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DATE

STUDY OF THE NEUROPROTECTIVE EFFECT OF TEN COMPOUNDS IN CULTURES OF MESENCEPHALIC NEURONS IMPAIRED BY Aß

Experiment II

The goal of the second stage of this project was to study the neuroprotective effects of several CLIENT compounds in primary cultures of mesencephalic dopaminergic neurons treated with exogenous β amyloid protein (Aβ). As defined in the first stage of the project, the extent of neuronal loss in each experimental condition was quantified using the variation in MAP2 surface labeling as specific neuronal index. According to the optimal conditions defined during the first stage of the project, the possible neuroprotective effect of the CLIENT compounds was assayed in 8-day-old neuronal cultures treated for 6 days with 10µM of Aβ (complete 1-42 peptide, Sigma No. A9810). The test system has been described in detail (Rouge Pont et al., *European J. Neuroscience,* 1999, 11:2343-2350). These cultures contain dopaminergic neurons taken from embryonic rat mesencephalon. These neurons fully differentiate *in vitro*, survive for at least 30 days, and release dopamine in response to different stimulations.

1. Methods

1.1 Treatments

Solutions of compounds were prepared as specified by CLIENT. All the solutions were freshly prepared in culture medium at the maximum

concentration used and not filtered, excepted for XXX2 (filtered) and XXX7 (the stock solution was done in water and was not diluted more than 100 times in culture medium).

Treatments were performed in a serum-free defined medium according to the recommendations of CLIENT with 30min pre-treatment before adding Aβ. The XXX1 compound was also tested in two others conditions: (i) 24h pre-treatment before adding Aβ and (ii) pre-mixing and pre-incubation of the compound with the Aβ in defined medium at room temperature for 24h, before treating the cells.

1.2 Measure of neuronal loss

Neuronal cell loss was quantified using confocal image analysis, specifically by measuring the decrease of the surface occupied by neurons, as revealed by MAP2 staining (green fluorescence). Using Metamorph 4.5 (Universal Imaging), the MAP2 area ("green thresholded area") was quantified using an optimized intensity threshold value discriminating MAP2 staining from the background.

Each culture well was divided into four fields and the entire surface occupied by the culture was analyzed. Three wells were used for each treatment condition. Thus, each plotted mean corresponds to the average of 12 values (3 wells x 4 fields per well) except for some controls (untreated and Aβ) which comprise up to 24 values.

The size of the surviving neuronal population was expressed as the percentage of the green thresholded area relative to the total area of the field.

This protocol was slightly altered in two conditions, for practical reasons. For the positive control (NDGA), the analyses were performed using two separate neuronal cultures in which NDGA was included each time. Also, the three conditions for the XXX1 compound were tested in a separate experiment. (See Results for further explanation.)

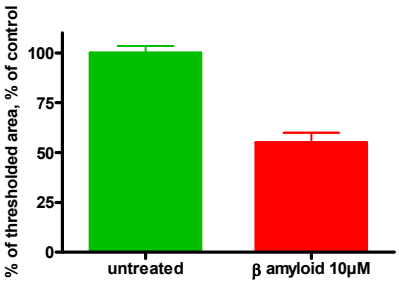
1.3 Statistical analysis

Each group was tested for the normality of the distribution of its values using the D'Agostino and Pearson test. Significant differences among the experimental conditions were determined by one-way ANOVA followed by Tukey or Bonferroni post-hoc tests, as appropriate, following significant ANOVA.

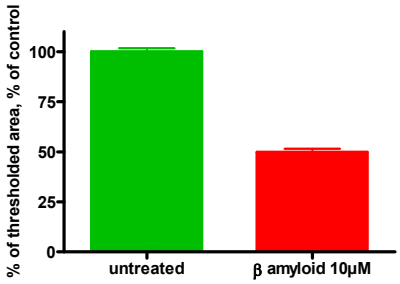
2. Results

2.1 Analysis of Aβ effects in controls

Neuronal loss induced by Aβ in control cultures (i.e. not treated with the CLIENT compounds or NDGA) was expressed as the percent decrease of the thresholded area relative to cultures that did not receive Aβ (untreated) (Fig.1). All the Aβ treatments yielded comparable results with about 45% neuronal loss. This value was deemed sufficiently close to that obtained in the first stage of this study (60% neuronal loss).

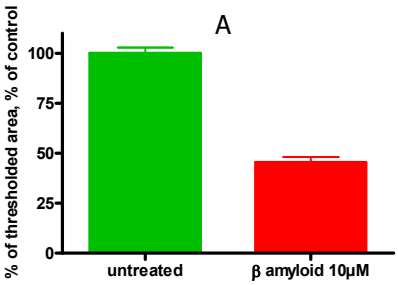


A

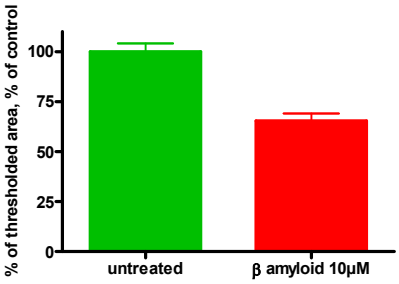


B

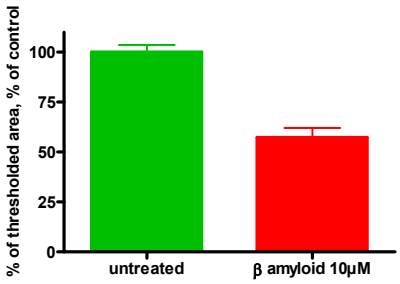
C



Control 1



Control 2



Control 3

Fig. 1: Aβ effects in control cultures. Data are presented as mean +/- SEM. Control 1: 6 day treatment. These controls were in experiments with a 30 min pre-treatment of the test compounds or NDGA before adding Aβ. A: XXX1 and NDGA controls; B: XXX2, XXX3, XXX4, XXX5 controls; C: XXX6, XXX7, XXX8, NDGA controls. Control 2: 24h in serum-free defined medium followed by 6 day treatment. This control occurred when testing XXX1 with a 24h pre-treatment before adding Aβ. Control 3: 24h pre-incubation of defined medium +/- Aβ at room temperature followed by 6 day treatment. This control occurred when testing XXX1 with 24h pre-mixing/pre-incubation before adding Aβ.

2.2 Effects of CLIENT compounds and NDGA

2.2.1 30 min of incubation

All the CLIENT compounds as well as the NDGA positive control were tested in the presence and in the absence of Aβ. There were two types of effects: 1. a neurotrophic effect, consisting of an increase in neuronal survival in the absence of Aβ; 2. a neuroprotective effect, consisting in an increase of neuronal survival in the presence of Aβ. The pattern of results showed that all compounds could be classified as either (A) those for which the neuroprotective effects were greater than the neurotrophic effects; or (B) those for which the neuroprotective effects and the neurotrophic effects were equivalent.

2.2.1.1 Effects of the NDGA control

Administration of NDGA in the absence of Aβ had neurotrophic effects that started at the 3μM dose and increased at higher doses. In the presence of Aβ, NDGA also showed a neuroprotective effect that became significant at 10μM.

The EtOH 0.02% control for NDGA at the latter's highest dose(i.e., the solvent used) produced an unexpected result, i.e. MAP 2 labelling was significantly increased in the EtOH 0.02% control. It is then difficult to

estimate how much of the increase in NDGA effects observed at the highest dose is actually the result of NDGA effects or of the EtOH 0.02% solution. Indeed subtraction of the EtOH effects would eliminate completely the further increase in NDGA action observed at the highest dose.

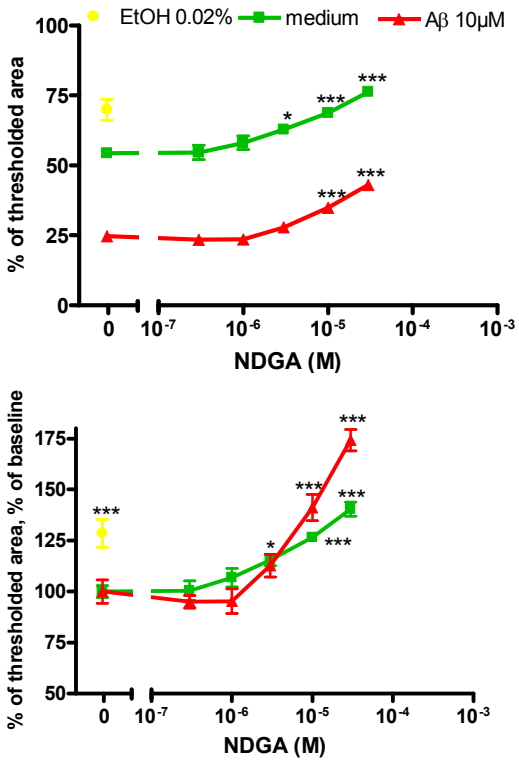


Fig. 2: Effects of the NDGA control. Data are presented as mean +/- SEM: raw data are presented on the left panel and percentages of the baseline on the right panel. ***= P<0.001, *= P<0.05 in comparison to baseline (0M dose).

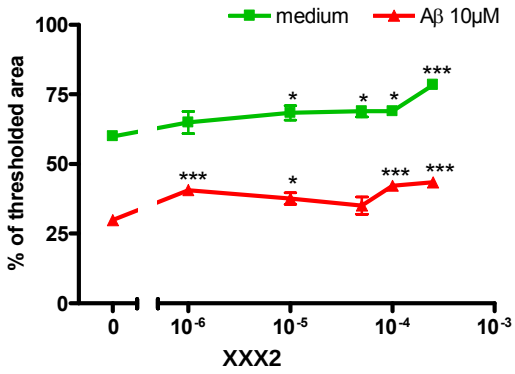
2.2.1.2 CLIENT compounds with greater neuroprotective than neurotrophic effects

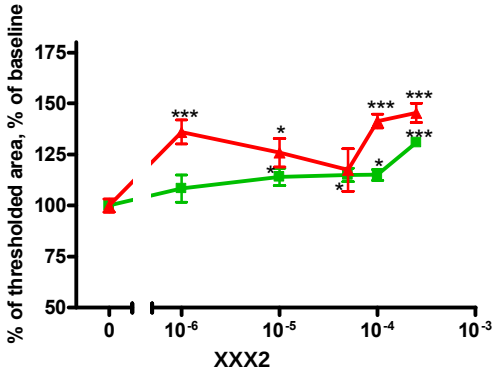
Dose-response functions of compounds in this class showed there were actually two further subclasses: a. compounds showing biphasic effects and b. compounds showing a bell-shaped dose response function.

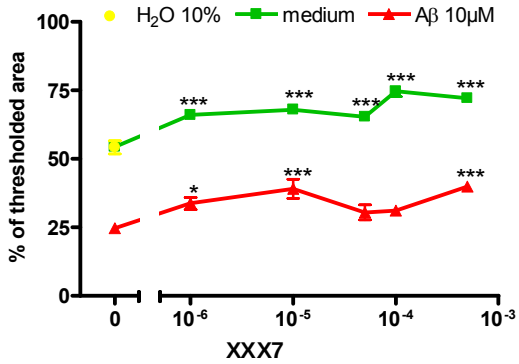
2.2.1.2.1 CLIENT compounds with comparatively greater neuroprotective effects showing a biphasic action

XXX2 and XXX7 (Fig.3) showed both neurotrophic and neuroprotective effects. However the neuroprotective effects were higher than the neurotrophic ones. Thus, in the presence of Aβ the two compounds induced an increase in neuronal survival of approximately 50%, while in the absence of Aβ the increase in neuronal survival reached a maximum of around 25%.

XXX2 and XXX7 also showed a biphasic dose response function with neuroprotective effects appearing at 10^{-6}M, decreasing between 10^{-5}M and 10^{-4}M and increasing again at higher doses. It is worth noting also that with XXX7 there was a slightly hypo-osmotic condition due to the presence of 10% distilled water in the treatment medium, following the solubilisation of XXX7, but that this effect appears innocuous.


medium
Aβ 10μM
% of thresholded area
XXX2


% of thresholded area, % of baseline
XXX2


H2O 10%
medium
Aβ 10μM
% of thresholded area
XXX7

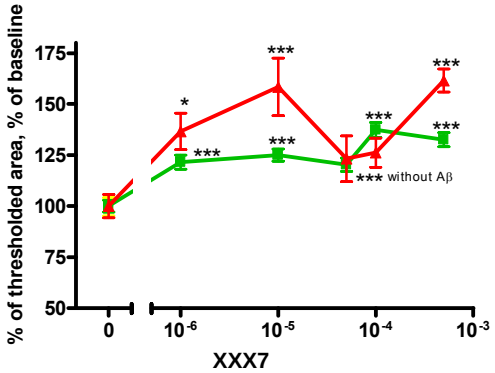

% of thresholded area, % of baseline
*** without Aβ
XXX7

Fig. 3: Effects of XXX2 and XXX7. Data are presented as mean +/- SEM: raw data are presented on the left side and percentages of the baseline on the right side. ***= P<0.001, *= P<0.05 in comparison to baseline (0M dose).

Comparison of the neurotrophic effect (in the absence of Aβ) of XXX7 and XXX2 with the appropriate NDGA control (Fig.4) revealed a similar maximal effect but this occurred at higher doses for the CLIENT compounds. In contrast, comparison of the neuroprotective properties (in the presence of Aβ) revealed an opposite picture with a higher potency for the CLIENT compounds compared to NDGA. This result pattern suggests that the neurotrophic and neuroprotective effects of the TEVA compounds are mediated by different mechanisms.

The maximal neuroprotective effect of XXX7 and NDGA did not differ significantly (Fig.4) while the maximal effect of XXX2 seemed lower. However, the range of doses used is only in the descending limb of the dose response function of XXX2. Consequently the maximal effect of this compound is probably observed for doses that are lower than 10^{-6}M. It is also important to note that the effects of the highest dose of NDGA are probably substantially due to the EtOH 0.02% solution.

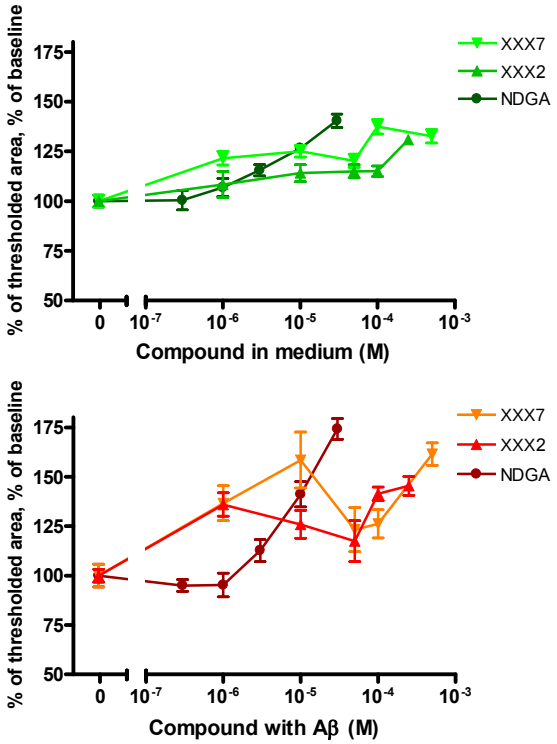
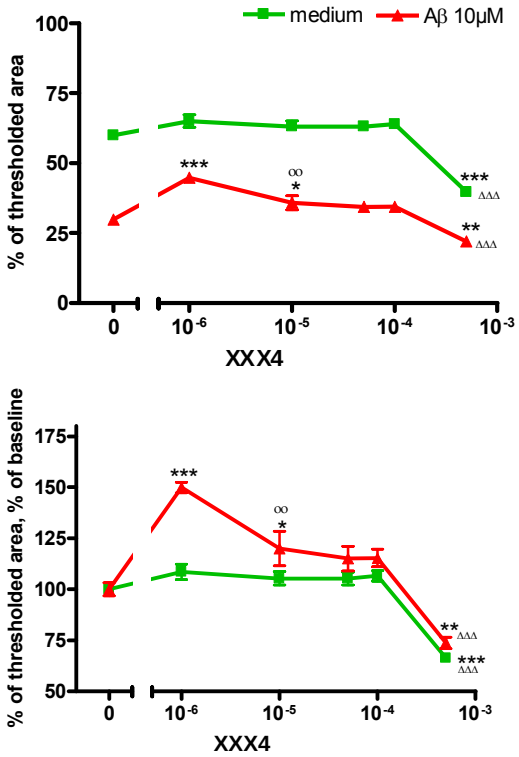


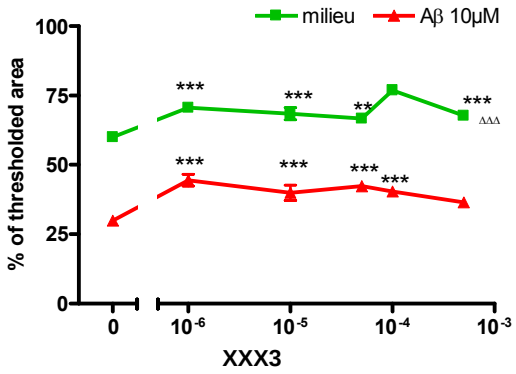
Fig. 4: Comparison of XXX7, XXX2 and NDGA effects. Data are presented as mean +/- SEM percentages of the baseline.

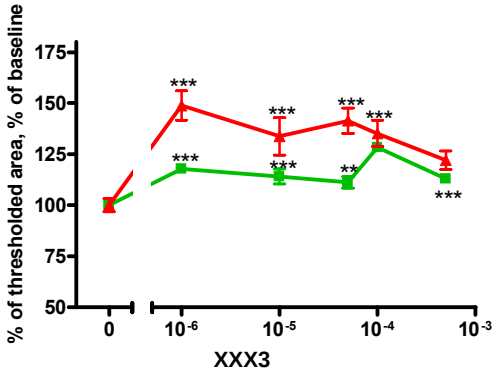
2.2.1.2.2 CLIENT compounds with comparatively greater neuroprotective effects showing a bell-shaped dose response function.

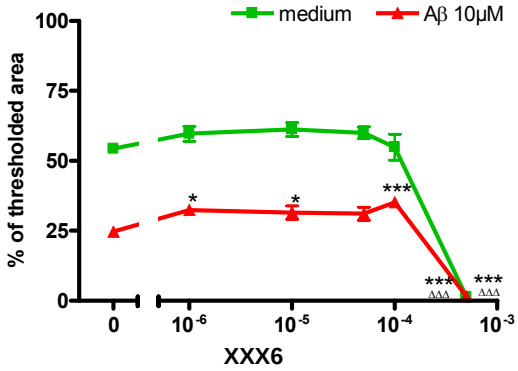
XXX4, XXX3 and XXX6 showed neuroprotective effects that were greater than their neurotrophic ones (Fig.5). Thus, the increase in neuronal survival in the presence of Aβ reached a maximum of 50 % for all the compounds. In contrast, in the absence of Aβ XXX3 induced a moderate increase in neuronal survival that did not exceed 25%, whilst XXX4 and XXX6 had no significant effects. Consequently XXX4 and XXX6 showed selective neuroprotective effects. Again these results suggest that neuroprotective and neurotrophic effects are probably mediated by different mechanisms. In addition, all these compounds were characterized by a bell-shaped dose response function. Indeed, after the maximal effect was reached, the neuroprotective effect progressively

decreased at higher doses with the appearance of neurotoxic effects for XXX4 and XXX6.




milieu
Aβ 10µM
% of thresholded area
100
75
50
25
0
***
**
***
ΔΔΔ
0
10-6
10-5
10-4
10-3
XXX3


% of thresholded area, % of baseline
175
150
125
100
75
50
***
**
0
10-6
10-5
10-4
10-3
XXX3


medium
Aβ 10µM
% of thresholded area
100
75
50
25
0
*
***
ΔΔΔ
0
10-6
10-5
10-4
10-3
XXX6

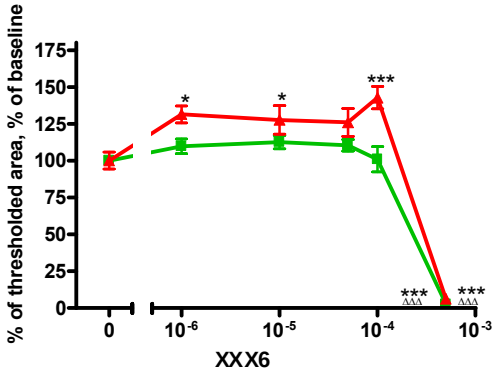

% of thresholded area, % of baseline
175
150
125
100
75
50
25
0
*
***
ΔΔΔ
0
10-6
10-5
10-4
10-3
XXX6

Fig. 5: Effects of XXX4, XXX3 and XXX6. Data are presented as mean +/- SEM: raw data are presented on the left side and percentages of the baseline on the right side. ***= P<0.001, ** = P<0.01, *= P<0.05 in comparison to base line (0M dose); ΔΔΔ= P<0.001 in comparison to 10^{-4}M; oo P<0.01 in comparison to 10^{-6}M.

Comparing the effects of XXX4, XXX3 and XXX6 indicates that for all the compounds except XXX6, the maximal neuroprotective effect was observed at the 10^{-6}M dose. XXX6 was the least potent of the CLIENT compounds tested, with a maximal effect observed at 10^{-4}M. The maximal effects of XXX4 and XXX3 were similar and comparable to NDGA. Again two considerations are worth noting. First, the effects of the highest dose of NDGA are probably due to the EtOH 0.02% solution. Second, the ranges of doses used for XXX4 and XXX3 are exclusively in the descending limb of the dose response function of these compounds. Consequently, their true maximal effect is probably at a dose lower than 10^{-6}M.

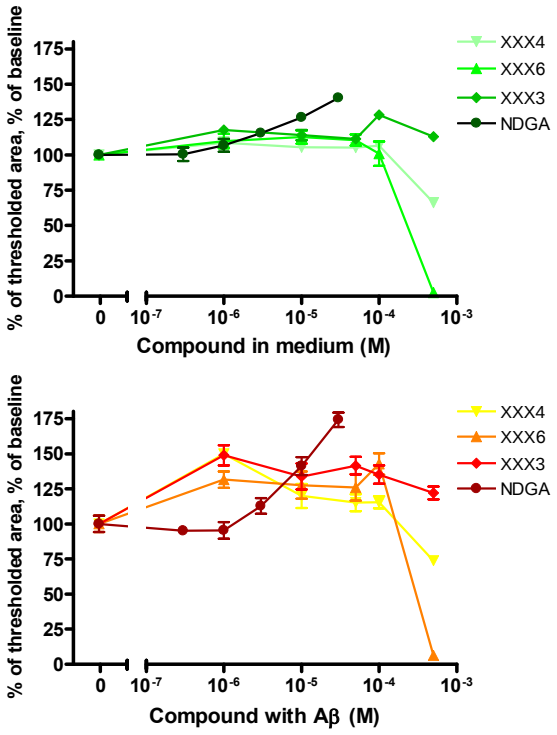
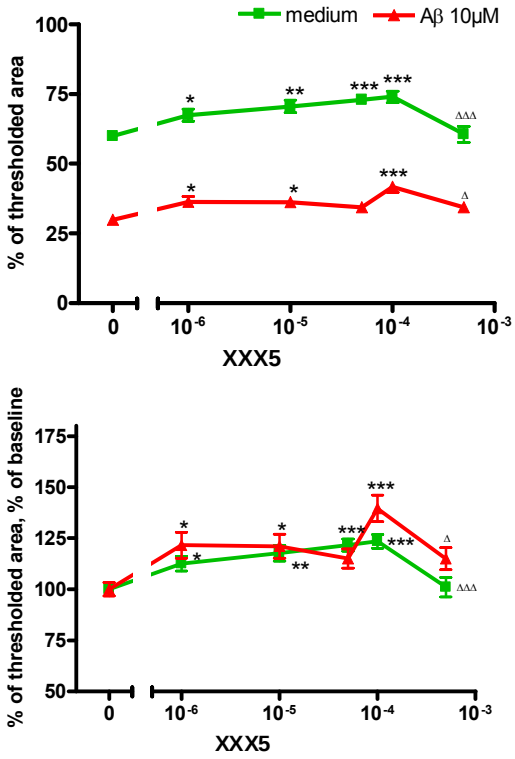


Fig. 6: Comparison of XXX4, XXX6, XXX3 and NDGA. Data are presented as mean +/- SEM of the percentages of the baseline.

2.2.1.3 CLIENT compounds with similar neuroprotective and neurotrophic effects

The compounds in this class, XXX5 and XXX8, both showed a bell-shaped dose response function. In general, the effects of these compounds on neuronal survival were lower (between 25 and 30 % increase) than the ones of the previous class (50 % increase). XXX5 reached a maximal effect at 10^{-4}M and XXX8 at 10^{-5}M. After these doses neuroprotective and neurotrophic effects progressively decreased with a very strong neurotoxic effect being observed for XXX8. Furthermore the effect of XXX8 was not significant in the presence of Aβ.



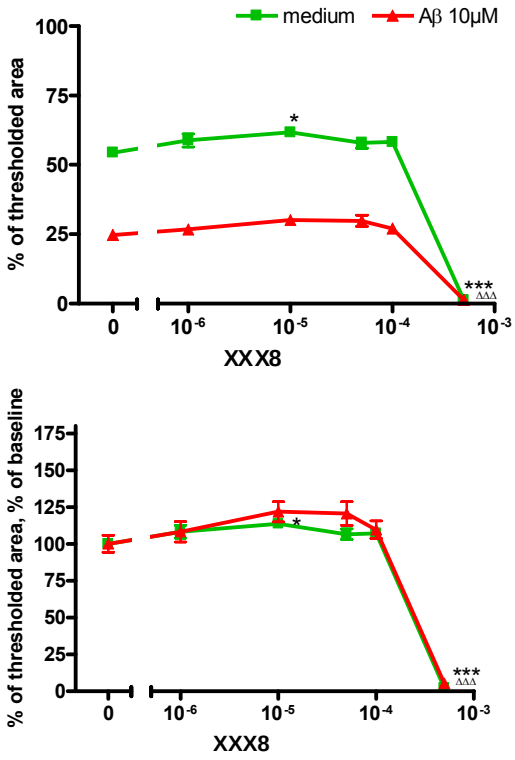


Fig. 7: Effects of XXX5 and XXX8. Data are presented as mean +/- SEM: raw data are presented on the left side and percentages of the baseline on the right side.

***= P<0.001, **= P<0.01,*= P<0.05 in comparison to baseline (0M dose); ΔΔΔ= P<0.001, Δ =P<0.05 in comparison to 10^{-4}M.

Comparison of the effects of these two compounds with NDGA showed a lower maximal effect for both CLIENT compounds. However for XXX5 neuroprotective effects were observed at lower doses than NDGA.

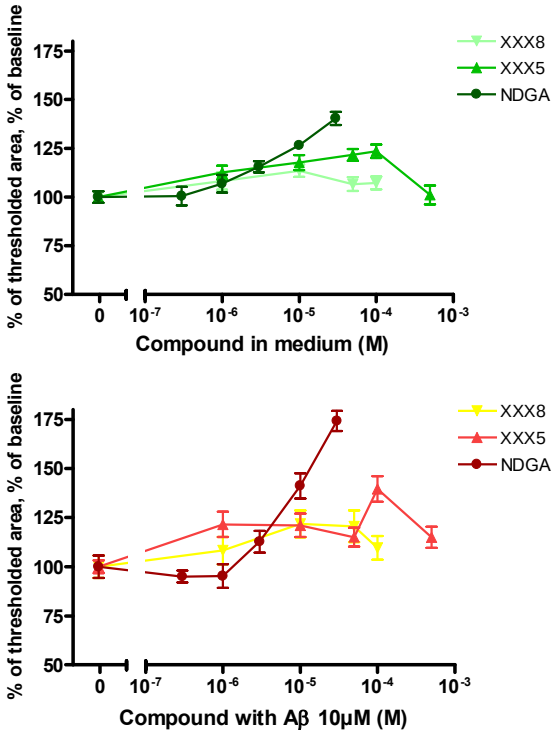
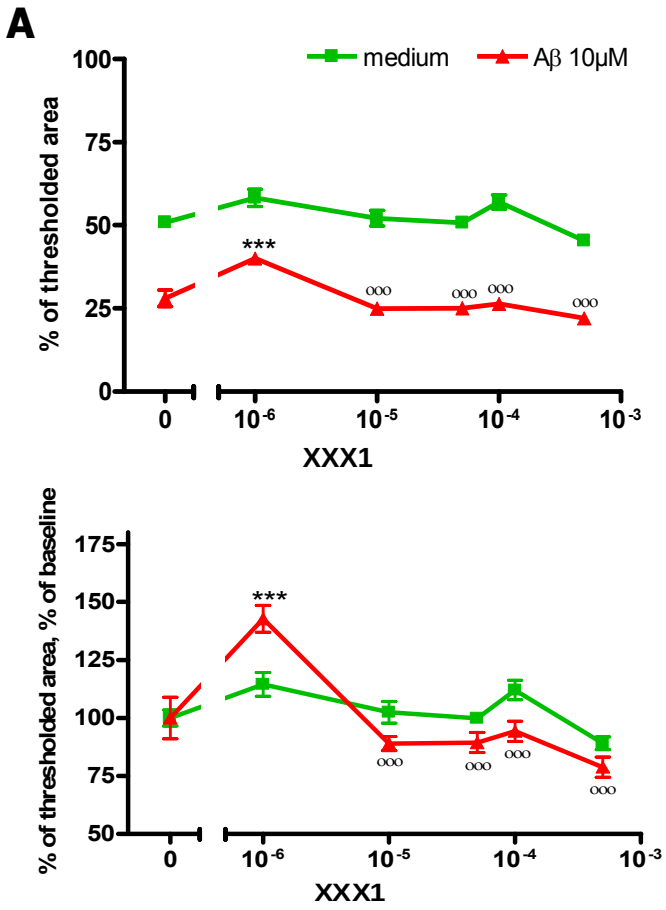


Fig. 8: Comparison of XXX8, XXX5 and NDGA. Data are presented as mean +/- SEM percentages of the baseline.

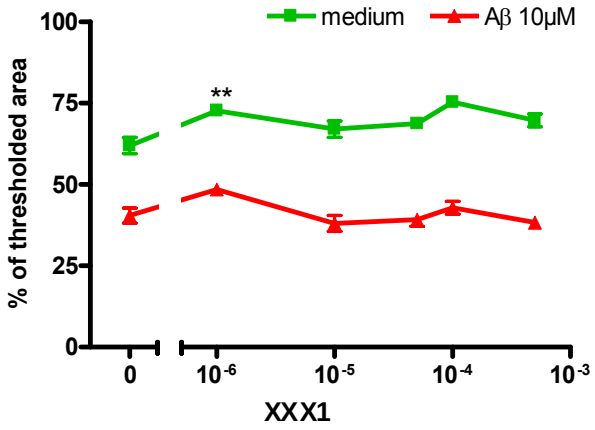
2.2.2 Separate incubation conditions (XXX1)

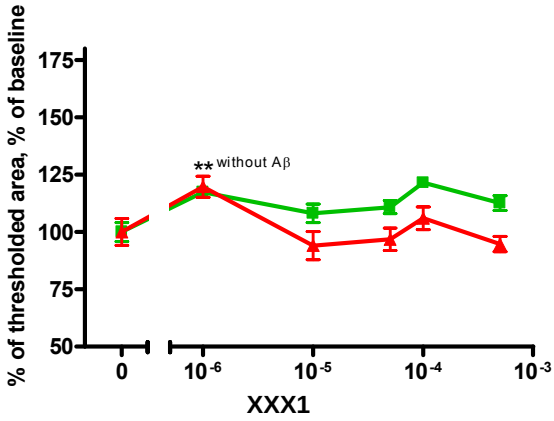
As seen in Fig. 9, the 30 min pre-incubation condition highly modified the effects of XXX1. Thus when the treatment with XXX1 was performed 30min before treatment with Aβ (Fig.9A), this compound showed a bell-shaped dose response function and a profile similar to those of the other CLIENT compound in this class: a maximal effect at 10^{-6}M and a higher neuroprotective than neurotrophic effect. However, when the treatment with XXX1 was performed 24h before the exposure to Aβ (Fig.9B), the neuroprotective effect of this compound decreased. In addition, pre-mixing with Aβ (Fig.9C) completely abolished both the neuroprotective and the neurotrophic effect of XXX1. The NDGA control of this experiment (Fig.9D) confirmed the neuroprotective and neurotrophic effects of this compound. However, in this experiment the neuroprotective and

neurotrophic effects of NDGA become equal, and appeared at lower doses (3.10^{-7}M) than in the previous experiment (3.10^{-6}M). Furthermore the maximal response to NDGA and XXX1 (30 min pre-incubation) was of similar amplitude (Fig.10).

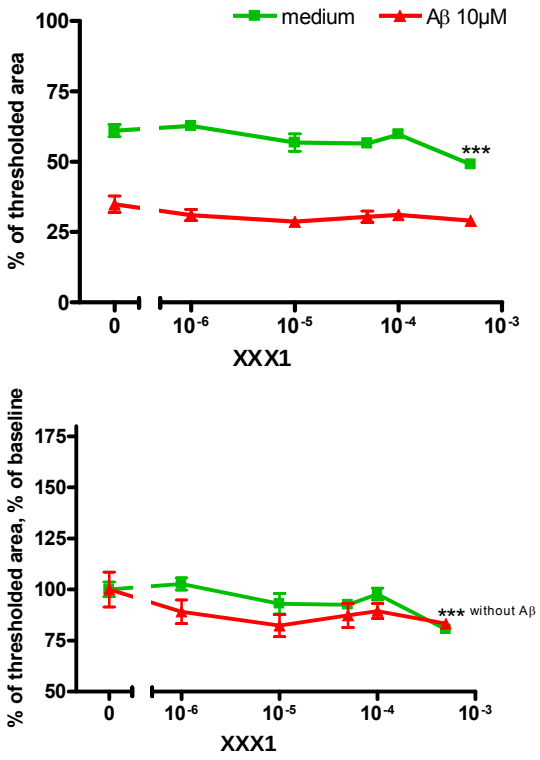


B


medium
Aβ 10μM
% of thresholded area
100
75
50
25
0
**
0
10-6
10-5
10-4
10-3
XXX1


% of thresholded area, % of baseline
175
150
125
100
75
50
** without Aβ
0
10-6
10-5
10-4
10-3
XXX1

C


medium
Aβ 10μM
% of thresholded area
100
75
50
25
0
***
0
10-6
10-5
10-4
10-3
XXX1
% of thresholded area, % of baseline
175
150
125
100
75
50
*** without Aβ
XXX1

D

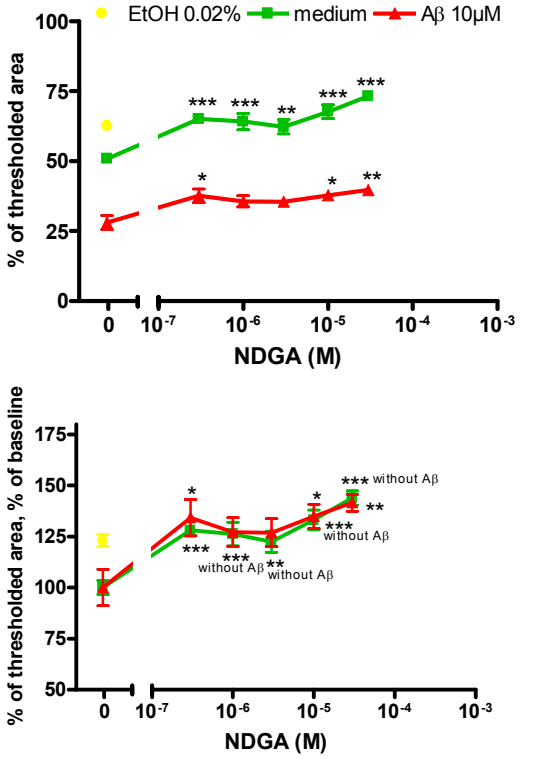

EtOH 0.02%
medium
Aβ 10μM
% of thresholded area
100
75
50
25
0
***
***
**
***
***
*
*
**
10-7
NDGA (M)
% of thresholded area, % of baseline
175
150
125
100
75
50
*** without Aβ
** without Aβ
*** without Aβ
*** without Aβ
** without Aβ
NDGA (M)

Fig. 9: Effects of XXX1 and NDGA in different incubation conditions. A: 30min pre-treatment, B: 24h pre-treatment, C: 24h pre-mixing +/- before Aβ treatment, D: 30min pre-treatment. Data are presented as mean +/- SEM: raw data are presented on the left side and percentages of the baseline on the right side. ***= P<0.001, **= P<0.01,* =P<0.05 compared to base line; ooo= P<0.001 compared to 10^{-6}M.

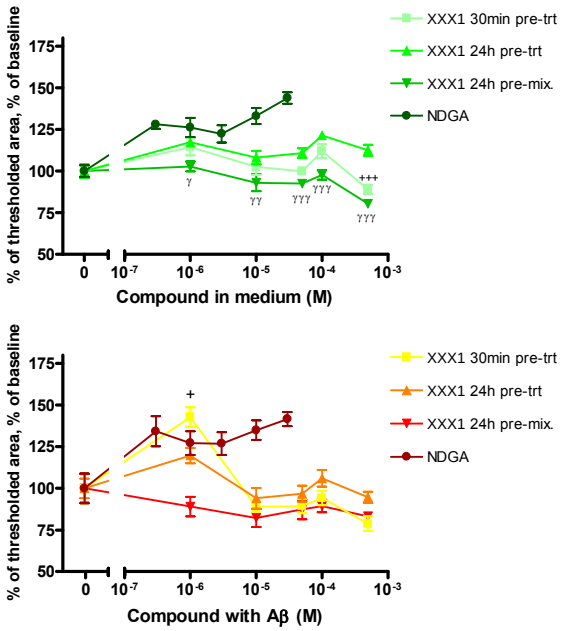
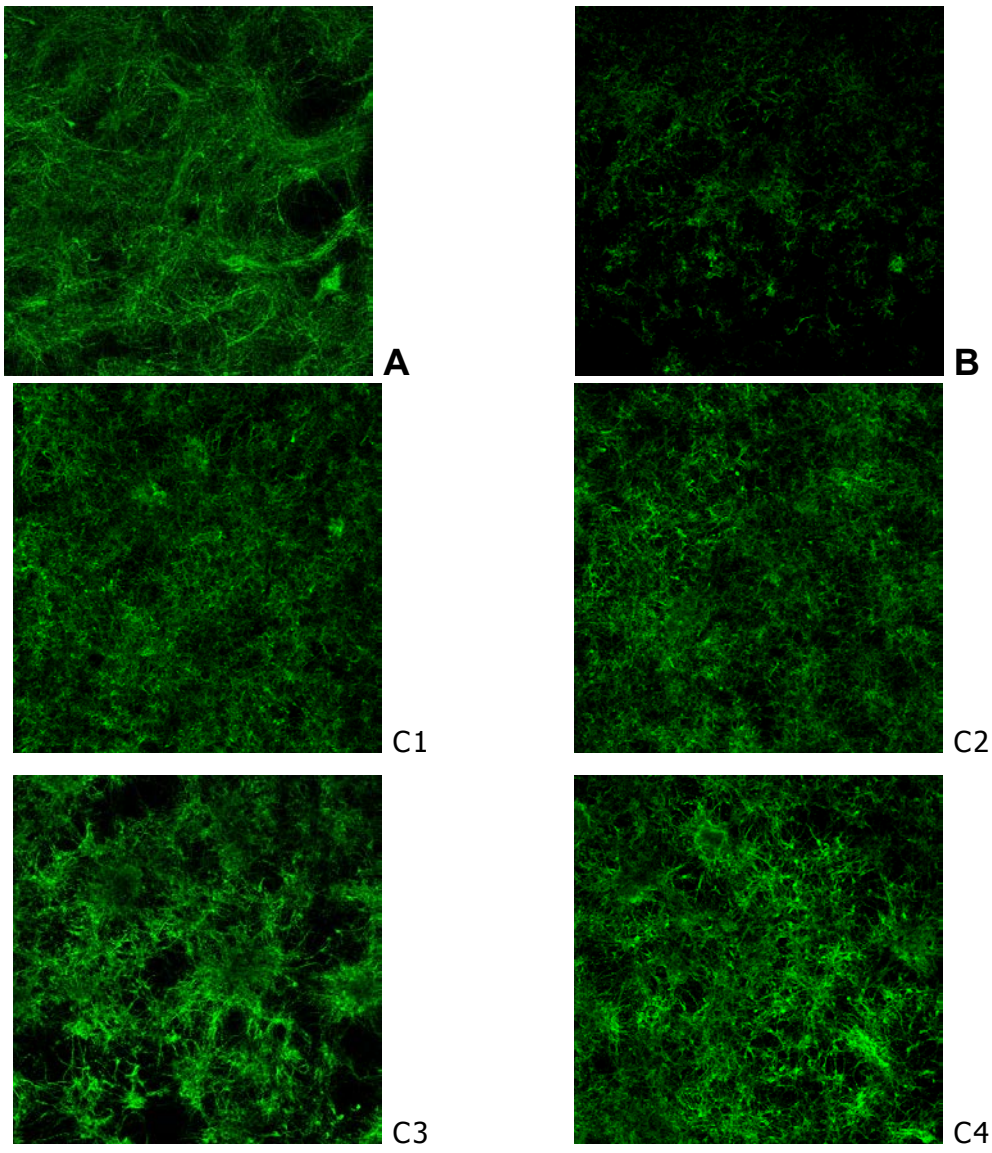
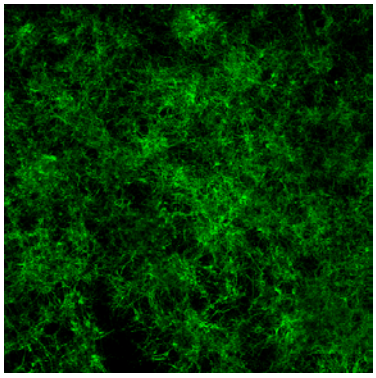


Fig. 10: Comparison of the effects of XXX1 in three incubation conditions. Data are presented as mean +/- SEM of the percentages of the baseline. +++= P<0.001, += P<0.05 compared to 24h pre-treatment; γγγ = P<0.001, γγ = P<0.01, γ= P<0.05 comparing 24h pre-treatment to 24h pre-mixing.

2.3 Image analysis

Untreated, neurons are well differentiated and a dense network of neurites connects different clusters (Fig11A). Following Aβ treatment the neurons have a large, shrunken dendritic network (Fig.11B). After treatment with several CLIENT compounds (Fig.11 C1-6), a partial recovery can be observed as shown by the re-establishment of a significant neuronal network.




C5

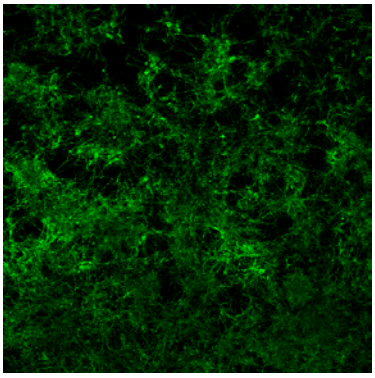

C6

Fig. 11: Examples of MAP2 labelling following several treatments. A: untreated, B: Aβ treated, C: Aβ treated plus: C1 XXX7 10μM, C2 XXX2 1μM, C3 XXX1 (30min pre-treatment) 1μM, C4 XXX4 1μM, C5 XXX3 1μM, C6 NDGA control 2 10μM

3. Conclusion

As in Experiment I of this study, Aβ treatment induced a significant neuronal loss in all the experiments. Most of the CLIENT compounds (the exception being XXX8) showed neuroprotective effects by decreasing Aβ induced toxicity (see table 1). Most of the compounds (with the exceptions of XXX1, XXX4 and XXX6) also had neurotrophic effects. However these were of lower magnitude (25%) than the neuroprotective effects (50%).

The profile of the CLIENT compounds was different from the NDGA positive control. That is, most of the CLIENT -compounds had greater neuroprotective than neurotrophic effects, and some of them had selective neuroprotective effects (XXX1, XXX4, XXX6). On the other hand, NDGA had neurotrophic and neuroprotective effects that were of comparable magnitude. Furthermore, in the conditions in which most compounds were tested (30 min pre-incubation), the potency of most of the CLIENT compounds (XXX1, XXX2, XXX3, XXX4, XXX5; XXX6; XXX8) was higher than that of NDGA. Finally, many of the CLIENT compounds

showed either a biphasic or a bell-shaped dose response function, and some of them also had strong neurotoxic effects at the highest doses (XXX4, XXX6 and XXX8).

Table1

Compound	Neurotrophic	Neuroprotective
XXX1	-	1µM
XXX1 24h pre-treatment	1µM	-
XXX1 pre-incubation	-	-
XXX2	10µM-250µM	1µM, 10µM, 100µM, 250µM
XXX3	1-500µM	1-100µM
XXX4	-	1µM and 10µM
XXX5	1-100µM	1-100µM
XXX6	-	1µM, 10µM and 100µM
XXX7	1-500µM	1µM, 10µM and 500µM
XXX8	10µM	-
NDGA (24 hours incub)	0.3-30µM	0.3µM, 10µM and 30µM
NDGA (30 min incub)	3-30µM	10µM and 30µM

4. Perspectives

Most of the CLIENT compounds tested had either a biphasic or a bell-shaped dose response function, and for many of them (XXX1, 3, 4, 2) the highest effect was observed for the lowest dose tested (10^{-6}M). As a consequence it may be important to test these compounds at lower doses than 10^{-6}M. Indeed, it seems likely that the dose of 10^{-6}M is already in the descending limb of the dose response function and that higher neuroprotective effects could be observed at lower doses.

In addition, it may be revealing to test the compounds having either a neuroprotective or neurotrophic effect in other models of neurodegeneration. For example the effective CLIENT compounds may prevent the selective loss of dopaminergic neurons in mesencephalic cultures such as the ones used here, when these neurons are exposed to other toxins more relevant to Parkinson's disease.