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RESEARCH ARTICLE

Pharmacological characterization of NMDA-like receptors in the single-celled organism *Paramecium primaurelia*

Paola Ramoino[1,*], Simona Candiani[1], Anna Maria Pittaluga[2], Cesare Usai[3], Lorenzo Gallus[1], Sara Ferrando[1], Marco Milanese[2], Marco Faimali[4] and Giambattista Bonanno[2,5,6]

ABSTRACT

Paramecium primaurelia is a unicellular eukaryote that moves in freshwater by ciliary beating and responds to environmental stimuli by altering motile behaviour. The movements of the cilia are controlled by the electrical changes of the cell membrane: when the intraciliary Ca^{2+} concentration associated with plasma membrane depolarization increases, the ciliary beating reverses its direction, and consequently the swimming direction changes. The ciliary reversal duration is correlated with the amount of Ca^{2+} influx. Here, we evaluated the effects due to the activation or blockade of *N*-methyl-D-aspartic acid (NMDA) receptors on swimming behaviour in *Paramecium*. Paramecia normally swim forward, drawing almost linear tracks. We observed that the simultaneous administration of NMDA and glycine induced a partial ciliary reversal (PaCR) leading to a continuous spiral-like swim. Furthermore, the duration of continuous ciliary reversal (CCR), triggered by high external KCl concentrations, was longer in NMDA+glycine-treated cells. NMDA action required the presence of Ca^{2+}, as the normal forward swimming was restored when the ion was omitted from the extracellular milieu. The PaCR and the enhancement of CCR duration significantly decreased when the antagonists of the glutamate site D-AP5 or CGS19755, the NMDA channel blocker MK-801 or the glycine site antagonist DCKA was added. The action of NMDA+glycine was also abolished by Zn^{2+} or ifenprodil, the GluN2A and the GluN2B NMDA-containing subunit blockers, respectively. Searches of the *Paramecium* genome database currently available indicate that the NMDA-like receptor with ligand-binding characteristics of an NMDA receptor-like complex, purified from rat brain synaptic membranes and found in some metazoan genomes, is also present in *Paramecium*. These results provide evidence that functional NMDA receptors similar to those typical of mammalian neuronal cells are present in the single-celled organism *Paramecium* and thus suggest that the glutamatergic NMDA system is a phylogenetically old behaviour-controlling mechanism.

KEY WORDS: Glutamatergic NMDA system, NMDA receptor pharmacology, Swimming behaviour, Ciliated protozoa

INTRODUCTION

Glutamate is the most widespread excitatory neurotransmitter in vertebrates, controlling both peripheral and central neurotransmission. Glutamatergic inputs are mediated by receptors subdivided into ionotropic and metabotropic receptor families. On the basis of their pharmacological profile, the ionotropic glutamate receptors are further subdivided into three subtypes: the α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA)-preferring receptor, the kainate-preferring receptor and the *N*-methyl-D-aspartate (NMDA) receptor (Watkins and Collingridge, 1994; Dingledine et al., 1999). Glutamate and ionotropic glutamatergic receptors are present in vertebrates as well as in prokaryotes and plants, suggesting that the glutamatergic system is archaic and phylogenetically conserved (Tikhonov and Magazanik, 2009).

The NMDA receptor mediates the vast majority of excitatory neurotransmission. In vertebrates, most of the NMDA receptors are heteromeric, composed of GluN1 subunits (which bind glycine) in combination with GluN2 subunits (which bind glutamate). As a consequence, the activation of these receptors requires the presence of both glutamate and glycine. The GluN1 subunit arises from one single gene with eight functional splice variants, whereas GluN2 is encoded by four different genes (Mori and Mishina, 1995; Zukin and Bennett, 1995). Recently, a glycine-binding GluN3 subunit, encoded by two different genes, has been identified. The GluN3 subunit can assemble with GluN1 to express a functional glycine receptor that is insensitive to glutamate (Chatterton et al., 2002), but it can also contribute to the expression of native GluN1/GluN2/GluN3 receptors, the activation of which results in an NMDA+glycine-evoked current, which is less sensitive to Mg^{2+} (Chatterton et al., 2002).

An atypical NMDA receptor-like complex composed of four proteins – the glutamate-binding protein (GBP), the glycine/*N*-[1-(2-thienyl)cyclohexyl]piperidine(TCP)-binding protein (Gly/TCP-BP), the (+)-3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid-binding protein (CPP-BP) and the phencycline-binding protein – has also been described (Ly and Michaelis, 1991; Kumar et al., 1994). This complex is able to induce the expression of glutamate-activated ion channels; its activity depends on the presence of glycine and permits the influx of Ca^{2+} ions (Aistrup et al., 1996). However, the exact stoichiometry of subunit assembly is still unknown and it has not yet been demonstrated whether these subunits can assemble with GluN subunits to express functional receptors.

The purpose of this study was to verify whether NMDA receptors are present in the ciliated protozoan *Paramecium primaurelia* (Sonneborn, 1975) by evaluating the effects of NMDA receptor activation on swimming behaviour. The locomotion of paramecia depends on ciliary movements, which are controlled by the electrical changes of the cell membrane (Eckert, 1972; Naitoh, 1974). It has been reported that membrane hyperpolarization is correlated with augmented ciliary beating and accelerated forward swimming (Naitoh and Eckert, 1973), whereas depolarization is correlated with ciliary reversal and backward swimming (Machemer and Eckert, 1973). Paramecia normally swim forward, except for episodic brief periods of backward swimming or whirling (Jennings, 1906). Upon

[1]Department of Earth, Environment and Life Sciences (DISTAV), University of Genoa, Corso Europa 26, 16132 Genoa, Italy. [2]Department of Pharmacy (DIFAR), University of Genoa, Viale Cembrano 4, 16148 Genoa, Italy. [3]Institute of Biophysics, CNR Genoa, Via De Marini 6, 16149 Genoa, Italy. [4]Institute of Marine Science (ISMAR), CNR Genoa, Via De Marini 6, 16149 Genoa, Italy. [5]Center of Excellence for Biomedical Research, University of Genoa, Viale Benedetto XV, 16132 Genoa, Italy. [6]National Institute of Neuroscience, Corso Raffaello 30, 10125 Torino, Italy.

*Author for correspondence (ramoino@dipteris.unige.it)

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List of abbreviations

CCR	continuous ciliary reversal
CGS19755	*cis*-4-phosphonomethyl-2-piperidine carboxylic acid
CPP-BP	(+)-3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid-binding protein
D-AP5	D-2-amino-5-phosphonopentanoic acid
DCKA	5,7-dichlorokynurenic acid
EGTA	ethylene glycol tetraacetic acid
GBP	glutamate-binding protein
GluN1	NMDA receptor subunit 1
GluN2	NMDA receptor subunit 2
GluN3	NMDA receptor subunit 3
Gly/TCP-BP	glycine/*N*-[1-(2-thienyl)cyclohexyl]piperidine(TCP)-binding protein
GRINA	glutamate receptor, ionotropic, *N*-methyl-D-aspartate-associated protein 1
MK-801	dizocilpine
NMDA	*N*-methyl-D-aspartate
PaCR	partial ciliary reversal
PCR	periodic ciliary reversal

electrical, chemical or mechanical stimulation, the ciliary Ca^{2+} channels open, giving rise to a depolarizing Ca^{2+} action potential and to a transient increase in intraciliary Ca^{2+} concentration (Dryl, 1974; Machemer, 1988; Preston and Saimi, 1990). This increase in internal Ca^{2+} ions triggers the ciliary reversal. Three forms of behaviour related to ciliary reversal can be observed: (1) a continuous ciliary reversal (CCR), yielding relatively long periods of fast backward swimming mediated by full ciliary reversal, (2) a periodic ciliary reversal (PCR), characterized by brief and repeated episodes of backward swimming, and (3) a partial ciliary reversal (PaCR), giving rise to a spiral-like movement and resulting from the reversal of only part of the somatic cilia of the cells (Dryl, 1974). Whereas weak stimuli cause a moderate Ca^{2+} influx and a short circular movement, strong stimuli can sustain backward swimming for tens of seconds or minutes. The duration of backward swimming is proportional to the duration of the action potential and the addition of drugs able to reduce the inward calcium current decreases the duration of stimulated backward swimming (Hennessey and Kung, 1984). Hence, the swimming behaviour of the cell serves as a visual correlate of the electrical state of the cell membrane.

Our study indicates that the swimming behaviour of paramecia is modified by NMDA and that these changes are controlled by classic receptor antagonists, suggesting the existence of NMDA-like receptors at the cell membrane level.

RESULTS

Effects of NMDA on swimming behaviour

Fig. 1A is a representative image depicting the normal cell swimming in NMDA-free solution. However, in the presence of 200 μmol l^{-1} NMDA and 1 μmol l^{-1} glycine, and in the absence of Mg^{2+}, cells exhibited a PaCR (Fig. 1B–D). Exposure to NMDA concentrations from 1 to 200 μmol l^{-1}, in the presence of 1 μmol l^{-1} glycine, produced a concentration-dependent rise in the percentage of cells that adopt this spiral-like movement from 13% at 50 μmol l^{-1} NMDA to 100% at 200 μmol l^{-1} NMDA (Fig. 2, Table 1). Fig. 2 also shows that the exposure of paramecia to increasing concentrations of NMDA in the absence of exogenously added glycine failed to induce the PaCR in a significant percentage of cells. In addition, glycine (1 μmol l^{-1}), in the absence of NMDA, did not lead to any modification of swimming behaviour in the 80 observed cells (Fig. 2, Table 1).

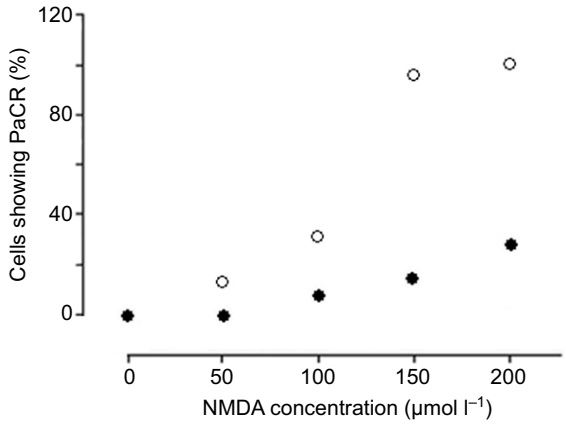

Fig. 2. Dose-dependent effect of NMDA on partial ciliary reversal (PaCR). Percentage of *Paramecium primaurelia* cells showing a PaCR when exposed to NMDA in the presence (open circles) or the absence (filled circles) of glycine (1 μmol l^{-1}).

Ion dependence of the NMDA-induced swimming behaviour

In order to analyse the impact of Mg^{2+} ions on the NMDA-mediated changes in swimming behaviour, experiments were performed in the presence of 0.5–3.0 mmol l^{-1} $MgCl_2$. Regardless of the concentration used, $MgCl_2$ was unable to trigger ciliary reversal and 2.0 mmol l^{-1} $MgCl_2$ completely counteracted the NMDA effect (Table 2). The NMDA+glycine-induced spiral-like *Paramecium* swimming relies on the influx of Ca^{2+} ions. Indeed, paramecia exposed to 200 μmol l^{-1} NMDA+1 μmol l^{-1} glycine in medium without Ca^{2+} and containing 1 mmol l^{-1} EGTA normally swim forward (Table 2). In contrast, the omission of Na^{+} from the extracellular milieu, replaced by an isosmotic amount of *N*-methylglucamine, failed to modify the spiral-like swimming behaviour induced by 200 μmol l^{-1} NMDA+1 μmol l^{-1} glycine (Table 2).

Effects of NMDA receptor antagonists on swimming behaviour

The 200 μmol l^{-1} NMDA+1 μmol l^{-1} glycine-induced PaCR was prevented by 1 μmol l^{-1} MK-801, a non-competitive NMDA receptor

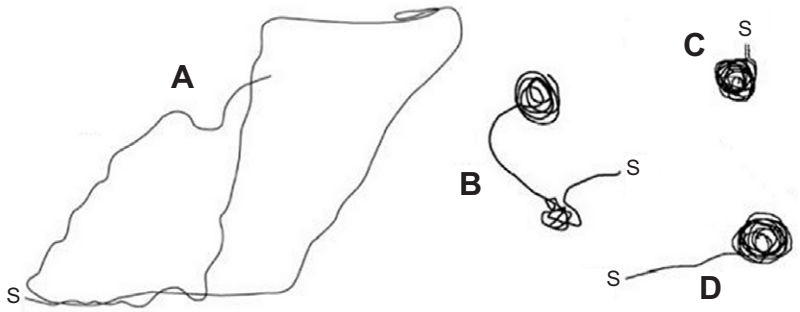

Fig. 1. *Paramecium* swimming behaviour alteration after NMDA and glycine exposure. Paramecia usually swim forward (A) but exhibit a spiral-like swimming behaviour due to a partial ciliary reversal when exposed to 200 μmol l^{-1} NMDA+1 μmol l^{-1} glycine in the absence of Mg^{2+} (B–D). Cells can immediately start swimming in circles (C), or first swim forward and then describe circles or loops (B), or, alternatively, after a lasting ciliary reversal, which terminates as a turn on the spot, swim forward followed by reversion and a loop movement (D). The movement was digitally recorded for 60 s at 4 frames s^{-1}. Swimming registration starts at 'S'.

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Table 1. Percentage of *Paramecium primaurelia* cells showing a partial ciliary reversal (PaCR) when exposed to NMDA

NMDA ($\mu mol\ l^{-1}$)	Glycine	
	0 $\mu mol\ l^{-1}$	1 $\mu mol\ l^{-1}$
0	0% (60)	0% (80)
1	0% (60)	0% (70)
10	0% (50)	0% (60)
50	0% (48)	13% (70)
100	7% (60)	31% (75)
150	15% (100)	95% (110)
200	29% (80)	100% (140)

The number of cells tested in four independent experiments is given in parentheses.

channel blocker (Fig. 3, Table 3). Fig. 3 and Table 3 also show that the spiral-like swimming was largely reduced by 40 $\mu mol\ l^{-1}$ CGS19755 and by 200 $\mu mol\ l^{-1}$ D-AP5, two selective antagonists at the glutamate-binding site located on the GluN2 subunits, as well as by the selective glycine antagonist DCKA (1 $\mu mol\ l^{-1}$). Prevention of the NMDA+glycine-induced changes in *Paramecium* swimming by the above NMDA receptor antagonists is predictive of the involvement of GluN2-containing NMDA receptors. Chemical agents able to discriminate between GluN2A- and GluN2B-containing NMDA receptors in mammals are available. In particular, ifenprodil has been found to preferentially inhibit the mammalian GluN2B-containing NMDA receptor complex (Williams et al., 1993), while nanomolar Zn^{2+} was shown to act as a selective GluN2A-containing NMDA receptor antagonist (Paoletti et al., 1997). We investigated the effects of ifenprodil (10 $\mu mol\ l^{-1}$) and $ZnCl_2$ (100 $nmol\ l^{-1}$) on the 200 $\mu mol\ l^{-1}$ NMDA+1 $\mu mol\ l^{-1}$ glycine-evoked cell spiral-like behaviour and found that both compounds were able to counteract NMDA effects (Fig. 3, Table 3). None of the antagonists administered in the absence of NMDA+glycerine were effective at the concentration used.

Effects of NMDA on swimming behaviour induced by membrane depolarization

When *P. primaurelia* membranes were depolarized using 40 $mmol\ l^{-1}$ KCl, a CCR reaction was triggered. The average duration of the KCl-induced backward swimming amounted to 44.5±1.25 s (*N*=120 cells). The duration of backward swimming was dose-dependently increased by NMDA, with an augmentation of up to 20% by 200 $\mu mol\ l^{-1}$ NMDA+1 $\mu mol\ l^{-1}$ glycine (Fig. 4). Fig. 4 also shows that the CCR duration increment induced by NMDA was smaller in the absence of glycine. As high NMDA concentrations could induce receptor desensitization, we then used higher glycine concentrations (10 and 100 $\mu mol\ l^{-1}$) to prevent NMDA desensitization. Indeed, it has been found that desensitization decreases with increasing glycine concentration in cultured hippocampal neurons as well as in oocytes injected with rat brain mRNA (Benveniste et al., 1990; Lerma et al., 1990; Mayer et al., 1989; Nahum-Levy et al., 2001; Vyklický et al., 1990). Our data indicate that in *Paramecium* the effects of 1–200 $\mu mol\ l^{-1}$ NMDA are not modified by increasing glycine concentration from 1 to 10 or 100 $\mu mol\ l^{-1}$ (Table 4).

Co-administration of NMDA and D-serine, a potent agonist at the glycine-binding site (Mothet et al., 2000), resulted in a significant increase in CCR duration (Table 5).

As Fig. 5 illustrates, the rise in the duration of the 40 $mmol\ l^{-1}$ KCl-induced backward swimming behaviour brought about by 200 $\mu mol\ l^{-1}$ NMDA+1 $\mu mol\ l^{-1}$ glycine was counteracted by the NMDA receptor antagonists MK-801 (1 $\mu mol\ l^{-1}$), CGS19755 (40 $\mu mol\ l^{-1}$), D-AP5 (200 $\mu mol\ l^{-1}$), DCKA (1 $\mu mol\ l^{-1}$), ifenprodil (10 $\mu mol\ l^{-1}$) and $ZnCl_2$ (100 $nmol\ l^{-1}$).

Identification of NMDA receptor-like complex subunit encoding genes in the *Paramecium* genome

We scrutinized the *Paramecium* genome to see whether it contains sequences encoding NMDA subunit proteins. The results suggest that an NMDA-like receptor, with the ligand-binding characteristics of an NMDA receptor-like complex purified from rat brain synaptic membranes (Hui et al., 2009) and also found in some metazoan genomes (see supplementary material Tables S1, S2), is present in *Paramecium*. Indeed, searches of the current *Paramecium tetraurelia* genome database indicate that *Paramecium* harbours at least 20 sequences related to a large family of proteins known as the transmembrane Bax inhibitor 1 protein family (BI1), some of which contain seven putative transmembrane helices (Fig. 6; supplementary material Table S1). In the *Paramecium* genome we also identified homologues of glycine-binding protein (GlyBP). The *Paramecium* genome carries at least two different sequences in contrast to the single sequence found in *Tetrahymena* and in other organisms (Fig. 7; supplementary material Table S2).

DISCUSSION

Some ciliates have been shown to be sensitive to a variety of neurotransmitters and neuropeptides, to which they would not usually be exposed (Le Roith et al., 1980; Nomura et al., 1998). Treatment of *Paramecium* with a β-adrenergic antagonist affects its behavioural responses to depolarization in a time- and dose-dependent manner, and the shortening of ciliary reversal duration occurs concomitantly with impairment of phagocytic activity (Wyroba, 1989; Ucieklak et al., 1993). In *Tetrahymena*, the swimming pattern and the rate of phagocytosis are altered in response to nanomolar concentrations of opiate endorphins (Renaud et al., 1991; Renaud et al., 1995). We have previously reported that $GABA_B$ receptors modulate swimming behaviour in *Paramecium* by inhibition of dihydropyridine-sensitive calcium channels via G-proteins (Ramoino et al., 2003) and that the number of $GABA_B$ receptors at the plasma membrane is regulated by endocytosis into clathrin-coated and -uncoated vesicles and by recycling back to the cell surface (Ramoino et al., 2005; Ramoino et al., 2006).

Glutamate is an important chemo-attractant for paramecia, probably because it signals the presence of bacteria that are food for this unicellular organism. This observation therefore implies that

Table 2. Ion dependence of percentage of *P. primaurelia* cells showing a PaCR when exposed to NMDA

Drugs	%Total cells	*N*
200 $\mu mol\ l^{-1}$ NMDA + 1 $\mu mol\ l^{-1}$ glycine	100	140
200 $\mu mol\ l^{-1}$ NMDA + 1 $\mu mol\ l^{-1}$ glycine + 2 $mmol\ l^{-1}$ $MgCl_2$	0	90
200 $\mu mol\ l^{-1}$ NMDA + 1 $\mu mol\ l^{-1}$ glycine + 1 $mmol\ l^{-1}$ EGTA + 0 $mmol\ l^{-1}$ $CaCl_2$	0	80
200 $\mu mol\ l^{-1}$ NMDA + 1 $\mu mol\ l^{-1}$ glycine + 8 $mmol\ l^{-1}$ *N*-methylglucamine + 0 $mmol\ l^{-1}$ $NaCl_2$	100	60

N, number of cells tested in three independent experiments.

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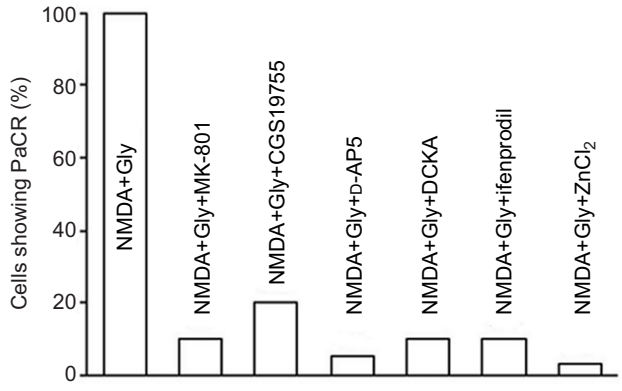

Fig. 3. Effects of NMDA receptor antagonists on PaCR. The 200 µmol l^{-1} NMDA+1 µmol l^{-1} glycine (Gly)-induced PaCR was prevented by 1 µmol l^{-1} MK-801, 40 µmol l^{-1} CGS19755, 200 µmol l^{-1} D-AP5, 1 µmol l^{-1} DCKA, 10 µmol l^{-1} ifenprodil and 100 nmol l^{-1} $ZnCl_2$.

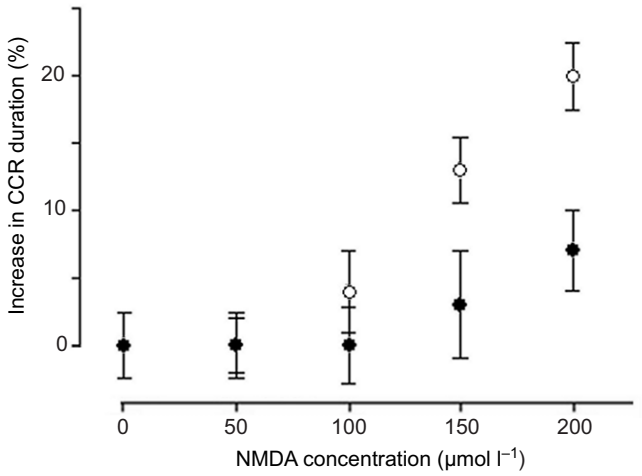

Fig. 4. Dose-dependent effect of NMDA on continuous ciliary reversal (CCR) duration evoked by 40 mmol l^{-1} KCl. An increase of 13% or 20% CCR duration was induced when 150 or 200 µmol l^{-1} NMDA with 1 µmol l^{-1} glycine was used, respectively. Data are given as means ± s.e.m. (*N*=60) from four separate experiments and are normalized to control (40 mmol l^{-1} KCl) values, taking the control CCR duration as 100 s. Filled circles, NMDA; open circles, NMDA+glycine.

paramecia are endowed with glutamate sensors, possibly receptors. It is well known that paramecia swim smoothly and quickly in the presence of glutamate, which indicates a relatively hyperpolarized membrane potential (Preston and Usherwood, 1988). The mechanism of hyperpolarization has not been fully elucidated yet but it probably involves the activation of a hyperpolarizing K^+ conductance and shares some interesting characteristics with umami taste (Van Houten et al., 2000), as a sustained hyperpolarization induced by glutamate was observed in some rat taste cells (Bigiani et al., 1997). Glutamate is a broad spectrum agonist that binds all the glutamate receptors, so that the effects caused by glutamate may be the result of several events, including the metabotropic receptor-mediated modification of cell metabolism and the ionotropic receptor-induced changes of cation balance (Nakanishi et al., 1994; Traynelis et al., 2010). Therefore, the respective roles that the different glutamate receptors exert in controlling *Paramecium* functions remain a challenging issue. As to NMDA receptors, this question can be feasibly addressed through the use of selective NMDA receptor agonists and antagonists.

Evidence is accumulating to suggest the presence and functional roles of NMDA receptors in invertebrates. They have been implicated in insect and crustacean neuromuscular transmission (Ultsch et al., 1993; Feinstein et al., 1998), in insect hormone production (Chiang et al., 2002), in neuroendocrine function in *Ciona* (D'Aniello et al., 2003) and in synaptic activity in the leech (Grey et al., 2009). Moreover, NMDA receptors mediate learning and memory in *Drosophila* (Wu et al., 2007; Xia et al., 2005) and the honeybee, *Apis mellifera* (Si et al., 2004), while their role in synaptic plasticity in *Caenorhabditis elegans* is still controversial (Kano et al., 2008; Rose et al., 2005). Disrupting NMDA receptor function prevents long-term potentiation and leads to changes in learning and memory in *Aplysia* (Ezzeddine and Glanzman, 2003; Murphy and Glanzman, 1997) and *Lymnaea* (Rosenegger and Lukowiak, 2010). In addition, an immunocytochemical study reported a specific localization of the GluN1 subunit in dissociated neurons, the nematocytes and epithelial cells of the hypostomal region, as well as in the tentacle cells of the freshwater polyp *Hydra vulgaris* (Cnidaria, Hydrozoa), one of the first species to develop a nervous system (Scappaticci et al., 2004). Biochemical and behavioural experiments have provided strong evidence for the presence of NMDA receptors in hydra tissue and for their involvement in hydra feeding behaviour (Pierobon et al., 2001; Pierobon et al., 2004), nematocyst discharge in the tentacles (Scappaticci and Kass-Simon, 2008) and electrical tentacle activity (Kay and Kass-Simon, 2009). An NMDA-like receptor has also been described in the ciliated protozoon *T. pyriformis* (Fillingham et al., 2002). It was demonstrated that this NMDA receptor plays a crucial role in the perception of chemoeffectors (Nam et al., 2007) and that its stimulation induces an up to threefold increase of intracellular Ca^{2+} (Nam et al., 2009). It was found that 1 mmol l^{-1} NMDA functions as a repellent, whereas NMDA at 10 µmol l^{-1} and 10 nmol l^{-1} functions as a strong attractant, and that 10 µmol l^{-1} NMDA-induced chemotaxis and intracellular Ca^{2+} increase are suppressed by both U73122, an inhibitor of phospholipase C activity, and wortmannin, an inhibitor of phosphatidylinositol-3-kinase (Nam et al., 2009).

Table 3. Percentage of *P. primaurelia* cells showing a PaCR when exposed to NMDA and NMDA receptor inhibitors

Drugs	%Total cells	*N*
200 µmol l^{-1} NMDA + 1 µmol l^{-1} glycine	100	140
200 µmol l^{-1} NMDA + 1 µmol l^{-1} glycine + 1 µmol l^{-1} MK-801	10	70
200 µmol l^{-1} NMDA + 1 µmol l^{-1} glycine + 40 µmol l^{-1} CGS19755	20	50
200 µmol l^{-1} NMDA + 1 µmol l^{-1} glycine + 200 µmol l^{-1} D-AP5	5	80
200 µmol l^{-1} NMDA + 1 µmol l^{-1} glycine + 1 µmol l^{-1} DCKA	10	60
200 µmol l^{-1} NMDA + 1 µmol l^{-1} glycine + 10 µmol l^{-1} ifenprodil	10	70
200 µmol l^{-1} NMDA + 1 µmol l^{-1} glycine + 10 nmol l^{-1} $ZnCl_2$	3	60

N, number of cells tested in four independent experiments

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Table 4. Continuous ciliary reversal (CCR) duration (s) in *P. primaurelia* cells when exposed to NMDA and glycine

NMDA	Glycine			
(μmol l^{-1})	0 μmol l^{-1}	1 μmol l^{-1}	10 μmol l^{-1}	100 μmol l^{-1}
0	100.00±2.52 (120)	97.69±3.25 (45)	99.69±3.52 (45)	101.21±2.87 (45)
1	101.21±3.04 (45)	102.70±2.14 (45)	98.98±2.55 (45)	96.42±3.16 (45)
10	101.80±2.60 (45)	100.40±2.32 (45)	99.65±3.80 (45)	96.39±2.71 (45)
50	100.62±3.76 (60)	101.02±2.34 (45)	98.98±2.55 (45)	102.21±4.59 (45)
100	101.04±1.93 (60)	97.45±3.75 (60)	100.70±2.70 (45)	97.77±2.77 (45)
150	103.38±2.87 (60)	113.51±1.66 (60)	110.89±2.82 (45)	112.64±2.84 (45)
200	107.24±2.10 (120)	120.08±2.11 (120)	117.38±2.49 (45)	118.14±2.68 (45)

Values (means ± s.e.m.) were normalized to controls (40 mmol l^{-1} KCl), taking the control CCR duration as 100 s. The number of cells tested in three to five different experiments is given in parentheses.

The major novelty of the present investigation is that *Paramecium* is able to sense and respond to the NMDA present in the environment by modifying its swimming behaviour. Indeed, in the absence of external Mg^{2+}, NMDA+glycine causes marked functional changes in *Paramecium* movements and this action is enhanced by membrane depolarization. Addition of K^+ at a high concentration to the medium elicits membrane depolarization, thus causing backward swimming through the opening of voltage-dependent Ca^{2+} channels in the ciliary membrane (Oami and Takahashi, 2002). NMDA affects the behavioural response to membrane depolarization by increasing backward swimming duration. These changes were relieved by the selective NMDA receptor channel blocker MK801, indicative of the involvement of NMDA receptors in the observed effects. Notably, the modification of *Paramecium* swimming was also counteracted by D-AP5 or by DCKA, selective antagonists of the glutamate-binding sites located at the GluN2 subunits and of the glycine-binding sites at the GluN1 subunits, respectively. Our findings imply that the co-presence of the two agonists is required for NMDA-induced reversal of swimming direction. Hence, NMDA alone (i.e. in the absence of glycine) or glycine alone (i.e. in the absence of NMDA) produced little or no change in *Paramecium* movement. D-Serine, a glial-derived endogenous ligand for the glycine-binding site of NMDA receptors in mammals (Mothet et al., 2000; Oliet and Mothet, 2009; Schell et al., 1997), behaved similar to glycine thus strengthening the role of this binding site in the machinery of the NMDA-like receptors in *Paramecium*.

The above-mentioned NMDA-induced changes were observed in the absence of external Mg^{2+}, an ionic condition widely used to force NMDA receptor activation. Accordingly, in our experiments the introduction of Mg^{2+} abolished the effects of NMDA. NMDA receptors are channels permeable to both Na^+ and Ca^{2+} (Ascher and Nowak, 1986; MacDermott et al., 1986; Mayer and Westbrook, 1987), and usually blocked by Mg^{2+} (Ault et al., 1980; Nowak et al., 1984). In particular, GluN2-containing receptors are Ca^{2+} permeant and highly sensitive to Mg^{2+} (Paoletti and Neyton, 2007), while GluN3-containing receptors are to Na^+-permeable and Mg^{2+}-insensitive ion channels (Chatterton et al., 2002). Therefore, our results with Mg^{2+}, along with the Ca^{2+} dependency of the effect, indicate the presence of GluN2 receptor subunits in the *Paramecium* NMDA-like receptor.

With regard to the GluN2 subunit, the blockade of NMDA-induced changes in swimming behaviour by Zn^{2+} and ifenprodil implies the co-presence of the GluN2A and GluN2B subunits (Neyton and Paoletti, 2006). This condition can be suggestive of a receptor assembled in a heterotrimeric fashion (GluN1/GluN2A/GluN2B) although, on the basis of the present results, the presence of two different receptor populations (GluN1/GluN2A and GLUN1/GluN2B) cannot be ruled out.

In apparent contrast with our hypothesis, however, searches of the available *Paramecium* genome database did not produce sequences with homology matches to this ionotropic glutamate receptor family, but revealed the existence of several homologous sequences of the BI-1-like protein family as well as of the NMDA receptor-like complex, the GlyBP. In mammals, the BI-1 family consists of at least six members: TMBIM1/RECS1, TMBIM2/FAIM2, TMBIM3/GBP (also known as GRINA, glutamate receptor, ionotropic, *N*-methyl D-aspartate-associated protein 1), TMBIM4/GAAP, TMBIM5/GHITM and TMBIM6/TEGT (see supplementary material Table S1). GlyBP is a receptor protein that differs, in terms of length and amino acidic sequence, from the classic GluN subunits but is able to assemble with

Table 5. CCR duration (s) in *P. primaurelia* cells when exposed to D-serine

D-Serine	NMDA	
(μmol l^{-1})	0 μmol l^{-1}	200 μmol l^{-1}
0	100.00±2.52 (120)	107.24±2.10 (120)
1	99.69±3.12 (45)	113.88±2.88 (45)
10	100.05±2.85 (45)	114.94±2.52 (45)
100	98.75±3.64 (45)	116.44±2.72 (45)

Values (means ± s.e.m.) were normalized to controls (40 mmol l^{-1} KCl), taking the control CCR duration as 100 s. The number of cells tested in three to five different experiments is given in parentheses.

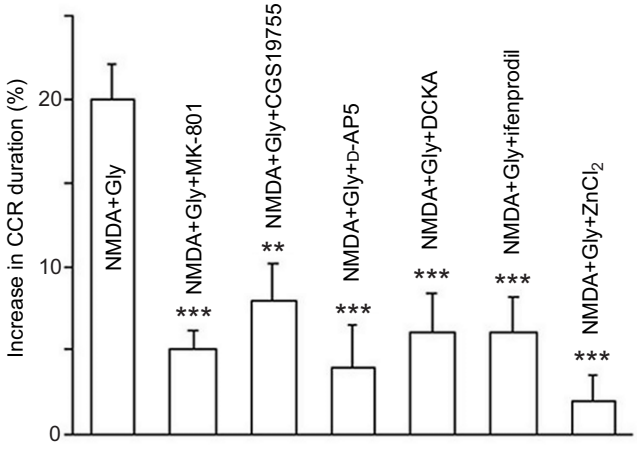

Fig. 5. Effects of NMDA receptor antagonists on CCR. The NMDA+glycine-induced enhancement of CCR duration evoked by 40 mmol l^{-1} KCl in *P. primaurelia* was prevented by exposure to NMDA receptor antagonists (1 μmol l^{-1} MK-801, 40 μmol l^{-1} CGS19755, 200 μmol l^{-1} D-AP5, 1 μmol l^{-1} DCKA, 10 μmol l^{-1} ifenprodil and 100 nmol l^{-1} $ZnCl_2$). Values are given as means ± s.e.m. (*N*=60) from four separate experiments and represent the percentage change from control (40 mmol l^{-1} KCl). ***P*<0.005, ****P*<0.0005 compared with control values by Student's *t*-test.

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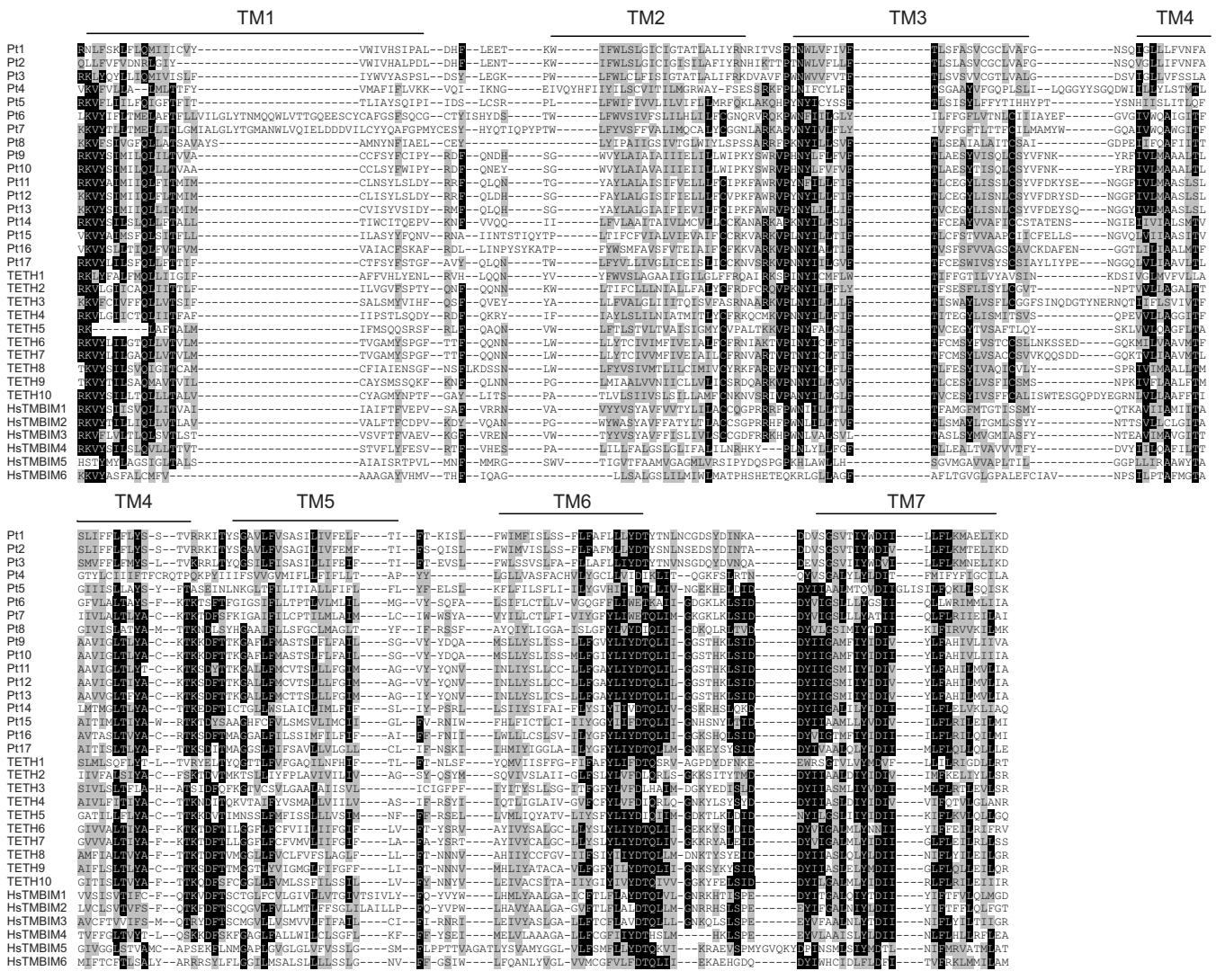

Fig. 6. Multiple alignment of transmembrane Bax inhibitor protein 1 family. Identical and similar residues in at least 50% of the species are indicated in black and grey, respectively. The alignment includes only the transmembrane (TM) helices and loops. The black bars indicate the predicted transmembrane helices of human TMBIMs (transmembrane Bax inhibitor motifs). Abbreviations for the taxa are: Pt, *Paramecium tetraurelia*; TETH, *Tetrahymena thermophila*; Hs, *Homo sapiens*.

the glycine/TCP-BP protein, the CPP-BP, and the phencyclidine-binding protein in mammalian brain synaptosomal membranes to express an NMDA receptor-like complex, with binding properties similar to those of classic NMDA receptors (Kumar et al., 1998). Indeed, the NMDA-like receptors elicit Ca^{2+}-dependent responses similar to those observed for classic NMDA receptors (Michaelis, 1998), which can be prevented by classic NMDA receptor antagonists (Kumar et al., 1998; Michaelis, 1998).

In conclusion, these results provide evidence that NMDA-like receptors, functionally resembling those identified in mammals, are present in the single-celled organism *Paramecium* and also suggest that the glutamatergic NMDA or NMDA-like system is an early evolved, phylogenetically old mechanism.

MATERIALS AND METHODS

Cells and culture conditions

Experiments were carried out on *P. primaurelia*, stock 90, cultured at 25°C in lettuce medium (pH 6.9) inoculated with *Enterobacter aerogenes* (Sonneborn, 1970). Cells were harvested in the late log phase of growth.

Cell movement analysis

Paramecia were transferred from culture medium into an adaptation solution containing 1 mmol l^{-1} $CaCl_2$, 4 mmol l^{-1} KCl, 4 mmol l^{-1} NaCl and 1 mmol l^{-1} Hepes (pH 7.2) and allowed to equilibrate for 30 min. Then, they were transferred into a recording camera (internal size: 21×14×3 mm) containing the indicated concentrations of drugs in the adaptation solution. Responses to drugs were determined by acquiring cell-swimming behaviour through an infrared video camera that recorded the swimming of a small sample of protozoa, i.e. one to five individuals at the same time. Protozoan movement was digitally recorded for 60 s at 4 frames s^{-1}. Records were analysed by means of image-analysis software (IToolTrack, e-magine IT s.r.l., Genoa, Italy) to reconstruct each individual path.

CCR induction

Cells were transferred from culture medium into the adaptation solution for 30 min and then to a bath containing the indicated concentrations of drugs in the test solution (40 mmol l^{-1} KCl, 1 mmol l^{-1} $CaCl_2$, 4 mmol l^{-1} NaCl, 1 mmol l^{-1} Hepes, pH 7.2). The presence of high KCl in the test solution depolarizes the cell membrane, triggering ciliary reversal and backward swimming. Responses to drugs were determined by transferring individual

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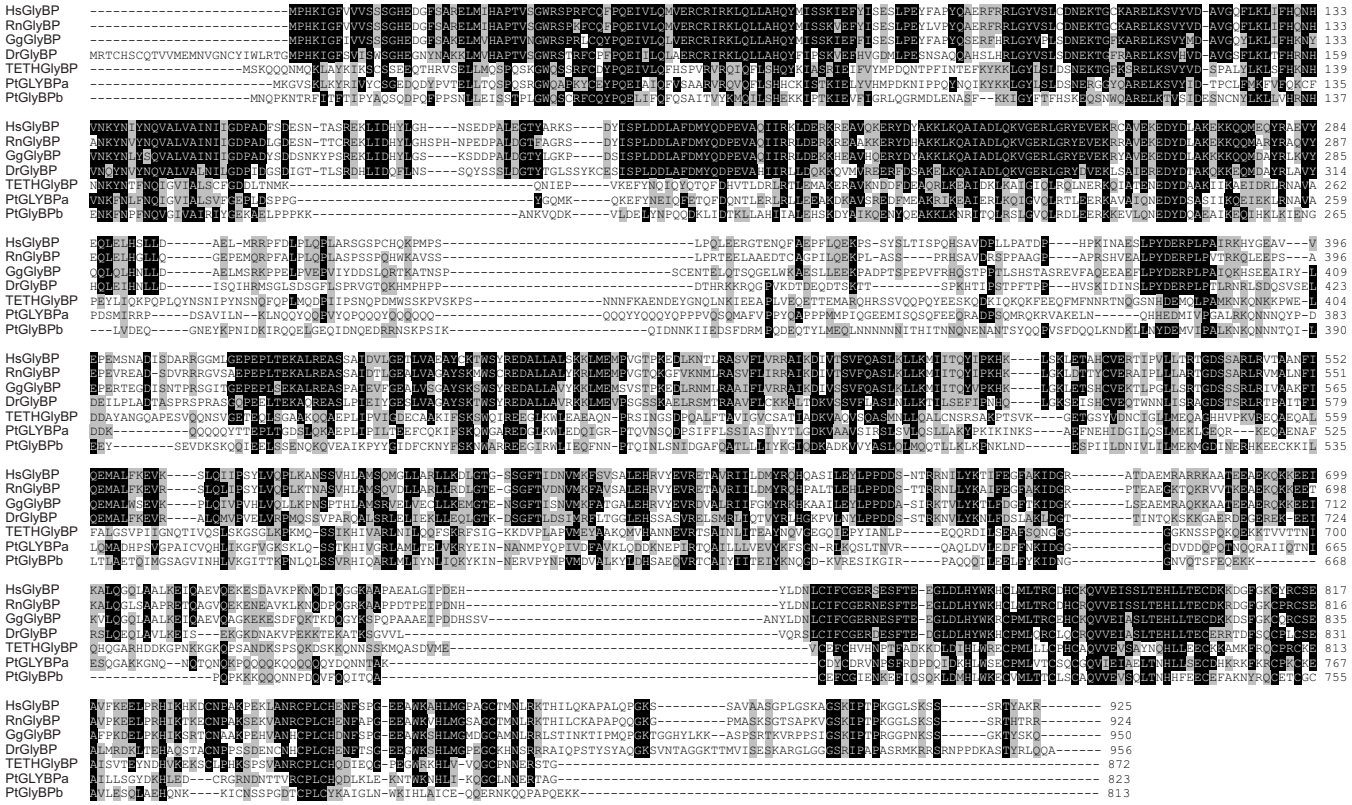

Fig. 7. Sequence alignment of glycine-binding protein (GlyBP) from several organisms. Identical and similar residues in at least 50% of the species are indicated in black and grey, respectively. The alignment includes full-length sequences for *Homo sapiens* (Hs), *Rattus norvegicus* (Rn), *Gallus gallus* (Gg), *Danio rerio* (Dr), *P. tetraurelia* (Pt) and *T. thermophila* (TETH).

cells with a micropipette into the test solution. Cell responses were recorded at low power magnification (12×), and then the duration of backward swimming was scored using a stopwatch.

Tests were carried out on 15–20 cells and were repeated on three to five different occasions over several weeks.

Drugs used

The drugs used were as follows: NMDA (1–200 μmol l^{-1}), glycine (1–100 μmol l^{-1}), D-serine (1–100 μmol l^{-1}), MK-801 (1 μmol l^{-1}), D-AP5 (10–200 μmol l^{-1}), CGS19755 (10–40 μmol l^{-1}), DCKA (1 μmol l^{-1}), $ZnCl_2$ (100 nmol l^{-1}), ifenprodil (100 nmol l^{-1} to 10 μmol l^{-1}), $MgCl_2$ (0.5–3.0 mmol l^{-1}), EGTA (1 mmol l^{-1}) and *N*-methylglucamine (8 mmol l^{-1}). MK-801, D-AP5, CGS19755, DCKA and ifenprodil were purchased from Tocris Bioscience (Bristol, UK). NMDA, glycine, D-serine, *N*-methylglucamine and all other chemicals (unless otherwise specified) were obtained from Sigma Chemical Co. (St Louis, MO, USA). Drugs were dissolved in milli-Q water.

Statistical analysis

Differences between means (±s.e.m.) were determined using Student's *t*-test (GraphPad Prism, GraphPad, San Diego, CA, USA). Statistical tests were performed on raw data but, to emphasize any changes in the cell responses to test solutions, backward swimming durations were normalized to control (40 mmol l^{-1} KCl) values (taking the control as 100 s).

Sequence analysis

TBlastN and BlastP searches (Altschul et al., 1997) using protein sequences of selected vertebrate, invertebrate and plant glutamate receptors were conducted on the genomic and protein databases of *P. tetraurelia* [*Paramecium* Genomics, http://paramecium.cgm.cnrs-gif.fr/] and of *Tetrahymena thermophila* [The TIGR *Tetrahymena thermophila* Genome Project, http://www.ciliate.org]. Similar searches were also performed using as queries the relative sequences of the mammalian NMDA receptor-like complex proteins. Multiple sequence alignments were performed using M-coffee (Edgar, 2004) and/or ClustalW [http://www.ebi.ac.uk/Tools/clustalw]. All the sequences used in the sequence alignments are described in supplementary material Tables S1 and S2. The putative membrane-spanning alpha helices were assigned using TMHMM software (http://www.cbs.dtu.dk/services/TMHMM).

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Competing interests

The authors declare no competing financial interests.

Author contributions

L.G., S.F., M.M. and M.F. performed the experiments. S.C. accomplished the genetical analysis. P.R., A.M.P., C.U. and G.B. analyzed and discussed the experimental results and wrote the manuscript.

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Supplementary material

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