Fragile X mental retardation protein (FMRP) and metabotropic glutamate receptor subtype 5 (mGlu5) control stress granule formation in astrocytes

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Abstract

Fragile X syndrome (FXS) is a common form of intellectual disability and autism caused by the lack of Fragile X Mental Retardation Protein (FMRP), an RNA-binding protein involved in RNA transport and protein synthesis. Upon cellular stress, global protein synthesis is blocked and mRNAs are recruited into stress granules (SGs), together with RNA-binding proteins including FMRP. Activation of group-I metabotropic glutamate (mGlu) receptors stimulates FMRP-mediated mRNA transport and protein synthesis, but their role in SGs formation is unexplored. To this aim, we pre-treated wild type (WT) and Fmr1 knockout (KO) cultured astrocytes with the group-I-mGlu receptor agonist (S)-3,5-Dihydroxyphenylglycine (DHPG) and exposed them to sodium arsenite (NaAsO2), a widely used inducer of SGs formation. In WT cultures the activation of group-I mGlu receptors reduced SGs formation and recruitment of FMRP into SGs, and also attenuated phosphorylation of eIF2α, a key event crucially involved in SGs formation and inhibition of protein synthesis. In contrast, Fmr1 KO astrocytes, which exhibited a lower number of SGs than WT astrocytes, did not respond to agonist stimulation. Interestingly, the mGlu5 receptor negative allosteric modulator (NAM) 2-methyl-6-(phenylethynyl)pyridine (MPEP) antagonized DHPG-mediated SGs reduction in WT and reversed SGs formation in Fmr1 KO cultures. Our findings reveal a novel function of mGlu5 receptor as modulator of SGs formation and open new perspectives for understanding cellular response to stress in FXS pathophysiology.

Keywords:
Fragile X syndrome
Astrocytes
Stress granules
FMRP
mGlu5 receptor

1. Introduction

Fragile X syndrome (FXS) is the most common form of inherited intellectual disability (ID) and a leading genetic cause of autism. FXS patients suffer from moderate to severe cognitive impairment, and can also exhibit autistic behavior, increased susceptibility to seizures, hyperactivity, anxiety, and hypersensitivity to sensory stimulation (Hagerman and Hagerman, 2002; Hagerman et al., 2017). FXS is caused by the amplification of CGG trinucleotide repeat in the 5’UTR of the Fragile X Mental Retardation gene 1 (FMR1). In patients this mutation is associated with the methylation of the FMR1 gene resulting into the transcriptional silencing of this gene (Verkerk et al., 1991; Pieretti et al., 1991; Devys et al., 1993) and the lack of the Fragile X Mental Retardation Protein (FMRP), an RNA binding protein involved in the regulation of translation and transport of its target mRNAs (Maurin et al., 2014; Maurin et al., 2018). FMRP acts mainly as a negative regulator of translation, although recent evidence indicates that it can also function as enhancer of translation (Bechara et al., 2009; Darnell et al., 2011;
A new aspect of FMRP function in the cytoplasm is related to its presence in peculiar structures called stress granules (SGs), cytoplasmic aggregates that are formed only under stress conditions, such as exposure to heat, oxidative agents, UV irradiation (Anderson and Kedersha, 2002). SGs are dynamic membrane-less structures composed of stalled preinitiation complexes, mRNAs and proteins, including initiation factors and RNA-binding proteins that scaffold untranslated mRNAs and interact with each other (Anderson and Kedersha, 2002; Buchan and Parker, 2009; Propper and Parker, 2016). SGs are reversible aggregates where mRNAs are recruited and temporarily stored during stress, and are dispersed upon stress resolution (Anderson and Kedersha, 2002).

They are thought to redirect protein translation during stress by limiting global protein synthesis while allowing the translation of stress-induced mRNAs. FMRP has been found to be associated with the pool of mRNAs that go into SGs upon cellular stress and can be involved in the inhibition of protein synthesis occurring during stress (Kim et al., 2006). Lack of FMRP in mouse fibroblasts has been reported to impair SGs formation (Didiot et al., 2009), although FMRP appears to be dispensable in Drosophila (Garaeau et al., 2013).

Several FMRP-mediated functions, such as mRNPs transport and protein synthesis, are crucially regulated by activation of group-I metabotropic glutamate (mGlu) receptors (mGlu1 and mGlu5 receptor subtypes) (Nicoletti et al., 2011; D’Antoni et al., 2014). Activation of group-I mGlu receptors increases the rapid translation of pre-existing mRNAs, including the Fmr1 mRNA (Weiler et al., 1997; Weiler et al., 2004). This mechanism underlies mGlu-mediated Long-Term Depression, a form of protein synthesis-dependent synaptic plasticity, which is abnormally exaggerated in the hippocampus and cerebellum of the Fmr1 knock out (KO) brain (Huber et al., 2000; Huber et al., 2002; Koekkoek et al., 2005). Furthermore, the activation of mGlu5 receptors is necessary for FMRP-containing mRNPs trafficking from the cell body into dendrites (Antar et al., 2004; Dichtenberg et al., 2008). However, the involvement of mGlu5 receptors in SGs formation has never been investigated.

FMRP is highly expressed in neurons, but is also expressed in glial cells although at lower extent (Bonaccorso et al., 2015; Gholidazeh et al., 2015). Accordingly, a growing number of recent studies highlights the contribution of astrocytes to synaptic defects in FXS and subsequently to the pathophysiology of this disorder (Pacey and Doering, 2007; Cheng et al., 2012; Cheng et al., 2016; Higashimori et al., 2016; Wallingford et al., 2017; Hodges et al., 2017). Importantly, mGlu5 receptor-mediated signaling in astrocytes modulates specific functions involved in synaptic transmission and may also directly participate to pathological events in different neurological disorders, including neurodevelopmental disorders (D’Antoni et al., 2008; Petrelli and Bezzi, 2018). Based on the premise that regulation of mRNA metabolism via mGlu5 receptors in astrocytes may give an insight into the mechanisms of contribution of this cell type to FXS pathophysiology, we report that upon stress primary cultured astrocytes from Fmr1 KO mice exhibit less SGs than wild type (WT) astrocytes. More importantly, the activation of mGlu5 receptors reduces the formation of SGs in WT, but has no effect in Fmr1 KO astrocytes, highlighting a link between mGlu5 receptor and translational regulation during stress in the presence and in the absence of FMRP.

2. Materials and methods

2.1. Astroglial cell cultures

Primary astroglial cultures were prepared from cortex of P0-P1 newborn pups of litters obtained from WT or Fmr1 KO mice (Dutch-Belgian Fragile X Consortium, 1994). All experiments were performed without previous knowledge of the genotype of the culture being tested. The mice genotype was defined by PCR (Musumeci et al., 2007). Cortices were dissected at stereomicroscope (STEMI DV4 ZEISS) and tissues were enzymatically dissociated into nutrient medium. The basal nutrient medium consisted of Dulbecco modified Eagle’s medium (DMEM, Sigma), containing 10% heat inactivated fetal bovine serum (FBS, Sigma), 1% penicillin and streptomycin. Cells were seeded into plastic flasks of 25 cm² at a plating density of 0.5 × 10⁵ cells/ cm² (one hemisphere/flask). Cultures were incubated at 37 °C in a humidified 5% CO₂ / 95% air atmosphere. After 10–12 days, cultures were treated with 5 mM leucin methyl ester (Sigma) and shaken (for 6–8 h, 180 rpm) to purify cell cultures from oligodendrocytes and microglia. Subsequently, 35 mm Ø dishes were prepared with 2000 cells/dishes to perform immunocytochemistry. To evaluate specific proteins by Western Blot analysis, cultures were seeded onto 100 mm Ø dish at density of 8 × 10⁵ cells/dish.

2.2. Treatments

To induce oxidative stress, astrocytes were first shifted in serum-free media for 16 h, then treated with sodium arsenite (500 µM NaAsO₂, Carlo Erba) or with hydrogen peroxide (500 µM H₂O₂, Fluka) for 60 or 90 min, and kept at 37 °C in a humidified 5% CO₂ / 95% air atmosphere. To induce heat shock, cells were maintained at 43 °C for 1 h in a humidified mix 5% CO₂ / 95% air atmosphere. A 5 min pre-treatment with the orthosteric group-I mGlu receptor agonist (S)-3,5-Dihydroxyphenylglycine (100 µM DHPG, Tocris) was carried out before the exposure to stress-inducing agents. The negative allosteric modulator (NAM) of mGlu5 receptor 2-methyl-6-(phenylethynyl)pyridine (3 µM MPEP, Tocris) was applied 10 min before DHPG and during DHPG treatment. In the absence of DHPG, MPEP treatment was carried out 10 min before and during NaAsO₂ exposure. Cycloheximide (30 µg/ml, Tocris) or puromycin (20 µg/ml, Tocris) were added 30 min after NaAsO₂ and maintained for the whole duration of stress exposure.

2.3. Immunocytochemistry and Image analysis

After treatments, cultures were fixed with 4% paraformaldehyde for 15 min at R.T. followed by additional 10 min with cold methanol. Then, cultures were permeabilized in PBS containing 0.2% Triton for 10 min, incubated for 20 min at R.T. with blocking solution containing 4% donkey or goat serum and subsequently with anti-TIA-1 antibody (goat, 1:250, Santa Cruz Biotechnology Cat#sc-1751) for 2 h at R.T. The expression of FMRP in astrocytes was studied by double-labelling immunocytochemistry using the anti-FMRP antibody (rabbit 1:50, Cell Signaling Cat#4317). After washing, cultures were incubated with donkey anti-goat Cy3-conjugated and goat anti-rabbit DyLight488 fluorescent secondary antibodies (Jackson Immunoresearch). Images were obtained with the Axio Imager. D2 (Zeiss) or LSM-510 Meta Confocal microscopes (Zeiss), and analyzed using the AxioVision Imaging System and the ImageJ softwares. A negative control (no primary antibodies) was used to properly set acquisition parameters.

We identified cells displaying SGs by observing cytoplasmic TIA-1 positive granular spots, which were clearly detectable above a diffuse background (See Fig. 1). We considered SG positive a cell displaying at least 2 TIA-1 positive cytoplasmic spots.

We used the ImageJ software for quantitative analysis of images. To quantify SG numbers and size, images were converted in binary format and processed by the Analyze Particles function of Image J. In order to
exclude nuclei, the size parameters of Analyze Particles were set on 0–1000 pixels. Then, we obtained the masks showing SGs that were used for SG quantification and figure preparation. For TIA-1 and FMRP SG colocalization, images were split in order to obtain an image per antibody. Subsequently, images were processed with JACoP colocalization tool using Objects Based Methods set on Centre of Mass with a range of 0–1000 pixel. SGs numbers resulting from the TIA-1/FMRP colocalization mask were used for quantification.

2.4. Western Blotting

Astrogial cell cultures (80% confluence) were harvested by scraping them on ice. Cells were homogenized in lysis buffer (Tris-HCl 40 mM pH 6.8, 1 × Protease Inhibitor Cocktail-Roche, 1 × Phosphatase Inhibitor Cocktail-Roche, 100 μM phenylmethanesulfonyl fluoride (PMSF), 1 mM ethylenediaminetetraacetic acid (EDTA), 5 mM EGTA and 2% SDS), centrifuged for 10 min at 15000 g at 4 °C. Cortices obtained from WT, Fmr1 KO and mGlu5 KO mice were allowed to thaw on ice, weighed, and then treated with the bicinchoninic acid method (BCA kit; Pierce Rockford, IL). Supernatant were collected and protein concentration was determined as compared with those found in the cortex of mice at post-natal day 7, when the expression of mGlu5 receptors in the brain is maximal (Catania et al., 1994; Catania et al., 2007).

Activation of mGlu5 receptors with the group-I mGlu receptor agonist (S)-3,5-Dihydroxyphenylglycine (DHPG, 100 μM for 5 min) before exposure of astrocytes to NaAsO₂ induced a significant reduction in the number of SGs per cell in WT cultures (Fig. 2A, B), but had no effect in Fmr1 KO astrocytes (Fig. 2C, D). Quantification of the size of SGs also revealed that SGs were smaller in Fmr1 KO than in WT cultures and that DHPG treatment before exposure to NaAsO₂ reduced SGs size in WT astrocytes, at similar levels as SGs size observed in Fmr1 KO astrocytes; however, DHPG treatment before exposure to NaAsO₂ did not modify the size of SGs in Fmr1 KO astrocytes (Fig. S2).

We also quantified the percentage of cells bearing SGs in both WT and Fmr1 KO stressed cultures and found that the pre-treatment with DHPG induced a significant reduction of SGs positive cells in stressed WT cultures only, with no effect in Fmr1 KO cultures (Fig. 3A, B). The DHPG-induced effect in WT cultures was antagonized by the highly selective mGlu5 receptor NAM 2-methyl-6-(phenylethynyl)pyridine (MPEP, 3 μM), clearly indicating an mGlu5 receptor – mediated effect (Fig. 3A). Interestingly, the stress-induced increase in the percentage of SG positive cells was not affected by application of MPEP alone before and during stress in WT astrocytic cultures, whereas it was further increased in Fmr1 KO cultures (Fig. S3).

In order to get an insight into the mechanisms underlying the effects of mGlu5 receptor activation on the modulation of SGs formation, we exposed both WT and Fmr1 KO cell cultures to NaAsO₂ and then treated them with the protein synthesis inhibitors puromycin and cycloheximide, which have different mechanisms of action. Puromycin destabilizes polyribosomes and facilitates SGs formation by making mRNAs available, while cycloheximide freezes ribosomes on translating mRNAs and therefore inhibits SGs formation (Kedersha et al., 2000). Indeed, puromycin induced a significant increase of cells bearing SGs, whereas cycloheximide induced a drastic reduction of SGs formation in both WT and Fmr1 KO astrocytes (Fig. 3). This result suggests that the basic mechanisms underlying SGs formation are not disrupted in the absence of FMRP. Interestingly, the exposure to DHPG for 5 min before stress
induction reduced the number of cells with SGs also in puromycin-treated WT cells (Fig. 3A), but had no effect in Fmr1 KO astrocytes (Fig. 3B). This would indicate that activation of mGlu5 receptors before stress induction counteracts the formation of SGs despite the availability of mRNAs in WT cultures, whereas does not trigger a similar mechanism in Fmr1 KO astrocytes (Fig. 3B).

3.3. Activation of mGlu5 receptors before stress induction reduces phosphorylation of translation initiation factor eIF2α in WT but not in Fmr1 KO astrocytes

Since the stress-induced phosphorylation of eIF2α factor is a major trigger of SGs formation, (Redersha et al., 1999, see discussion), we tested if mGlu5 receptor activation affects NaAsO₂-induced eIF2α
phosphorylation. Western Blots analyses showed that eIF2α was highly phosphorylated under stress condition, as expected, in both WT and Fmr1 KO astrocytes (Fig. 4A, B). Interestingly, while exposure to NaAsO2 induced a robust phosphorylation of eIF2α in both WT and Fmr1 KO (Fig. 4A, D), a 5 min pre-treatment with DHPG before stress induction differently affected eIF2α phosphorylation in the two genotypes. Semiquantitative analysis of phosphorylated-eIF2α revealed lower levels of eIF2α phosphorylation upon stress in WT than in Fmr1 KO cultures after activation of mGlu5 receptors (Fig. 4C, D, E).

3.4. Activation of mGlu5 receptors reduces recruitment of FMRP in SGs

Double-labelling immunocytochemistry and confocal microscopy revealed a remarkable co-localization of FMRP and TIA-1 protein in WT (reviewed in Bassell and Warren, 2008). Indeed, activation of mGlu5 receptors triggers protein translation in hippocampal slices of WT mice, but does not further increase the constitutively elevated protein synthesis in Fmr1 KO cells (reviewed in Bassell and Warren, 2008). Indeed, activation of mGlu5 receptors triggers protein translation in hippocampal slices of WT mice, but does not further increase the constitutively elevated protein synthesis in Fmr1 KO cells (reviewed in Bassell and Warren, 2008). Indeed, activation of mGlu5 receptors triggers protein translation in hippocampal slices of WT mice, but does not further increase the constitutively elevated protein synthesis in Fmr1 KO cells (reviewed in Bassell and Warren, 2008).

4. Discussion

Within the Central Nervous System, the function of FMRP has been principally investigated in neurons, whereas the biological significance of FMRP in other cell types has received scant attention until recently. Emerging evidence suggests that lack of FMRP in astrocytes contributes to FXS phenotype, i.e. abnormal dendritic spine morphology/dynamics and synapse development, through mechanisms that involve neuron-glia interaction (Cheng et al., 2016; Higashimori et al., 2016; Hodges et al., 2017; Wallingford et al., 2017). This can occur because the FMRP-regulated synthesis of both resident and secretory astrocytic proteins is disrupted in FXS. Therefore, SGs formation and its modulation in astrocytes is an important yet unexplored aspect of mRNA metabolism in FXS. Here we report that the activity of mGlu5 receptors, which regulate FMRP-dependent mRNA transport and translation in neurons, can also modulate SGs formation in astrocytes.

The activation of mGlu5 receptors reduced SGs formation in WT to a similar extent as in Fmr1 KO astrocytes. However, in the absence of FMRP, mGlu5 receptor activation did not further reduce SGs formation. In contrast, the NAM MPEP, which is known to inhibit the constitutive activity of mGlu5 receptors (Pagano et al., 2000), did not have any effect in WT cultures, but reversed the reduced SGs formation in Fmr1 KO astrocytes. These results resemble several observations reporting that activation of mGlu5 receptors mediates effects in WT, i.e. mRNA transport and translation, whereas it has no effect in Fmr1 KO cells (reviewed in Bassell and Warren, 2008). Indeed, activation of mGlu5 receptors triggers protein translation in hippocampal slices of WT mice, but does not further increase the constitutively elevated protein synthesis in Fmr1 KO mice, which, in contrast, is strikingly reversed by the pharmacological blockade of mGlu5 receptors or its genetic down-regulation (Dolen et al., 2007; Michalon et al., 2012).

To deepen the relationship between activation of mGlu5 receptors, SGs formation and mRNA translation, we carried out stress inducing experiments with/without DHPG in the presence of cycloheximide or puromycin. Using these drugs, it was established that SGs-associated mRNAs are in a dynamic equilibrium with polyribosomes (Kedersha et al., 2000). In line to what previously reported in other cell types, we observed that in both WT and Fmr1 KO astrocytes puromycin increased SGs formation upon stress, while cycloheximide completely reversed SGs formation. Interestingly, we observed that in the presence of puromycin SGs formation occurred in Fmr1 KO astrocytes to a similar extent as in WT, indicating that destabilization of polyribosomes makes available the initiation complex and mRNAs for SGs formation both in the presence and in the absence of FMRP. The reduced SGs formation in Fmr1 KO astrocytes is also restored by MPEP suggesting that the molecular phenotype could be due to an increased rate of mRNA recruitment in polyribosomes in the absence of FMRP. This is in agreement...
with the notion that the absence of FMRP leads to a constitutive mGlu5-dependent increased rate of protein synthesis (Dolen et al., 2007; Michalon et al., 2012). In other words, an altered balance between polyribosomes and SGs is possibly responsible for the reduced SGs formation in Fmr1 KO cells rather than the absence of the shuttling action of FMRP between the two ribonucleoproteic structures.

We also found that mGlu5 receptor activation differently affected eIF2α phosphorylation in stressed WT astrocytes and Fmr1 KO cultures, with lower levels in WT astrocytes. In stressed cells, activation of one or more eIF2α kinases (e.g. PKR, PERK/PEK, GCN2, HRI) results in the phosphorylation of eIF2α, an essential subunit of the eIF2-GTP-tRNAMet ternary complex required to initiate protein synthesis. Once phosphorylated eIF2α is no longer available to the canonical assembly of the translation initiation complex, and favours the formation of an abnormal 48S complex carrying mRNAs that were destined for translation and that take part in SGs (Anderson and Kedersha, 2002; Kedersha and Anderson, 2002).
Fig. 5. Activation of mGlu5 receptors reduces FMRP recruitment into SGs.
(A) Images show WT astrocytes stained with anti-TIA-1 and anti-FMRP primary antibodies. Drawings show TIA-1 positive SGs, FMRP positive SGs and double TIA/FMRP SGs as revealed by masks generated by the Analyze Particles module of Image J. Astrocytes were untreated, exposed to DHPG (100 μM for 5 min), treated with NaAsO₂ (500 μM for 30 min) or exposed to DHPG for 5 min and then to NaAsO₂ for thirty minutes. TIA-1 staining is shown in red and FMRP in green. Scale bar = 20 μm. Small panels show magnifications of the dashed-line boxed areas, scale bar 20 μm. (B) Fmr1 KO astrocytes stained with anti-TIA-1 and anti-FMRP primary antibodies as a negative control. (C) The graph represents the percentage of FMRP co-localization in TIA positive SGs calculated by JACoP colocalization plugin of Image J software. ***p < 0.0003 unpaired t-test. n = 27 cells (NaAsO₂) and 16 cells (DHPG NaAsO₂) from 1 to 2 cultures.
2009). After this crucial initial event, TIA-1 and then other RNA binding proteins including FMRP are recruited to SGs. Thus, mGlu5 receptor activation in WT may impair SG formation by reducing the number of abnormal pre-initiation complexes which represent the core of SGs essential for the subsequent recruitment of TIA-1 and FMRP. Accordingly, in WT astrocytes the activation of mGlu5 receptors before stress counteracts SGs formation even in the presence of puromycin, whereas this did not occur in Fmr1 KO cultures. On the other hand, it is also possible that mGlu5 activation may favour the rapid synthesis or post-translational modification of other proteins interfering with the subsequent aggregation of interacting RNA-binding proteins in SGs.

Intriguingly, elf2α phosphorylation was also increased upon stress in Fmr1 KO astrocytes, although it was not reduced by activation of mGlu5 receptors. This is not in contrast with our observation that Fmr1 KO astrocytes exhibit an impaired SGs formation. In fact, SGs formation is abolished even in the presence of continued phosphorylation of elf2α when the availability of free mRNAs is reduced by drugs such as cycloheximide or emetine (Kedersha et al., 2000, Fig. 4). The lack of a DHPG-induced effects on elf2α dephosphorylation and SGs formation might rather indicate that in the absence of FMRP mGlu5 receptors are insensitive to the orthosteric agonists and/or uncoupled from downstream signaling, as shown for DHPG stimulated mRNA translation of FMRP targets (Dolen et al., 2007; Bassell and Warren, 2008; Michalon et al., 2012).

Despite recent advancements in elucidating the SGs composition and mechanisms underlying their formation, the biological significance of SGs remains undefined. By providing a sink for pro-apoptotic signaling molecules SGs may play a role in promoting cell survival upon stress (Arimoto et al., 2008; Eisinger-Mathason et al., 2008). Therefore, the reduced SGs formation in the absence of FMRP argues for a further vulnerability of FXS phenotype in coping with different stressors, including oxidative stress. Several pieces of evidence indicate that oxidative stress is indeed increased in the Fmr1 KO mouse model and may play a role in FXS pathophysiology (El Bekay et al., 2007; Bechera et al., 2009; Davidovic et al., 2011; D’Antoni et al., 2020). The restored formation of SGs by MPEP suggests that antagonism of mGlu5 receptors could be a protective therapeutic strategy against the deleterious consequences of stress in FXS. Besides the pathophysiological relevance of our data, we believe that, highlighting the role of FMRP in SG formation and its modulation by mGlu5 receptors, our study contributes to a further understanding of the function of FMRP in the control of RNA metabolism.

To our knowledge, this is the first report that the activation of a neurotransmitter receptor has an impact on SGs formation, revealing a novel function of mGlu5 receptors in astrocytes. Our study adds relevant information to a complex biological problem involved in the mechanisms of cellular response to stress and may have critical implication for FXS pathophysiology. Furthermore, considering a possible link between SGs formation and cell survival (Arimoto et al., 2008; Eisinger-Mathason et al., 2008), our study may open new perspectives for pharmacological modulation of SGs in neurological disorders in which oxidative stress and endoplasmic reticulum stress contribute to cell death.

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Author contribution

Barbara Di Marco: Conceptualization, Validation, Formal Analysis, Investigation, Writing – Original Draft, Writing – Review & Editing, Funding Acquisition. Paola Dell’Albani: Formal Analysis, Investigation, Writing – Review & Editing, Simona D’Antoni: Formal Analysis, Investigation, Writing – Review & Editing, Visualization. Michela Spatuzza: Formal Analysis, Investigation. Carmela M. Bonaccorso: Investigation. Salvatore A. Musumeci: Funding Acquisition. Filippo Drago: Funding Acquisition. Barbara Bardoni: Conceptualization, Resources, Writing – Review & Editing. Maria Vincenza Catania: Conceptualization, Formal Analysis, Resources, Supervision, Writing – Original Draft, Writing – Review & Editing, Funding Acquisition.

Ethical approval

Animal care and handling were carried out in compliance with the European Council Directive (86/609/EEC) and the Italian Animal Welfare Act for the use and care of laboratory animals. All efforts were made to minimize animal suffering and to reduce the number of animals used.

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Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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