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### **► To cite this version:**

Baptiste Porte, Gwendolyn Marguerit, Sylvie Thomasseau, Claire Paquet, Jacques Hugon. Dose-dependent neuroprotective effect of the JNK inhibitor Brimapitide in 5xFAD transgenic mice. *Brain Research*, 2020, 1727, pp.146587 -. <10.1016/j.brainres.2019.146587>. <hal-03488388>

**HAL Id: hal-03488388**

**<https://hal.science/hal-03488388v1>**

Submitted on 21 Dec 2021

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## **Dose-dependent neuroprotective effect of the JNK inhibitor Brimapitide in 5xFAD transgenic mice**

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## **ABSTRACT**

Alzheimer's disease (AD) is a neurodegenerative disease mainly affecting old people. According to the "amyloid cascade hypothesis", the accumulation of A $\beta$  oligomers could lead to kinase activation and tau phosphorylation. Activated kinases include c-Jun N-terminal kinase (JNK) and previous studies highlighted the beneficial effects of the JNK-specific inhibitor Brimapitide (10 mg/kg) in 5xFAD transgenic mice. Our aim was to evaluate the effects of decreasing doses of Brimapitide on cognition and neurodegeneration in early treated 5xFAD mice.

Three month-old 5xFAD were intravenously treated for 6 months with either Brimapitide (3 mg/kg or 0.3 mg/kg) or NaCl. Cognition and amyloid burden, neuronal and synaptic impairments were evaluated. Low doses of Brimapitide (0.3 mg/kg) reduced neuronal degeneration and improved cognition in treated mice compared to non-treated mice. Amyloid burden and synaptic degeneration only decreased with the 3 mg/kg dose.

This JNK inhibitor can afford neuroprotection but with a differential effect on amyloid deposition in 5xFAD mice. Brimapitide might partially prevent ongoing neurodegeneration in 5xFAD mice.

**KEYWORDS:** Alzheimer's disease; mice; JNK inhibitor; dose ranging; Brimapitide

## 1. INTRODUCTION

Alzheimer's disease (AD) is the most common form of dementia mostly affecting aging population. In 2018, almost 44 million people in the world suffered from dementia (GBD 2016 Dementia Collaborators. 2019). This number is estimated to triple by 2050 (Patterson 2018). The three main pathological features of AD are: i) extra-neuronal accumulation of  $\beta$ -amyloid peptides ( $A\beta$ ) aggregating into senile plaques, ii) intra-neuronal accumulation of hyperphosphorylated tau proteins and iii) neuronal death associated with synaptic loss and neuroinflammation (Duyckaerts et al. 2009). Memory troubles are often the first clinical sign followed by aphasia, apraxia, agnosia and behavioral disturbances.

The "amyloid cascade hypothesis" describes the pathophysiological mechanism of AD linked to a dysregulation of  $A\beta$  peptides accumulation (Hardy et al. 1992). The amyloid precursor protein (APP) is first cleaved by  $\beta$ -site APP cleaving enzyme (BACE1) producing: i) a soluble form of APP and, ii) a membrane-linked fragment. Then,  $\gamma$ -secretase cleaves this fragment into  $A\beta$  peptides of either 40 ( $A\beta_{40}$ ) or 42 ( $A\beta_{42}$ ) amino acids.  $A\beta_{40}$  is the most common form of  $A\beta$  peptide whereas  $A\beta_{42}$  is present in lower quantity at physiological state. In familial AD cases, mutations identified in patients affect genes coding for APP or  $\gamma$ -secretase, modifying the balance between  $A\beta_{40}$  and  $A\beta_{42}$ . Accumulation of  $A\beta_{42}$  leads to the formation of neurotoxic  $A\beta$  oligomers that aggregate into insoluble senile plaques and cause ultimately a cascade of brain lesions responsible for cognitive decline (Lane et al. 2018). Amyloid-induced kinase activation is a common feature of this "cascade hypothesis" and could lead to tau phosphorylation and neuronal demise (Ebenezer et al. 2010, Kim et al. 2015, Hugon et al. 2017).

Several studies have demonstrated the implication of c-Jun N-terminal kinase (JNK), a serine-threonine kinase of the mitogen activated protein kinase family, notably in the pathological process of AD (Kim and Choi 2015). JNK participate in the development of inflammation and neurodegeneration (Morishima et al. 2001, Zhu et al. 2001, Savage et al. 2002, Tamagno et al. 2005, Colombo et al. 2009, Scip et al. 2011, Scip et al. 2014, Gourmaud et al. 2015). Among the three isoforms (JNK1, JNK2 and JNK3), JNK 3 is expressed rather specifically in the brain. Cerebrospinal fluid JNK3 as well as PKR levels are enhanced in AD patients and correlate with cognitive impairments (Dumurgier et al. 2013, Gourmaud et al. 2015). JNK is

activated in AD brains and can be associated with A $\beta$  accumulation (Savage et al. 2002). In addition, JNK can phosphorylate APP on threonine 668 leading to an augmented A $\beta$  production and can increase BACE1 mRNA expression (Standen et al. 2001, Tamagno et al. 2005).

Previous works have shown that JNK inhibition is an appropriate tool for studying signaling pathways at a physiological state (Wei et al. 2014, Jing et al. 2019) and for assessing therapeutic intervention in various pathologies (Morishima et al. 2001, Borsello et al. 2003, Scip et al. 2014, Yarza et al. 2015). Among specific JNK inhibitors, Brimapitide (XG-102) is a small inhibitory peptide already proposed in clinical development for other diseases (Chiquet et al. 2017, Eshraghi et al. 2018, Staecker et al. 2019). In preclinical studies, Brimapitide has demonstrated neuroprotective effects notably in cerebral ischemia (Borsello et al. 2003) and in AD models, by decreasing APP phosphorylation and A $\beta$  production (Colombo et al. 2009, Ploia et al. 2011, Scip et al. 2011). Moreover, *in vitro* studies have revealed that JNK inhibition, including with Brimapitide, was able to reduce A $\beta$  toxicity or neurodegeneration of cultured cortical neurons (Repici et al. 2009, Gourmaud et al. 2016). In 2018, Gourmaud et al. reported that a 6-month treatment with Brimapitide at 10 mg/kg improved cognition and reduce amyloid burden and neurodegeneration in 5xFAD mice (Gourmaud et al. 2018). Thus, a phase 1 study in healthy human volunteers has already shown that the intravenous (IV) infusion of Brimapitide at 10, 40 and 80  $\mu$ g/kg was well-tolerated (Deloche et al. 2014).

5xFAD mice are genetically engineered animals that express five human mutations, three on APP and two on presenilin-1 (PS1), one of the 4 units of  $\gamma$ -secretase. 5xFAD mouse is considered as an aggressive transgenic AD model, as A $\beta$  accumulation and plaque formation start earlier than in many other AD models (Webster et al. 2014). Indeed, Oakley et al. (2016) described in 5xFAD mice early cognitive impairments (at 3 months-old) associated with A $\beta$  accumulation from 1.5 month onward, synaptic markers progressively decreasing with aging (notably PSD-95) and a peak of neuronal loss at 6 month. Both increased A $\beta$  load and loss of PSD-95 labelling have been shown in various AD mice models (Dinamarca et al. 2012) and will be used in this study as markers of brain lesions.

The aim of our study was to evaluate the effect of Brimapitide at lower doses than 10 mg/kg in 5xFAD mice. We wanted to carry out a dose-effect study to determine the most appropriate starting concentrations for future human trials. The 5xFAD mice were IV treated

for 6 months with Brimapitide at 3 mg/kg, 0.3 mg/kg or with NaCl. The results show that 3 mg/kg Brimapitide efficiently decreases A $\beta$  burden while 0.3 mg/kg reduces the neuronal degeneration in association with improved cognition in 5xFAD mice.

## **2. RESULTS**

### **2.1. Brimapitide improves cognition of 5xFAD mice at 0.3 mg/kg**

The effect of Brimapitide on cognition was assessed with the Y-maze test. Mice were placed in the center area of the Y-maze and allowed to explore freely for 8 minutes. Entries in each arm and in the center area were automatically recorded. These data were used to calculate alternation percentage based on the ability of animal to do triades of entries without re-entering in a just-visited arm. The task requires the animal to navigate in a maze (spatial memory) and remembering which arm it has previously visited (procedural working memory). 5xFAD mice treated with NaCl revealed a statistically lower alternation percentage than control mice. Regarding the effect of Brimapitide, only 0.3 mg/kg treated 5xFAD mice showed a statistically significant improved cognition compared both to NaCl and 3mg/kg treated counterpart (Fig. 1). Treatment at 3 m/kg failed to improve cognition.

In addition to Y-maze, we also used contextual fear conditioning, assessing associative memory: association of an aversive stimuli (electrical foot shock) with a particular context (a test chamber with black and white stripes on the walls). Cognition was evaluated by the number of freezing episodes and total freezing time. No effect of Brimapitide treatment was observed in this test (Supp data and Supp Fig 1)

### **2.2. Brimapitide is able to attenuate neuronal degeneration**

Fluor Jade (FJ) is a green fluorescent dye that labels degenerating neurons. Effect on neurodegeneration (labeled neurons) was evaluated by fluorescence imaging in the subiculum that is the most and earliest affected region of the hippocampus in 5xFAD mice. No FJ staining was detected in control mice (Fig 2A, picture only). Only in 0.3 mg/kg treated mice a statistically lower FJ labeling was detected as compared to NaCl treated mice (Fig 2A).

This significant effect of the lowest dose was also observed when results were compared to 3 mg/kg treated mice (Fig. 2A)

We then choose to analyze synaptic degeneration by assessing the levels of PSD95, a post synaptic protein, known to have decreased brain levels during the pathology evolution in AD mice models (Dinamarca et al. 2012). We realized immunoblots on extracts of total cortex and hippocampus of each mice group with an antibody directed against PSD-95. Only in total cortex of 3 mg/kg treated mice PSD 95 levels were significantly higher than in NaCl treated mice (Fig. 2B). In the hippocampus, no statistically significant changes were observed with treatment (data not shown).

### **2.3. Amyloid burden is reduced by Brimapitide at 3 mg/kg.**

Finally, the effect of Brimapitide on amyloid burden, a characteristic feature of AD pathology, was assessed by quantified immunohistochemistry using an antibody against the A4 epitope of APP which is specific for soluble A $\beta$ . No staining was found in control mice (Fig 3A and 3B, pictures only). In 5xFAD mice a significantly higher labeling was observed compared to control mice, both in the cortex and in the subiculum (data not shown). The amyloid load was statistically reduced in 3 mg/kg treated 5xFAD mice compared to NaCl-treated and to 0.3 mg/kg treated 5xFAD mice. This effect was found in the cortex (Fig. 3A) and the subiculum (Fig. 3B).

## **3. DISCUSSION**

Our study demonstrates that in 5xFAD mice, Brimapitide can modulate cognitive decline and improved AD brain lesions at lower doses than previously described (Gourmaud et al. 2018). In details, the 3 mg/kg dose was able to reduce synaptic loss (PSD95) in the cortex and the amyloid load in the cortex and in the subiculum. 5xFAD mice treated with 0.3 mg/kg dose showed improvement in the Y-maze task (alternation percentage) and decreased neuronal degeneration revealed by FJ. Treatment started at 3 months of age, a pathological stage with limited brain lesions which could correspond to a human MCI due to AD stage and the

pharmacological processes could somewhat correspond to a preventive approach of mildly affected 5xFAD mice.

The first step of our study was to determine the appropriate minimum doses able to ensure protective effect of Brimapitide in 5xFAD mice as an initial phase to envision future human trials with this drug (Gourmaud et al. 2018). We have determined two concentrations at 3 mg/kg and 0.3 mg/kg, according to a practical guide for dose conversion (Nair et al. 2016), that are able to modulate cognition, amyloid load, synaptic and neuronal damages in 5xFAD mice. The first question that can be addressed is why the 3 mg/kg dose did not reduced cognitive alteration or synaptic loss as observed with the 0.3 mg/kg dose ? Although all mice were similar and genetically controlled as in our previous report, one explanation could be linked to the known genetic and/or epigenetic heterogeneity in 5xFAD mice (Grinan-Ferre et al. 2016). We have assessed repetitively the genetic background of the 5xFAD mice but we could not evaluate in all mice the five inserted mutations responsible for brain lesions as well as epigenetic modifications. It is possible that due to this heterogeneity, a genetic drift may have modified the magnitude of abnormal metabolisms leading to neuropathological lesions and altered cognition. Using the same compound in TgCRND8 AD mice, the dose of 22 mg/kg was needed to be efficient at modulating cognition and brain lesions suggesting that a variability of response to Brimapitide occurs as well in various AD transgenic models (Sclip et al. 2011). Moreover, epigenetics factors can also modulate the pathological features of AD mice model. Devi et al. (2010) reported that behavioral stress can modulate A $\beta$  production in hippocampus. So it is not impossible that variable stress may have altered the level of A $\beta$  in 5xFAD brain and so can be accounted for the observed heterogeneity.

No effect on cognition was detected with the fear conditioning test. The doses used could be too low for inducing any effect in this paradigm as we previously described a reduction of cognitive deficit with a 10 mg/kg treatment (Gourmaud et al. 2018).

The second step of our research was to determine if Brimapitide was able to reduce neurodegeneration at reduced doses. We have shown that 0.3 mg/kg could reduce the FJ staining after 6 months of administration in the subiculum of transgenic animals. A neuroprotective action of Brimapitide was demonstrated in earlier works but with higher concentrations at 22 and 10 mg/kg in AD models (Sclip et al. 2011, Gourmaud et al. 2018). The role of JNK in neuronal death has been already well documented in various models of



brain disorders and our results emphasize the concept that JNK inhibition is a future possible target in AD (Sclip et al. 2014, Yarza et al. 2015).

The third notable point of our study is the dissociation between the effect of Brimapitide on amyloid burden and cognitive improvement in 5xFAD mice. This finding was already detected in our previous report using a 10 mg/kg dose for 6 months (Gourmaud et al. 2018) and was confirmed in this report using a 0.3 mg/kg dose for 6 months. With this concentration, the neuroprotective effect was not associated with a reduction of the amyloid burden. This result implies that Brimapitide can attenuate detrimental neuronal pathways independently of the concomitant neurotoxic role of A $\beta$  species. It is known that JNK is implicated in neuronal death, synaptic plasticity, inflammation and memory and the observed effects of Brimapitide on neurodegeneration and cognition may well be related to the direct consequences of JNK inhibition by Brimapitide in neurons but also in glial cells (Kaminska et al. 2009, Coffey 2014). Nevertheless, this dissociation can also be interpreted in keeping with the involvement of JNK in the pathological cascade of AD (Kim and Choi 2015). Moreover, as factors like mitochondrial dysfunction through calcium efflux impairments can also contribute to disease evolution (Jadiya et al. 2019), JNK inhibition alone may not be sufficient to rescue all abnormal signalings present in 5xFAD mice.

A previous works has shown that Brimapitide is able to cross the blood brain barrier after intraperitoneal (IP) injection and is present in the brain for at least one week with an increased concentration as compared to 24 h post injection (Davoli et al. 2014). To our knowledge no information is available concerning the brain long lasting presence of Brimapitide beyond one week after IV or IP administration. A limitation of our study is that mice were IV injected every 3 weeks to comply with our previous study. This rhythm of administration could explain the absence of modification of the ratio phosphorylated JNK/total JNK observed in total cortex and hippocampus of treated d 5xFAD mice (Supplementary data and Supplementary Fig 2). The remaining action of Brimapitide could be drastically decreased after 3 weeks. Future evaluations of the concentrations of Brimapitide in the brain of experimental animals will be necessary to determine the slowly decreasing cerebral levels after a single IV or IP administration. Similarly, an assessment of the cerebrospinal concentrations of Brimapitide after IV infusion will be needed in human trials.

In conclusion, our study has demonstrated that low doses of Brimapitide can have an effect on neurodegeneration, amyloidosis and cognition in the 5xFAD mice. 5xFAD mice were treated at an early stage of the evolution of brain lesions and Brimapitide seems to partially prevent ongoing abnormal neuronal processes leading to neurodegeneration. Future researches will be needed to determine if the early reduction of JNK activity, experimentally and in humans, is able to interfere with the course of abnormal brain lesions.

## 4. EXPERIMENTAL PROCEDURE

### 4.1. Mice and treatment

This study project (#9035 2017020611339643) was submitted to n°3 CECCO Committee at Cryopreservation, Distribution, Transgenesis and Archiving (license D-45-234-6) and n°5 Charles Darwin Committee at Brain and Spine Institute, Paris, France (license A75-13-19). Committees and the Higher Education, Research and Innovation French Ministry green lighted it (agreement APAFIS #9035-2017020611339643 v2). All experiments followed animal handling guidelines from the French Agriculture and Forestry Ministry (decree 87849) and EC Directive 86/609/EEC for animal experiments.

5xFAD are C57Bl6 mice with an artificial transgene expressing the K670N/M671L, I716V, and V717I mutations of human APP gene, and the M146L and L286V mutations of human PS1 gene. Three-month-old 5xFAD males were randomly assigned to a treatment group (n= 7-12 animals): NaCl, XG-102 (Brimapitide) at 3 mg/kg or at 0.3 mg/kg. Each group was treated during 6 month, reaching a total of 9 injections in the caudal vein. Brimapitide (XG-102 peptide, Xigen S.A., Switzerland) was reconstituted in NaCl (0.9%, B. Braun, France) and diluted at the corresponding concentrations. Doses were calculated according to dose conversion guide between humans and animals (Nair et al. 2016). C57Bl6 wild type mice were used as control mice (CT, Janvier Laboratories, Saint Brethevin, France).

Animals were housed in standard cages (5 or less mice per cage) in a controlled environment (23°C, 53% humidity, and 12h/12h light cycle). Mice had *ad libitum* access to food and water. All the breeding and injections were done in Cryopreservation, Distribution, Transgenesis and Archiving (CDTA, Orleans, France).

## **4.2. Behavioral tests**

Behavioral assessments took place 3 weeks after the last injection due to the sanitary requirements of the preclinical functional exploration unit PHENOPARC (Brain & Spine Institute, Pitié-Salpêtrière Hospital, Paris, France). ANY-Maze software (v5.2, Stoeling) was used to recorded and analyzed behavior.

The Y-maze test evaluated spatial and procedural working memory (hippocampus specific). It consisted in a Y shaped maze (27 cm length, 7 cm width and 20 cm height for each arm). Each mouse explored it freely during 8 minutes. Memory was evaluated by the alternation percentage:  $\text{number of consecutive entry in 3 different arms} / (\text{total number of entry} - 2) * 100$ .

## **4.3. Brain tissue and protein preparation**

Mice were anesthetized with 0.15 ml of sodium pentobarbital (Ceva, Paris, France). A quick intra-cardiac perfusion of phosphate buffer saline (PBS, Merck, Darmstadt, Germany) was performed for cleaning the circulatory system. Brain was obtained through dissection and separated in two hemispheres.

The left one was fixed with paraformaldehyde 4% diluted in PBS (PFA, Merck, Darmstadt, Germany) for 24 hours at 4°C, then post-fixed in paraformaldehyde 0.4% for a week at 4°C. The right hemisphere was dissected to obtain the total cortex and the hippocampus. Both structures were flash-freeze in liquid nitrogen and stored at -80°C. Proteins were extracted from total cortex and hippocampus samples with completed RIPA buffer (RIPA added with 1µM calyculin A, 1mM Na<sub>3</sub>VO<sub>4</sub> and a protease inhibitor cocktail; Merck, Darmstadt, Germany) then sonicated (1 min, 55% of maximal intensity, Sonopuls, Bandelin, Berlin, Germany). Protein concentration was measured with the BiCinchoninic acid assay.

## **4.4. Western blots**

Western blots were carried out using primary antibodies directed against PSD95 (Rabbit, D27E11, Cell Signaling, Leiden, The Netherlands) and Vinculin (Mouse, V9131, Merck KGaA,

Darmstadt, Germany) as housekeeping protein. Secondary antibodies coupled with horseradish peroxidase were used for revelation with enhanced chemiluminescence. They were directed against rabbit for PSD95 (7074, Cell Signaling, Leiden, The Netherlands) and mouse for Vinculin (HAF007, R&D Systems Inc., Minneapolis, USA).

#### **4.5. Immunohistochemistry**

Fixed hemispheres were dehydrated, included in paraffin and sagittally cut with a microtome at a 5 µm thickness. Brain sections were incubated with a primary antibody directed against APP (A4, Cell Signaling, Danvers, USA) and a secondary biotinylated anti-rabbit antibody (Vector Laboratory, Eurobio Scientific, Les Ulis, France). Neurodegeneration was evaluated with Fluoro-Jade B (Merck Millipore, Darmstadt, Germany). Labeling either visible or fluorescent was quantified with automated algorithm on ImageJ 1.48v (National Institute of Health, USA).

#### **4.6. Statistical analyses**

The aim of our study was to evaluate the effect of low doses of Brimapitide on pathological features of an AD mice model. As we wanted to find the lowest effective dose, we choose to compare separately each Brimapitide groups to Nacl counterpart and/or control animals. Non-parametric Mann-Whitney U-test was used for comparing each groups (GraphPad Prism, San Diego, USA). For each comparison, P value < 0.05 or inferior were considered statistically significant.

## **ACKNOWLEDGEMENTS**

The authors thank the XIGEN S. A. and INSERM for funding this work. The authors thank the staff from CDTA-TAAM and PHENO-ICMICE for their technical support.

## **DISCLOSURE**

Pr. J. Hugon is a consultant for Xigen S.A. and Pr. J. Hugon, Dr. B. Porte and G. Marguerit have received fundings from Xigen S.A.

All authors have approved the final article.

## **FUNDING SOURCE**

This work was supported by XiGen S.A *via* INSERM. The company also provided Brimapitide for this study and took part in the decision to submit this work for publication.

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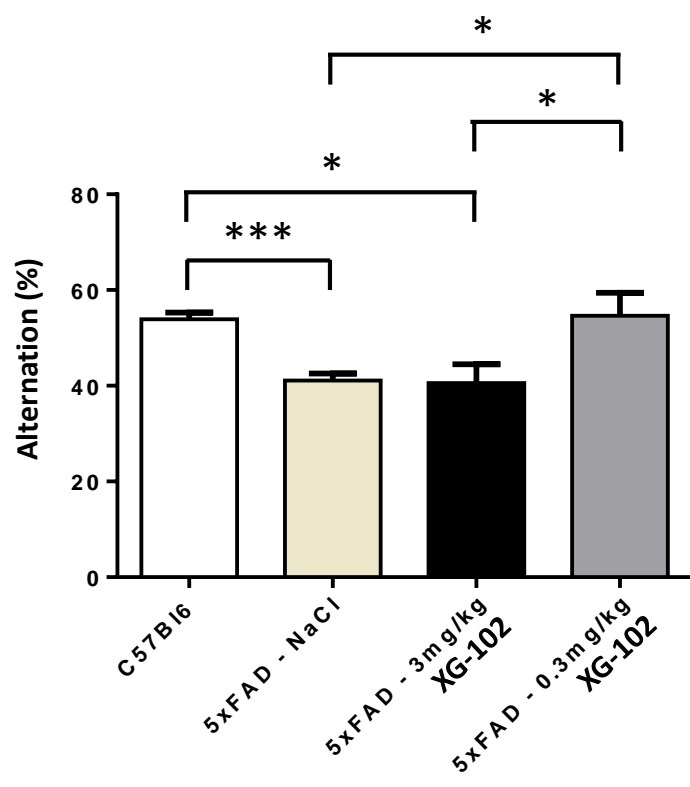


## FIGURES LEGENDES

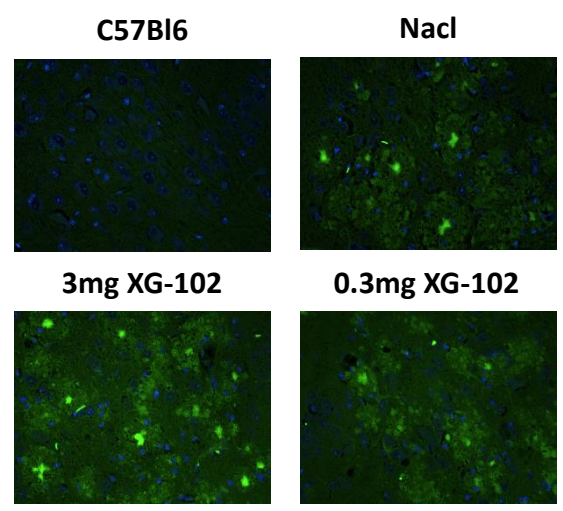
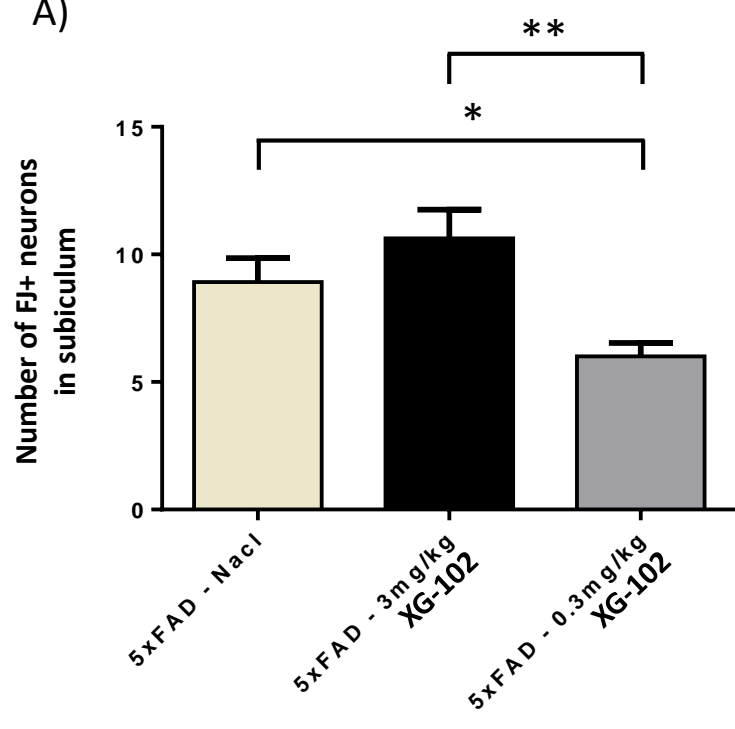
**Figure 1.** Effect of Brimapitide treatment on spatial and procedural working memory in the Y-maze test. Data are represented as means  $\pm$  SEM. \* $P < 0.05$ , \*\*\* $P < 0.001$ , Xg-102 = Brimapitide

**Figure 2.** Effect of Brimapitide treatment on neuronal impairments. A) Quantification of FluoroJade (FJ) labelling of degenerating neurons in subiculum. B) Level of PSD95 in the hippocampus. Data are represented as means  $\pm$  SEM. \* $P < 0.05$ , \*\* $P < 0.01$ , Xg-102 = Brimapitide

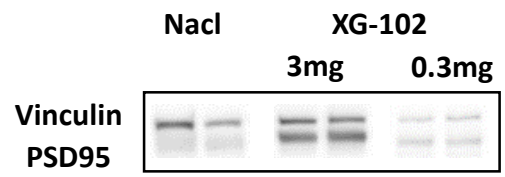
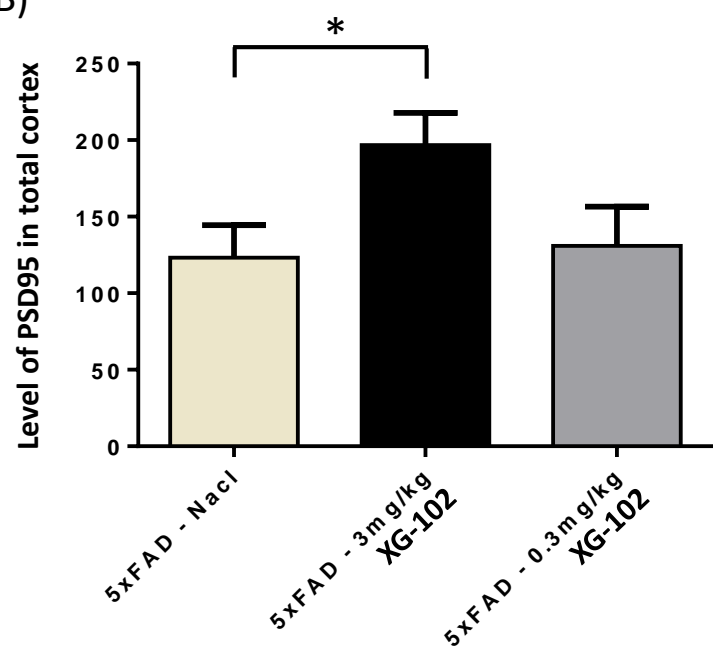
**Figure 3.** Effect of Brimapitide treatment on amyloid burden. Quantification of A $\beta$ 42 clone A4 labelling in A) the cortex and B) the subiculum. Data are represented as means  $\pm$  SEM. \* $P < 0.05$ , \*\*\*  $P < 0.001$ , \*\*\*\* $P < 0.0001$ , Xg-102 = Brimapitide



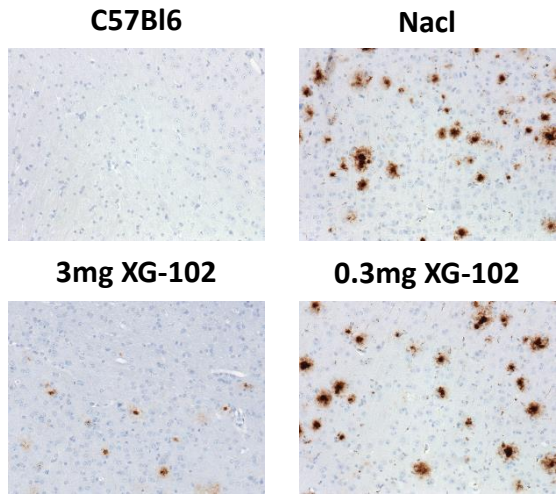
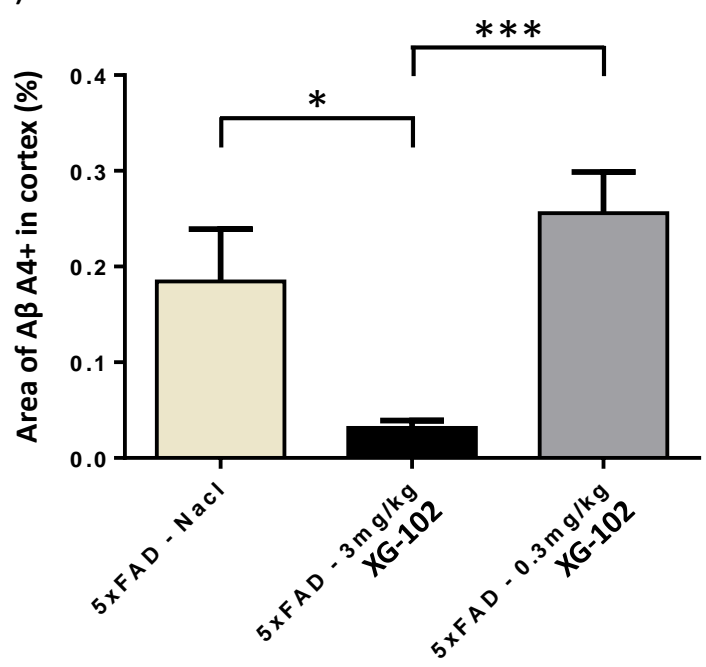
A)



B)



A)



B)

