Research report

Crucial role of kainate receptors in mediating striatal kainate injection-induced decrease in acetylcholine M₁ receptor binding in rat forebrain

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Accepted 15 August 2000

Abstract

We investigated the roles of kainate-, α-amino-3-hydroxy-5-methylisoxazol-4-propionate (AMPA)- and N-methyl-D-aspartate (NMDA)-receptors in mediating striatal kainate injection-induced decrease in the binding of acetylcholine M₁ receptors in rat forebrain. After unilateral intrastriatal injection of kainate (4 nmol), the bindings of [3H]kainate (10 nM), [3H]MK-801 (4 nM) and [3H]pirenzepine (4 nM) in the rat ipsilateral forebrain membranes declined, reaching the lowest on day 2 to 4 and recovering on day 8. Saturation binding studies, performed on day 2 post-injection, showed that kainate (1, 2, 4 nmol) dose-dependently decreased B max and K d of the three ligands. (+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine (MK-801), a selective NMDA receptor channel blocker, antagonised (from a dose of 4 nmol) kainate-induced decreases in the bindings of [3H]kainate (up to ~20%), [3H]MK-801 (up to ~90%) and [3H]pirenzepine (up to ~70%). In contrast, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), a selective non-NMDA receptor antagonist, almost completely abolished (from a dose of 12 nmol) kainate-induced decreases in the bindings of all the three ligands (up to ~95–98%). Cyclothiazide, a selective potentiator that enhances AMPA receptor-mediated responses, significantly enhanced (from a dose of 4 nmol) kainate-induced decrease in the binding of [3H]kainate but not that of [3H]pirenzepine or [3H]MK-801. In summary, these results indicate that striatal kainate injection-induced decrease in the binding of acetylcholine M₁ receptors in rat forebrain is dependent on activation of kainate receptors and, to a certain extent, a consequent involvement of NMDA receptors. These and previous studies provide some evidence showing that kainate receptors might play a crucial role in regulating excitatory amino acids (EAA)-modulated cholinergic neurotransmission in the central nervous system (CNS). © 2000 Elsevier Science B.V. All rights reserved.

Theme: Neurotransmitters, modulators, transporters, and receptors

Topic: Excitatory amino acid receptors: physiology, pharmacology and modulation

Keywords: Cholinergic neurotransmission; CNQX; Cyclothiazide; Kainate injection; [3H]Pirenzepine binding

1. Introduction

L-Glutamate, the principal excitatory neurotransmitter in the mammalian central nervous system (CNS), mediates most of the excitatory neurotransmission through activation of three classes of ionotropic glutamate receptors [31,34], named after the agonists N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methylisoxazol-4-propionate (AMPA) and kainate [39]. Among the three subtypes, kainate receptors have been shown to play a distinct role in mediating excitatory amino acids (EAA)-induced acetylcholine release in rat striatum in vitro [16], whereas a demonstration of kainate receptor-mediated control of cholinergic neurotransmission in the CNS under in vivo conditions is still lacking.

Local injection of a glutamate analogue, such as kainate, is widely used as a method for destroying neurones in vivo [34]. Previous studies have documented that local injection of kainate in rat brain induced massive neuronal loss and over-expression of amyloid precursor protein (APP) [7,12,21] as well as corresponding decreases in the expressions of choline acetyltransferase (ChAT) and glutamate decarboxylase [12,14] and in the bindings of ionotropic...
glutamate- and acetylcholine muscarinic-receptors [15,25]. However, the roles of NMDA-, AMPA- and kainate-receptors in these mechanisms are poorly understood. Generally, the effect of kainate is thought to be indirect through innervations of glutamatergic neurons [10]. It is the release of glutamate [11,26] that leads to neuronal death through activation of postsynaptic NMDA receptors [27,34]. These studies focusing on rat hippocampus suggested an excitatory role for presynaptic kainate receptors on glutamatergic terminals [27]. On the other hand, conflicting observations regarding the roles of presynaptic kainate- and postsynaptic NMDA-receptors have also been reported [5,6,12].

We have recently observed that striatal kainate injection induced a loss in cholinergic neuron and an increase in APP expression in rat forebrain. These effects are dependent on the activation of kainate receptors with a consequent involvement of NMDA receptors [42]. In the present studies, we, therefore, investigated the roles of kainate-, AMPA- and NMDA-receptors in mediating striatal kainate injection-induced decreases in the bindings of ionotropic glutamate- and acetylcholine M₁-receptors by examining the characteristics of the specific \[^{3}H\]kainate, \[^{3}H\]MK-801 and \[^{3}H\]pirenzepine binding to the membranes prepared from the rat ipsilateral forebrain.

2. Materials and methods

2.1. Animal treatment

Male Sprague–Dawley rats (200–250 g) were housed under controlled conditions with a 12-h day-night cycle and with food and water available ad libitum. Rats were anesthetized with chloral hydrate (400 mg/kg, i.p.) and mounted in a stereotaxic apparatus (Stoelting, Wood Dale, IL, USA). One µl of phosphate buffer (4 mM, pH 7.4) with kainate was slowly injected unilaterally into the striatum (AP=+0.7 mm, ML=±3.0 mm, DV=−6.5 mm from bregma), with 5 min allowed for local diffusion before removing the microsyringe. The antagonists (+)-5-Methyl-10.11-dihydro-5H-dibenzo[a, d]cyclohepten-5,10-imine (MK-801) or 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) was injected 15 min before the kainate injection. Cyclothiazide was co-injected with kainate.

2.2. Membrane preparation

Rats were decapitated without prior stunning or anaesthesia, and the forebrains were rapidly removed on ice. Membrane preparations were prepared as previously described [22]. In brief, the ipsilateral forebrains were homogenized in ten volumes of ice-cold sucrose (300 mM) containing 1 mM EGTA (pH 7.4) in a glass/Teflon homogenizer. The homogenate was centrifuged at 1000×g for 10 min, and then the supernatant was recentrifuged at 30 000×g for 20 min. The pellet was suspended in hypotonic buffer (1 mM EGTA buffered by Tris–HCl to pH 8.0) and left on ice for 30 min. The lysed membranes were collected by a 30-min centrifugation at 40 000×g, and then the lysis/centrifugation step was repeated one more time. The membranes were then suspended and washed twice in a Tris–acetate buffer (100 mM, pH 7.4). After each centrifugation, the membrane suspension was sonicated with a tip sonicator at high intensity. The final pellet was suspended in the assay buffer (100 mM HEPES containing 50 µM EGTA buffered to pH 7.4 by Tris–HCl). The membrane suspension was stored in aliquots at −80°C.

2.3. Protein analysis

On the day of the binding assay, the frozen membrane preparations were thawed on ice and their protein contents were determined by Bradford’s method [4]. Bovine serum albumin was used for the standard curve. The membrane preparations (protein 0.4 mg/ml) were kept on ice until used in \[^{3}H\]kainate, \[^{3}H\]MK-801 and \[^{3}H\]pirenzepine binding assays.

2.4. Radioligand binding assays

\[^{3}H\]Kainate, \[^{3}H\]MK-801 and \[^{3}H\]pirenzepine bindings were studied, using the filtration method [8,23,38]. All the binding incubations were performed in disposable sterile 96-well plates in a total volume of 300 µl and terminated by rapidly filtering the incubation medium through Whatman GF/B glass fiber filters under suction by means of a Skatron cell harvester.

For \[^{3}H\]kainate binding, aliquots of the membrane suspension (in a final concentration of 200 µg/ml) were incubated with \[^{3}H\]kainate (10 nM) on ice for 1 h. Non-specific binding, determined by inclusion of 1 mM l-glutamate, amounted to 10–12% of the total binding. For the saturation experiments, the membranes were incubated with 0.8–400 nM \[^{3}H\]kainate. Non-specific binding amounted to 10–40% of the total binding. The Skatron cell harvester was set at 111 which meant the filters were washed with three 1-ml aliquots of ice-cold 50 mM Tris–HCl buffer, pH 7.4 (total filtering and washing time was 3 s).

For \[^{3}H\]MK-801 binding, aliquots of the membrane suspension (in a final concentration of 200 µg/ml with 10 µM glutamate and 10 µM glycine) were incubated with \[^{3}H\]MK-801 (4 nM) at 25°C for 2.5 h. Non-specific binding, determined by inclusion of 100 µM MK-801, amounted to 5–8% of the total binding. For the saturation experiments, the membranes were incubated with 0.5–150 nM \[^{3}H\]MK-801. Non-specific binding amounted to 5–30% of the total binding. The Skatron cell harvester was set at 333 which meant the filters were washed with three 3-ml aliquots of ice-cold 50 mM Tris–HCl buffer, pH 7.4 (total filtering and washing time was 9 s).
For [3H]pirenzepine binding, aliquots of the membrane suspension (in a final concentration of 200 μg/ml with 1 mM KCl and 1 mM MgCl2) were incubated with [3H]pirenzepine (4 nM) at 25°C for 3 h. Non-specific binding, determined by inclusion of 1 μM atropine, amounted to 6–8% of the total binding. For the saturation experiments, the membranes were incubated with 0.5–100 nM [3H]pirenzepine. Non-specific binding amounted to 6–45% of the total binding. The Skatron cell harvester was set at 333 which meant the filters were washed with three 3-ml aliquots of ice-cold 50 mM Tris–HCl buffer, pH 7.4 (total filtering and washing time was 9 s).

2.5. Scintillation counting

The filters were transferred to scintillation vials and soaked in 3 ml of BCS (Amersham, Arlington Heights, IL, USA) scintillation cocktail. The vials were shaken and left overnight at room temperature, and shaken again before being counted in a Beckman LS 3801 liquid scintillation counter with an efficiency of 56% for tritium.

2.6. Calculation and statistical analysis

Specific binding was calculated as the difference between total and non-specific binding and expressed on a protein basis (pmol/mg protein). Receptor density (Bmax) and dissociation constant (Kd) values were determined from saturation binding data by a computerized non-linear regression analysis (GRAPHPAD PRISM 2.0, GraphPad Software, San Diego, CA, USA).

Scatchard analysis of the data was not used for the determination of binding parameters (Kd and Bmax), but plotted primarily to visualize the different binding models, curvilinearity for multiple binding sites and linearity for a single binding site. The best-fit model was determined using the F-test.

Total binding and non-specific binding for each sample were determined in triplicate. All data were the results of at least three independent experiments using at least three rats per experiment and given as means±S.E.M. One-way analysis of variance (ANOVA) was employed followed by Bonferroni post-hoc analysis for multiple comparisons. A probability level of <0.05 was considered significant.

2.7. Chemicals

[1H]Vinylidene]kainate (specific activity: 58 Ci/mmol), [3H](+)-MK-801 (specific activity: 22.5 Ci/mmol), and [3H]pirenzepine (specific activity: 85.6 Ci/mmol) were purchased from Research Biochemicals (RBI, Natick, MA, USA). All other chemicals were of analytical purity and of commercial origin.

3. Results

3.1. Time-courses of [3H]kainate, [3H]MK-801 and [3H]pirenzepine binding

After unilateral intrastriatal injection of kainate (4 nmol), the specific bindings of [3H]kainate (10 nM), [3H]MK-801 (4 nM) and [3H]pirenzepine (4 nM) to the membranes prepared from the ipsilateral striatum (results not shown), cortex (results not shown) and forebrain (Fig. 1) of rat clearly declined. Among them, the specific bindings of [3H]kainate and [3H]MK-801 declined to the lowest (about 33% and 19%, respectively) on day 2 and recovered on day 8 post-injection (Fig. 1A and 1B), whereas the specific binding of [3H]pirenzepine declined to the lowest (about 38%) on day 4 and did not recover until day 8 post-injection (Fig. 1C).

3.2. Saturation-curves of [3H]kainate, [3H]MK-801 and [3H]pirenzepine binding

Saturation binding experiments were performed on day 2 after unilateral intrastriatal injections of kainate (1, 2, 4 nmol). Kainate, in a dose-dependent manner, declined saturation-curves of the specific [3H]kainate, [3H]MK-801 and [3H]pirenzepine binding to the rat ipsilateral forebrain membranes (Fig. 2(1)). Their Kd and Bmax values are shown in Table 1.

The Scatchard plot of the saturation binding of [3H]kainate was curvilinear, indicating more than one population of receptor binding sites [Fig. 2(2A)]. F-test indicated that a two-site fit of the data was significantly better than a one-site fit (P<0.05). In contrast, the Scatchard plot of the saturation binding data of [3H]MK-801 [Fig. 2(2B)] or [3H]pirenzepine [Fig. 2(2C)] was linear, indicating only one apparent population of receptor binding site. F-test indicated that a two-site fit of the data was not significantly better than a one-site fit (P>0.05).

3.3. Differential antagonism by MK-801 and CNQX of kainate-induced decreases in [3H]kainate, [3H]MK-801 and [3H]pirenzepine binding

MK-801 and CNQX dose-dependently antagonised kainate (4 nmol)-induced decreases in the specific bindings of [3H]kainate (10 nM), [3H]MK-801 (4 nM) and [3H]pirenzepine (4 nM) to the rat ipsilateral forebrain membranes (Fig. 3). The antagonism by MK-801, from a dose of 4 nmol, reached a maximum of only about 20% for the specific [3H]kainate binding, but about 90% for the
Fig. 1. Time-courses of the bindings of \[^{3}H\]kainate (10 nM, A), \[^{3}H\]MK-801 (4 nM, B) and \[^{3}H\]pirenzepine (4 nM, C) to the rat ipsilateral forebrain membranes after kainate (4 nmol) was unilaterally injected into the striatum. Each point is the mean±S.E.M of nine to 14 observations from at least three independent experiments, using at least three rats per experiment. A significant difference (by one-way ANOVA with Bonferroni) vs. corresponding uninjected controls is represented by: * \(P<0.05\), ** \(P<0.01\), *** \(P<0.001\).
Fig. 2. (1) Saturation-curves of the bindings of [3H]kainate (0.8–400 nM, A), [3H]MK-801 (0.5–150 nM, B) and [3H]pirenzepine (0.5–100 nM, C) to the rat ipsilateral forebrain membranes. Saturation binding assays were performed on day 2 after kainate (1, 2, 4 nmol) was unilaterally injected into the striatum. Control curves come from uninjected rats. Their $K_d$ and $B_{max}$ are shown in Table 1. Each point is the mean±S.E.M of nine to 12 observations from at least three independent experiments, using at least three rats per experiment. In some cases, the low variability resulted in error bars that were too small to plot.
specific $[^3]$HMK-801 binding and about 70% for the specific $[^3]$Hpirenzepine binding. On the other hand, CNQX at a dose of 12 nmol almost completely abolished kainate (4 nmol)-induced decreases in the specific bindings of all the three ligands (about 95–98%).


Cyclothiazide, in a dose-dependent manner, significantly enhanced kainate (4 nmol)-induced decrease in the specific binding of $[^3]$Hkainate (10 nM) but not that of $[^3]$HMK-801 (4 nM) or $[^3]$Hpirenzepine (4 nM) to the rat ipsilateral forebrain membranes (Fig. 4). The potentiation by cyclothiazide, from a dose of 4 nmol, reached a maximum of about 15% for the specific $[^3]$Hkainate binding.

4. Discussion

4.1. Kainate-induced decrease in acetylcholine M$_1$ receptor binding

The present studies demonstrated that kainate injected unilaterally into the striatum dose-dependently decreased the bindings of ionotropic glutamate- and acetylcholine M$_1$-receptors in the ipsilateral forebrain of rat, particularly on day 2 to 4 post-injection. These results are in agreement with previous observations, using immunohistochemistry, histochemistry, histofluorescence, and electron microscopic techniques [7,42], that on day 2 after intrastratial injection of kainate, there were a massive neuronal loss and a significant reduction of ChAT expression in the ipsilateral forebrain of rat. Considered together, these findings suggest that the decrease in the binding of acetylcholine M$_1$ receptors in the present experimental model, at least to a certain extent, reflects a loss of cholinergic neurons [28,40].

4.2. Roles of NMDA-, AMPA- and kainate-receptors

Saturation binding experiments of radiolabeled kainate, MK-801 and pirenzepine were performed to mainly assess kainate-, NMDA- and acetylcholine M$_1$-receptor binding sites. The selective NMDA receptor channel blocker, MK-801, and the selective non-NMDA receptor antagonist, CNQX, were used to distinguish between NMDA- and non NMDA-receptor-mediated responses. The present results showed that CNQX and MK-801 differentially antagonised the kainate-induced decrease in acetylcholine M$_1$ receptor binding. We have further extended this observation to include the kainate-induced neuronal loss and decrease in ChAT expression [42]. Cyclothiazide is a selective potentiator that enhances AMPA receptor-mediated responses.
In the present studies, cyclothiazide was used to differentiate the involvement of the AMPA- and kainate-receptors since CNQX was able to almost completely block the kainate-induced decreases in all three bindings. The decrease in the binding of radiolabeled kainate induced by cyclothiazide shows the potentiation of AMPA receptor-mediated responses, which is in agreement with previous findings [22]. However, cyclothiazide did not enhance kainate-induced decrease in the binding of acetylcholine M₁ receptors.

These results indicate that the activation of NMDA receptors accounts for a substantial fraction of kainate-induced decrease in the binding of acetylcholine M₁ receptors in rat forebrain, which support most of the previous studies regarding the involvement of NMDA receptors in kainate-induced brain injury [11,26,27,42]. Radioligand binding and immunohistochemical studies have shown that NMDA receptors are distributed throughout the rat brain but predominantly within the forebrain, and mainly localized at postsynaptic densities and the associated dendrites of most neurons [31], including cholinergic neurons [17]. The activation of postsynaptic NMDA receptors on target cells may trigger cytotoxic actions by opening calcium channels [3] and evoke excessive release of neurotransmitters including acetylcholine by depolarising cell membranes [17,18]. Both the cytotoxic actions and acetylcholine release induced by excessive activation of postsynaptic NMDA receptors might directly contribute to the effects of NMDA receptors in the present experimental model.

In addition, these results show that the potentiation of AMPA receptors by cyclothiazide has no significant effects on kainate-induced decrease in the binding of acetylcholine M₁ receptors in rat forebrain, in agreement with our previous observation that AMPA receptors apparently did not participate in mediating kainate-induced acetylcholine release in the rat striatum [16]. Immunolabelings with anti-GluR1, anti-GluR2/3 and anti-GluR4 in the rat cerebral cortex and hippocampus have established the localization of AMPA receptors at postsynaptic densities [33] as well as at the developed dendrites [9,31]. However, an observation using autoradiography indicated the localization of AMPA receptors on presynaptic endings of dopamine fibres [41], which is supported by our previous studies regarding dopamine release from the presynaptic endings of dopamine fibres [16,17] and by a previous finding that dopamine positively mediated only GluR1 gene expression [1]. To date, there is no evidence showing the presence of excitatory presynaptic AMPA receptors on cholinergic and glutamatergic neurons.

More importantly, these results demonstrate a key role of kainate receptors in mediating kainate-induced decrease in the binding of acetylcholine M₁ receptors in rat forebrain, consistent with our other findings regarding the distinct role of kainate receptors in mediating kainate-induced acetylcholine release in rat striatum [16] and cholinergic neuron loss in rat forebrain [42]. Immunocytochemical studies, using antibodies against kainate receptor subunits, have clearly shown the presence of kainate receptors at both the presynaptic and postsynaptic densities in the rat brain [32,35]. In the present experimental model, the involvement of kainate and NMDA receptors suggest that both the pre- and post-synaptic kainate receptors might participate in mediating the effects of kainate. The mechanism underlying the link between the activation of kainate receptors and the involvement of NMDA receptors is a controversial issue. It might be attributed to a presynaptic excitation on glutamatergic neurons [27], a presynaptic disinhibitory effect on GABAergic neurons [6], or a postsynaptic excitatory action on target cells [5] through kainate receptors. In the latter case, it might be necessary to assume the presence of a direct functional interaction between the postsynaptic kainate- and NMDA-receptors on the same target cells, like that between adenosine A₂A- and dopamine D₂-receptors [19,30].

Table 1

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a Saturation assays were performed on day 2 after kainate (1, 2, 4 nmol) was unilaterally injected into rat striatum. Data are expressed as the mean±S.E.M of nine to 12 observations from at least three independent experiments, using at least three rats per experiment.

b A significant difference P<0.05 (by one-way ANOVA with Bonferroni) vs. corresponding uninjected control.

c A significant difference P<0.01 (by one-way ANOVA with Bonferroni) vs. corresponding uninjected control.

d A significant difference P<0.001 (by one-way ANOVA with Bonferroni) vs. corresponding uninjected control.
Fig. 3. Effects of increasing doses of MK-801 (filled symbols) or CNQX (unfilled symbols) on kainate (4 nmol)-induced decreases in the bindings of \(^{[3]H}\text{kainate (10 nM, A), }^{[3]H}\text{MK-801 (4 nM, B) and }^{[3]H}\text{pirenzepine (4 nM, C) to the rat ipsilateral forebrain membranes. Binding assays were performed on day 2 after kainate (4 nmol) was unilaterally injected into rat striatum. MK-801 or CNQX was injected into the ipsilateral striatum 15 min before the kainate injection. Responses are expressed as a percentage of kainate-induced decreases in the bindings of the three ligands. Each point is the mean±S.E.M of nine to 18 observations from at least three independent experiments, using at least three rats per experiment. A significant difference (by one-way ANOVA with Bonferroni) vs. corresponding control is represented by: * \(P<0.05\), ** \(P<0.01\), *** \(P<0.001\). In some cases, the low variability resulted in error bars that were too small to plot.
Fig. 4. Effects of increasing doses of cyclothiazide on kainate (4 nmol)-induced decreases in the bindings of \(^{[3]H}\)kainate (10 nM), \(^{[3]H}\)MK-801 (4 nM) and \(^{[3]H}\)pirenzepine (4 nM) to the rat ipsilateral forebrain membranes. Binding assays were performed on day 2 after kainate (4 nmol) with or without cyclothiazide was unilaterally injected into rat striatum. Each bar is the mean ± S.E.M of nine to 16 observations from at least three independent experiments, using at least three rats per experiment. A significant difference (by one-way ANOVA with Bonferroni) vs. corresponding control (kainate 4 nmol) is represented by: * \(P < 0.05\), ** \(P < 0.01\), *** \(P < 0.001\).

4.3. Kainate receptors and the cholinergic neurotransmission in the CNS

The present receptor binding results are consistent with those obtained in neurotransmitter release [16] and immunohistochemistry [42] studies. These results indicate that kainate receptors are predominantly involved in the EAA-induced increase in acetylcholine release, decrease in M, receptor binding and ChAT immunoreactivity, increase in APP immunoreactivity, and neuronal loss. They might suggest a crucial role for kainate receptors in regulating EAA-modulated cholinergic neurotransmission in the CNS as well as a similar role for the receptors in the CNS diseases associated with cholinergic disorders e.g. Alzheimer’s disease on the basis of the cholinergic hypothesis of this neurodegenerative disease [13,24].

The main functions ascribed to cholinergic pathways are related to arousal and learning, and motor control. Blockade of glutamatergic transmission by CNQX and NBQX (another antagonist at non-NMDA receptors) has been shown to produce cognitive deficits, which are attributed to blockade of AMPA receptors [3,43]. An involvement of kainate receptors could not be ruled out because of the lack of high selectivity of NBQX and CNQX on AMPA- and kainate-receptors.

Loss of cholinergic neurons in the basal forebrain is believed to account for much of the learning and memory deficits in Alzheimer’s disease. It was reported that domoate, a glutamate analogue produced by mussels, caused a syndrome with features that resemble those of Alzheimer’s disease in a group of Newfoundlanders after consuming domoate-contaminated mussels in 1987 [29,36,37]. Domoate has been established to be a relatively selective agonist at kainate receptors although it can still act on AMPA receptors [20]. The present studies may help to understand the mechanism of neurodegeneration underlying the domoate-induced syndrome.

5. Conclusion

In summary, the present studies demonstrated that striatal kainate injection decreased the binding of acetylcholine \(M_2\) receptors in rat forebrain. This decrease is
dependent on the activation of kainate receptors and, to a certain extent, a consequent involvement of NMDA receptors. These results support the possibility that both the pre- and post-synaptic kainate receptors participate in mediating the effects of kainate in the present experimental model. These and previous findings provide some evidence that kainate receptors may play a crucial role in regulating EAA-modulated cholinergic neurotransmission in the CNS.

Acknowledgements

This work was supported by grants (3981319/3981320) to PTHW from The National University of Singapore.

References


