (ventral root axons) and a significant loss of large alpha motor neurons in the ventral horn of the spinal cord. In paralell to our behavior and survival study, we have investigated the potential neuroprotective effict of PK in ALS mouse tissue from the neuromuscular junction, along the ventral roots and the spinal cord.

Neuromuscular junction (NMJ) quantification: in order to investigate the efficacy of PK in the protection of the NMJs, tibialis anterior (TA) muscles were dissected from mutant mice at 8-9 weeks of age. Mice were perfused transcardially with saline solution and 4% PFA. Muscles were post fixed in 4% PFA and cryopreserved in sucrose. Whole TA muscles were then embedded in OCT media (Tissue-Tek), frozen on dry-ice and stored at −80°C. A Leica cryostat was used to cut 25 µm longitudinal sections of frozen TA muscles which were subsequently collected onto 1 mm superfrost slides for immunostaining. Primary antibodies used were: mouse monoclonal anti-synaptic vesicle SV2 (Developmental Studies Hybridoma Bank), rabbit monoclonal anti-neurofilament H (200 KDa) (EMD Millipore). α-Bungarotoxin-647 was used to label post-synapsis and sections mounted. Images were acquired on a confocal microscope (Leica DFC 420C). Between 300-500 NMJs from each of 3 vehicle and 3 PK mice were manually counted. NMJ types (I, II and III) were defined by the extent of overlap between AChRs stained with labeled α-bungarotoxin (α-BTX) and nerve terminals stained with anti-synaptic vesicle SV2. Please note that we are currently counting 1 more Veh and 1 more PK animal per dose to complete the study.

Figure 20 illustrates the NMJ integrity criteria that were used to define ***three classes (fully innervated—class I, partially innervated—class II and fully denervated—class III) of neuromuscular synapses.*** Following this classification we have compared distal motor unit profiles of PK and Veh controls. NMJ synapse integrity quantification is presented in **Figure 21.** Results are expressed as the percentage of total NMJs of each class/muscle. This comparative analysis ***reveals a striking dose-dependent protective effect of PK on NMJ integrity in 8-9 week old pre-paralytic mutant SOD1 mice.*** **Figure 22** illustrates images of vehicle, PK 10 and PK 100 TA muscles at 8 weeks. NMJs that exhibited an overlap of red (α-BTX) and green (SV2) were considered innervated, while those that exhibited only α-BTX expression were considered denervated. Here we

Figure 20

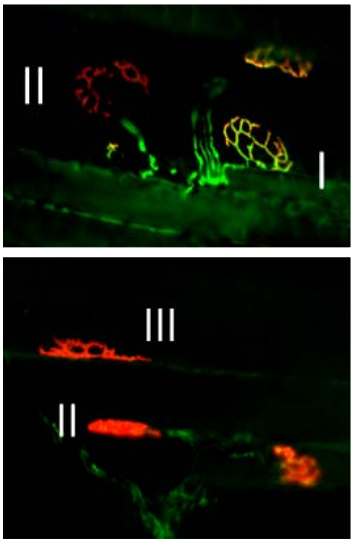
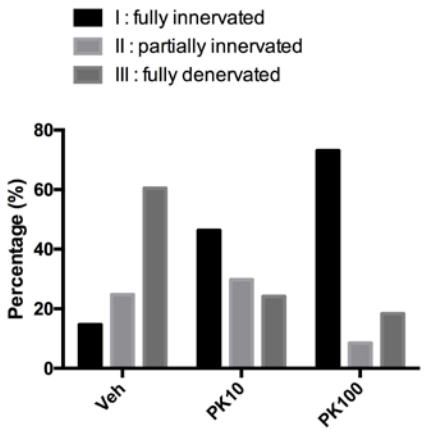


Figure 21



observed an ***obvious denervation in mouse vehicle whereas in PK 10 and PK 100 animals NMJ integrity is mostly preserved.*** This preservation of NMJ structures explain at the tissue level the motor functional benefit that we observed at the same stage (8-12 weeks) in PK-treated ALS mice performing the loaded grid test (**Figure 16**). Therefore, early in the disease process PK is capable to delay the loss of hind limb NMJs and improve the maintenance of grip strength in declining transgenic SOD1 ALS mice.

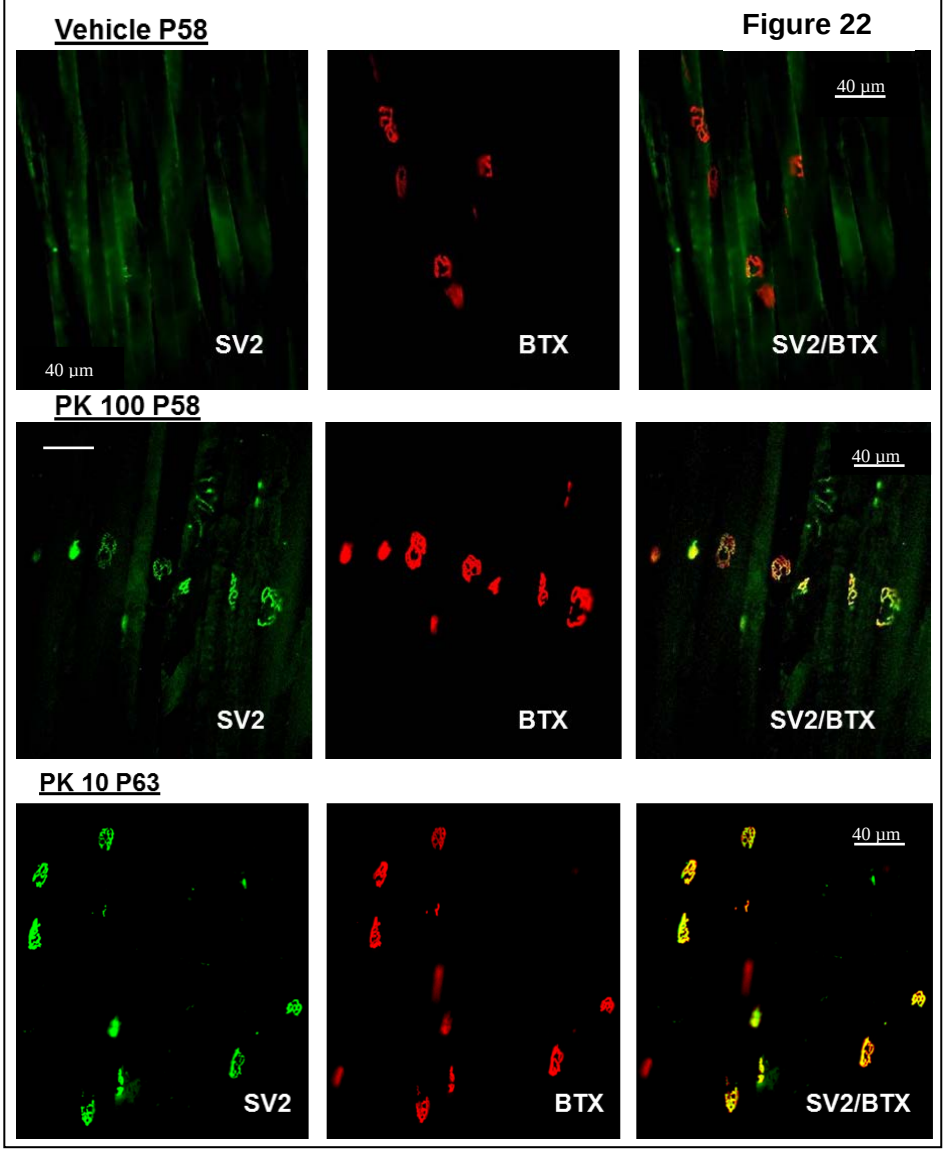
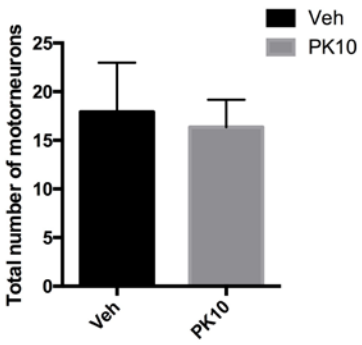


Figure 22

Counts of motor neuron (MN) cell bodies in the lumbar spinal cord (L4–L5) of 14-weeks-old mice:

Loss of motor neuron soma in the ventral horn of the spinal cord of SOD1G93A mice has previously been reported to be a late-stage event in the disease process. Significant decrease of large alpha-motor neuron cell body number begins at around post-natal day (P)90 (Fischer et al. 2004; Chiu & Zhai 1995). Here, spinal cords from ~P100 (14-weeks-old) vehicle and PK mice were embedded in 5% (wt/vol) agar and then were cut into 70-µm cross-sections with a Leica vibratome VT 1000S. For each experimental group, counts of motor neurons were performed on the lateral motor column of the lumbar spinal cord segments L4 through L5, which are the most affected in ALS mice and in most of ALS patients. Motor neuron were counted from z-series of confocal optical sections obtained at a magnification of 20× (0.9× optical zoom; zstep of 1 µm). Motor neurons labeled with ChAT (ChAT+ MNs) were imaged in each stack and were outlined in the confocal plane and counted. The histograms **presented in Figure 23** show that there is ***no change in the average number of MNs in the ventral horn of vehicle and PK10*** of 12 vibratome sections pooled from the L4/L5 spinal segments of a minimum of three different animals of similar age and genotype (we are currently counting more animals per group to complete the study). Ventral horn was defined as gray matter ventral to the central canal. Only MNs with a clearly identifiable nucleus were counted every 4th section. (Please note that these counts do not represent total motoneuron count and is a sampling approach). Depending on the group ~13–20 MNs were counted per ventral horn. In the histograms, error bars always indicate the SEM. Some spinal cord sections were photographed with a Leica confocal microscope **(Figure 24)**. These images are representative of the L4-L5 segment from a Veh **(figure 24a)** and a PK10 **(figure 24b)** mouse at 14 weeks. Motor neurons were immunolabeled for the marker choline acetyl-transferase (ChAT) in green. Scale

Figure 23



bars: 250 µm (left panel and) and 25 µm (right panel). While more motor neuron counts are currently ongoing for supplementary animals (at this stage, end-stage and for the treatment group PK 100), these results suggest that ***at later disease stages (14 weeks) for which the improvement in ALS mouse grip strength is lost (loaded grid; Figure 16) but PK still offers a significant beneficial effect on general motor performance (rotarod measures coordination and balance; Figure 17), the clinical benefit is not corroborated at the tissue level by a preservation of the number of motor neuron soma in the ventral horn of the spinal cord.*** We are currently assessing whether the motor benefit could be explained by preservation of neuromuscular junctions probably via sprouting of motor nerves adjacent to the lost ones. We are also examining the integrity of the motor nerves in the L4 and L5 ventral roots of the lumbar spinal cord by toluidine blue staining, as we previously described (Wu et al. 2006). This analysis is ongoing right now but as illustrated in **Figure 25,** we do not observe any obvious difference between the treatment groups: degenerative nerves with loss of or myelin debris are observed, as well as the presence of numerous small lipid droplets and autophagosomes. Quantification of large versus small motor nerves will confirm whether we do not observe any protective effects at this level similar to our observation in the spinal cord.

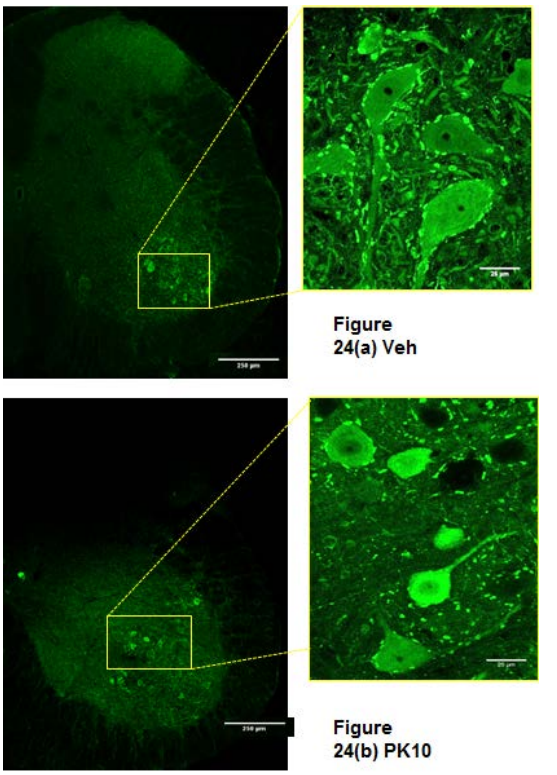
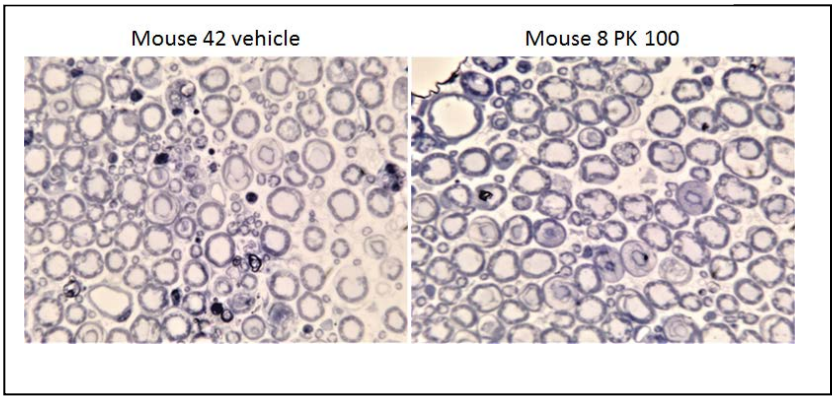
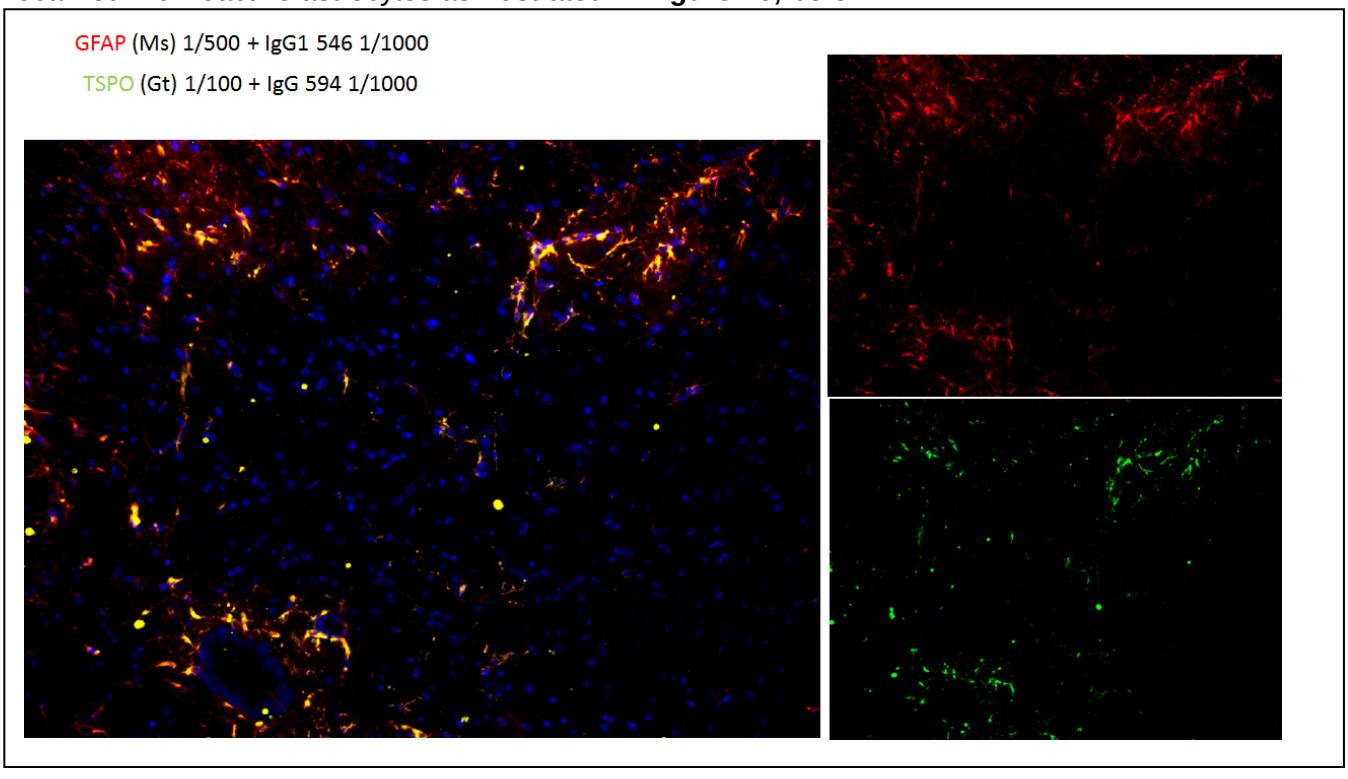


Figure 25: Toluidine blue-stained L5 anterior root sections



Finally, we are currently assessing the effect of PK on gliosis in the spinal cord and we found that TSPO is highly co-localized with reactive astrocytes as illustrated in **Figure 26, below.**



Conclusion and Perspectives:

Overall we found that both doses of PK significantly improve grip strength, motor performance, and muscle innervation of ALS mice in the early phase of the disease. However, PK does not delay the onset of paralysis and does not appear to protect motor neuron survival or extend mouse lifespan at later disease stages. In diseased ALS mouse spinal cord, we found that TSPO expression was primarily co-localized with inflammatory reactive astrocytes. Our future studies will have to determine whether over time PK's neuroprotective effect, which acts via an increase of motor neuron defense against astrocytes is not overruled by the continuous and possibly exacerbated detrimental effect of astrocytes. ***This supports the view that only the combination of therapies targeting neuroprotection and lessening of glial cell neurotoxicity will hold some promise for clinical efficacy in ALS.***

What opportunities for training and professional development has the project provided?

This project is fully supporting the post-doctoral training of Dr. Teresa Obis who is a very promising young scientist from Spain. Dr. Obis joined the laboratory of Dr. Re in mid-January 2015. She already had some experience in animal models of neuromuscular diseases and in investigations of neurodegeneration and pathology of the neuromuscular junction. However, over the 24 months of work on the project, under the mentorship of Dr. Re, she has been fully trained in *in vitro* investigations and disease modeling (mouse primary brain cells and mouse embryonic stem cell cultures). She also acquired a solid expertise in drug extraction process, pharmacokinetics, drug stability study and in HPLC analysis. Finally, Dr. Obis has now a vast knowledge on TSPO biology and on translational research for amyotrophic lateral sclerosis.

This first year of the project, has also allowed the PI, Dr. Re, to attend the Society for Neuroscience Annual Meeting in Washington (Nov 15-19 2014), where she met with the co-authors of one of the groundbreaking papers showing that TSPO knockout is not embryonic lethal and that TSPO may not be as important as previously thought for cholesterol transport and steroid synthesis (Banati et al. 2014). This finding had an important impact on the project, notably on **SA-1**.

This year (2017) Dr. Obis will present the results described in this report to the Society for Neuroscience Annual Meeting that will be held in Washington (Nov 11-15 2017).

How were the results disseminated to communities of interest?

Nothing to report.

What do you plan to do during the next reporting period to accomplish the goals?

Nothing to report.

IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

The findings that we made over this two years of the project will have a significant impact in the field of translocator protein (TSPO) research and associated therapeutic development. This protein was thought to have a role exclusively in non-neuronal cells which are responsible for inflammatory processes in the brain (astrocytes and microglia). Accordingly, TSPO was used as a biomarker of brain inflammation and TSPO ligands were developed for imaging purposes. Several other recent studies have suggested that TSPO ligands could be neuroprotective in the context of multiple sclerosis (Daugherty et al. 2013) and Alzheimer's disease (Barron et al. 2013). ***However, our study is the first to reveal that TSPO ligand neuroprotective effects could be mediated by targeting the neurons themselves and not the inflammatory glial cells. Furthermore, we provide novel insights into a differential localization of the protein TSPO in neurons as compared with astrocytes and microglia. This suggests that a new role of TSPO in neurons could be related to its presence in a different***

cell compartment than in other cells. Also, our data are pointing to the fact that neurons with the highest level of expression of TSPO are those most resistant to ALS astrocyte-mediated toxicity. These open new perspectives of using neuronal TSPO as a marker of resistance to the degenerative process in ALS.

Regarding therapeutic perspectives, our work indicates that TSPO ligands are very stable molecules at body temperature but they disappear very quickly from the brain after a single injection. Therefore, ***we propose for the first time to deliver a TSPO ligand continuously to animals modeling the paralytic disorder ALS, thanks to the use of mini-pumps placed under the skin that are designed to release in the body a certain amount of TSPO constantly for 4-6 weeks. The data we generated in this project will be very useful for the therapeutic development of TSPO ligands at large, not only for ALS.***

In the context of ALS, overall with a rigorously executed and controlled preclinical study, we found that ***the TSPO ligand PK11195 tested at 2 doses that we confirmed to reach brain and spinal cord levels relevant to its affinity for TSPO, significantly improve grip strength, motor performance, and muscle innervation of ALS mice in the early phase of the disease.*** However, ***PK does not delay the onset of paralysis and does not appear to protect motor neuron survival or extend mouse lifespan at later disease stages***. In contrast with too many studies in the field which do not measure the drug levels reach in mouse CNS, we know that the mitigated success of PK in ALS mice is not due to the incapacity of PK to reach the CNS as we have controlled that at end-stage, PK was still very efficiently going to the brain of our animals. In diseased ALS mouse spinal cord, we found that TSPO expression was primarily co-localized with inflammatory reactive astrocytes. Our future studies will have to determine whether over time PK's neuroprotective effect, which acts via an increase of motor neuron defense against astrocytes is not overruled by the continuous and possibly exacerbated detrimental effect of astrocytes. ***This supports the view that only the combination of therapies targeting neuroprotection and lessening of glial cell neurotoxicity will hold some promise for clinical efficacy in ALS. Therefore, even if PK is not the miraculous drug we are all looking for to cure ALS, our group and others may be interested in the future in testing the combination of PK with drugs mitigating glial cell toxicity in ALS, or drugs increasing TSPO expression selectively in motor neurons.***

What was the impact on other disciplines?

As just stated above, the use of mini-pumps to deliver TSPO ligands could offer therapeutic perspectives not only for ALS but for an array of neurodegenerative diseases, psychiatric disorders and even for cancer and metabolic dysfunctions where TSPO has been suggested to be involved (Batarseh & Papadopoulos 2010).

What was the impact on technology transfer?

Nothing to Report.

What was the impact on society beyond science and technology?

Nothing to Report.

CHANGES/PROBLEMS:

Changes in approach and reasons for change.

As mentioned in **Specific aim 1/Major Task 5** of the **Accomplishment** section, the experiments that we initially planned for our investigation of the mechanism of PK's neuroprotective effect have been revisited in light of our progresses in SA-1 and of some recent breakthroughs in the TSPO research field. Based on the finding that TSPO neuroprotective effect is mediated though its activity on motor neurons, we have entirely refocused this task on motor neurons. We are now trying to elucidate PK activation of a defensive or neuroprotective mechanism rather than TSPO involvement in a cell death cascade. Finally, our data showing a weak presence of TSPO at the level of the mitochondria in motor neurons suggest that the neuroprotective effect is unlikely to be mediated

through this organelle. However, before completely dismissing this possibility, it will be important to verify whether in motor neurons treated with PK, TSPO is not re-localized to the mitochondria. Currently, we are prioritizing experiments to understand which role TSPO may have in association with misfolded protein degradation pathway and vesicle trafficking in motor neurons.

Then, as mentioned in **Specific aim 2** of the **Accomplishment** section, before we started **Major Task 1**, data available from one of our previous clinical studies in ALS mice made us considerably revise our strategy. Indeed, in this previous study we found that after 100 days of daily gavage with 2 different JNK inhibitors (30 mg/kg/day), the brain concentrations of the inhibitors were~65% lower in Tg SOD1^{G93A} mice than in the NTg mice. We haven't clarified the origin of the difference in brain concentration between NTg and Tg SOD1^{G93A} mice, but ***we decided to evaluate a more direct route of administration that would allow us to bypass any potential intestinal absorption problem in Tg SOD1^{G93A} mice such as intraperitoneal injection (IP) or implanted subcutaneous osmotic pumps.*** As we found that PK was stable at 37^{0}C for several weeks, and based on our need of chronic administration of PK for months in ALS mice, we used mini-pumps instead of gavage in our preclinical study.

Actual or anticipated problems or delays and actions or plan to resolve them

The "full-speed" start of the project has been delayed of about three months knowing that on October 1st 2014, the PI of the project was promoted to Assistant Professor in the Department of Environmental Health Sciences (EHS) at Columbia University and she was allocated new laboratory space and a starter package to fully equip her new lab. Because of the time to interview staff and process necessary VISA and human resource paperwork, the post-doctoral student who is supported by this project did not start before mid-January 2015. But at that time the laboratory was fully equipped and "full speed" thanks to a lab technician supported by the starter package from the PI. From October 1st 2014 to mid-January 2015 Drs. Re, Guilarte and Guariglia were the only personnel actively working on the project. Dr. Guilarte made sure to provide access to any equipment and space from his lab that Dr. Re needed to start right away some experiments while her own lab was equipped and her staff was in the hiring process.

Another delay resulted from a necessary change in one of the key personnel on the project. Dr. Jackson-Lewis told the PI that she will not be able to fulfill her commitment to the project, due to personal reasons. Fortunately, a Staff Associate in the EHS Department, Vesna Ilievski, who possesses the same expertise as Dr. Jackson-Lewis in High Pressure Liquid Chromatography agreed with the consent of her PI to take over for Dr. Jackson-Lewis for the rest of the award performance. We requested approval for this change that was approved on April 23rd 2015.

Changes that had a significant impact on expenditures

3 types of changes need to be reported:

1. **Hiring staff delays:** for Dr. Obis, the post-doctoral scientist who started mid-January 2015 instead of September 30th 2014. And for Ms. Vesna Ilievski who started on April 23rd 2015 instead of September 30th 2014, as initially planned for Dr. Lewis.

2. **Animal cost delays**: all the work for specific aim 2 was delayed until Dr. Re had enough staff to take care of the animals in January. We are now ready to catch-up and some animals that were supposed to be ordered and housed in the first year of the grant will be used in the second year of the project as soon as it officially starts.

3. **Extra-cost for the mini-pumps versus gavage delivery of the drug**. One mini-pump costing ~$50 and knowing that we originally plan to treat with PK or vehicle 240 mice (120 mice for the pre-symptomatic study and 120 mice for the post-symptomatic study), and that for the pre-symptomatic study 2/3rd of them will need to receive a second pump (80 X 2) for the length of the study and 1/3rd of them (40 X 3) a third pump, and that for the post-symptomatic study half will need a second pump (60 X 2) and half only one pump (60), we will need to order for the [pre-symptomatic] and [post-symptomatic] studies a total of [(120)

+ (80 X 2) + (40 X 3)] 420 pumps X $50= $21,000

To meet this significant extra cost our options were to try to re-allocate part of our budget toward this new cost and to scale down our study. We obtained approval from our allocated science officer regarding these budget modifications.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of vertebrate animals

The only change to report is the new delivery method of the drug for the preclinical study (osmotic mini-pump versus gavage) which was approved by Columbia IACUC on 24-DEC-2014 protocol number AAAL2502 and reviewed and approved by the USAMRMC Animal Care and Use Review Office (ACURO) as of 10-SEP-2015.

Significant changes in use or care of human subjects

Nothing to Report.

Significant changes in use of biohazards, and/or select agents

Nothing to Report.

PRODUCTS

We are currently finalizing the confocal analysis of Major Task 1 in SA-1 as well as the counting of motor neuron and NMJs in SA-2 but we anticipate that a publication will be ready for submission in a highly respected neuroscience journal by July 2017.

Also we are now preparing an abstract to present the results described in this report to the Society for Neuroscience Annual Meeting that will be held in Washington (Nov 11-15 2017).

PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project

Name:	Diane Re aka Gourion-Arsiquaud
Project Role :	Principal Investigator
eRa Commons id :	Dr2240
Nearest person month worked :	3
Contribution to project :	Her role is to design, organize and supervise all of the experiments and review, analyze, and interpret all of the work performed by the scientists involved in this project.
Name:	Teresa Obis-Ibanez
Project Role :	Post-doctoral Scientist
Columbia University id :	Tvo2103
Nearest person month worked :	12
Contribution to project :	She is the prime mover on all of the *in vitro* and *in vivo* studies with the exception the electron and confocal microscopy work.

Name:	Sara Guariglia
Project Role :	Co-Investigator
eRa Commons id :	GUARIGLIAS
Nearest person month worked :	2.4
Contribution to project :	She undertook the sub-cellular characterization of TSPO by immuno electron and confocal microscopy.

Name:	Vesna Ilievski
Project Role :	Co-Investigator
Columbia University id :	Vi2101
Nearest person month worked :	2
Contribution to project :	She is sharing her experience in phamacokinetic/toxicology study and is also performing the HPLC measurement of PK11195 levels in tissue extracts.

Name:	Tomas Guilarte
Project Role :	Collaborator
eRa Commons id :	Tguilar1
Nearest person month worked :	0.24
Contribution to project :	Dr. Guilarte will provide his TSPO expertise to this proposal by co-supervising the work performed by Sara Guariglia and participating in the analyses and the interpretation of all of the generated results.

Has there been any change in the active other support of the PD/PI or senior key personnel since the last reporting period

Nothing to Report.

What other organization were involved as partners.

Nothing to Report.

2. Special Reporting Requirements: Not applicable.

Appendices

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