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Research Report

Reverse mode Na^+/Ca^{2+} exchangers trigger the release of Ca^{2+} from intracellular Ca^{2+} stores in cultured rat embryonic cortical neurons

Meng-Pei Wu[a], *Lung-Sen Kao*[b], *Hsin-Tzu Liao*[c], *Chien-Yuan Pan*[a,c,*]

[a]*Institute of Zoology, National Taiwan University, Taipei, Taiwan, ROC*
[b]*Department of Life Sciences and Institute of Genome Sciences, National Yang-Ming University, Taipei, Taiwan, ROC*
[c]*Department of Life Science, National Taiwan University, Taipei, Taiwan, ROC*

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ABSTRACT

The importance of Na^+/Ca^{2+} exchangers in the regulation of the physiological and pathological functions of the nervous system has been widely recognized. In this study, we used primary cultured E14.5 cortical neurons as a model system to study the possible roles of the reverse mode Na^+/Ca^{2+} exchange activity in neurotransmission. Using RT-PCR, several exchanger isoforms, *ncx1*, *ncx3* and *nckx2–4* were found to be expressed in freshly isolated and cultured cortical neurons. Expression of *ncx2* was undetectable in freshly isolated neurons but increased with time in culture. Neurons were treated with ouabain to increase the intracellular Na^+ concentration and the extracellular Na^+ was replaced by *N*-methyl-D-glucamine to activate reverse mode Na^+/Ca^{2+} exchange. During the maturation of the neurons, the exchange activity shifted from mostly K^+-dependent exchange to both K^+-dependent and K^+-independent exchange. The $[Ca^{2+}]_i$ rises were mostly suppressed by ryanodine and thapsigargin treatments, indicating contributions from the intracellular Ca^{2+} stores. This $[Ca^{2+}]_i$ elevation could propagate to the axon terminal and resulted in elevated $[Ca^{2+}]_i$ at the postsynaptic neurons based on the fact that the elevation in the postsynaptic neuron was inhibited by 6-cyano-7-nitroquinoxaline-2,3-dione and tetanus toxin. When neurons were stimulated by AMPA to increase the intracellular Na^+ concentration, the $[Ca^{2+}]_i$ elevations were significantly inhibited by thapsigargin pretreatment and by KB-R7943. These results demonstrate that, in cultured cortical neurons, the influx of Na^+ through the ionotropic glutamate receptor activates reverse Na^+/Ca^{2+} exchange, which then triggers the release of Ca^{2+} from intracellular Ca^{2+} stores to enhance Ca^{2+} signaling and neurotransmitter release.

* *Corresponding author*. Institute of Zoology, National Taiwan University, Rm730, Life Science Building, No. 1 Roosevelt Rd., Sec. 4, Taipei 106, Taiwan, ROC. Fax: +886 2 23636837.
E-mail address: cypan@ntu.edu.tw (C.-Y. Pan).

Abbreviations: AMPA, α-amino-5-hydroxy-3-methyl-4-isoxazole propionic acid; AP5, D-2-amino-5-phosphonopentanoate; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; CPA, cyclopiazonic acid; D.I.V., days *in vitro*; NCKX, K^+-dependent Na^+/Ca^{2+} exchanger; NCX, K^+-independent Na^+/Ca^{2+} exchanger; rNC(K)X, reverse mode Na^+/Ca^{2+} exchange; NMG, *N*-methyl-D-glucamine; Ry, ryanodine; RyR, ryanodine receptor; TBOA, DL-threo-β-benzyloxyaspartate; Tg, thapsigargin; TTX, tetrodotoxin

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1. Introduction

A change in the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) is a prerequisite of many cellular physiological activities. Therefore, precise spatial and temporal control of Ca^{2+} is an important issue for Ca^{2+} signaling. Intracellular organelles, including the endoplasmic reticulum (ER), mitochondria and secretory vesicles, have Ca^{2+} uptake mechanisms to remove Ca^{2+} from the cytosol and bring the $[Ca^{2+}]_i$ back to the resting level. In the plasma membrane, the Ca^{2+} pump and the Na^+/Ca^{2+} exchangers are the two main pathways for exporting Ca^{2+} out of the cell. Using these mechanisms, the $[Ca^{2+}]$ in various regions of the cell may be differentially modulated (Rizzuto and Pozzan, 2006; Schneggenburger and Neher, 2005; Verkhratsky, 2005).

The Na^+/Ca^{2+} exchangers play a dominant role in removing Ca^{2+} from the cytosol in cells that require extracellular Ca^{2+} for their physiological activities, such as neurons and cardiac muscle cells. Two families of Na^+/Ca^{2+} exchangers, the K^+-independent (NCX) and the K^+-dependent (NCKX), have been identified. Both exchangers are encoded by multigene families; three genes, *ncx1*, *ncx2* and *ncx3*, code for the NCX, (Canitano et al., 2002; Linck et al., 1998) and at least four genes, *nckx1*, *nckx2*, *nckx3* and *nckx4* code for the NCKX (Kiedrowski et al., 2002; Kip et al., 2006). NCX is found in almost every type of cell; while NCKX1 was originally identified in retinal cells and NCKX2–4 were later found to be expressed in the brain. It has been shown that the expression profiles of these isoforms change during brain development (Lytton et al., 2002; Papa et al., 2003).

The exchangers act in a reversible and electrogenic way with a stoichiometry of 3 Na^+ in exchange for 1 Ca^{2+} for the NCX system and 4 Na^+ in exchange for 1 Ca^{2+} and 1 K^+ for the NCKX system (Blaustein and Lederer, 1999). The reversal potential of the exchangers under physiological conditions is about +100 mV, much more positive than the resting potential. Therefore, the exchangers usually work in the forward mode to remove Ca^{2+} from the cell. It is possible that on stimulation, because the opening of the ionotropic receptors and membrane depolarization can cause the collapse of the cell Na^+ and K^+ gradients, there is an induction of the exchangers to function in the reverse mode (rNC(K)X) in order to bring Ca^{2+} into the cell (Schnetkamp, 2004). It has been shown that Ca^{2+} influx through the rNCX in trout atrial cardiomyocyte elicits Ca^{2+} release from sarcoplasmic reticulum and activates contraction (Hove-Madsen et al., 2003). However, the functions of this Ca^{2+} influx elicited by reverse mode exchange activity in modulating Ca^{2+} signaling in neurons are not very clear.

The importance of Na^+/Ca^{2+} exchangers in the regulation of the physiological and pathological functions of the central nervous system has been widely recognized. In this study, we used primary cultured embryonic cortical neurons as a model system to study the expression profile of the two gene families *ncx* and *nckx*. Furthermore, we explored the possible roles of reverse mode exchange in neurotransmission. Our results show that expression of the *ncx* and *nckx* isoforms changes with the time that the cortical neurons have been in culture. Further, we found that ionotropic α-amino-5-hydroxy-3-methyl-4-isoxazole propionic acid (AMPA) receptor-induced Na^+ influx led to the activation of rNC(K)X systems and triggered the release of Ca^{2+} from intracellular Ca^{2+} stores to enhance $[Ca^{2+}]_i$ elevation and subsequently glutamate release at the axon terminal. These results suggest that, in addition to electric action potential firing, the activation of the rNC(K)X systems provides another pathway whereby there is triggering of neurotransmitter release, which then activates the postsynaptic neurons. For neurons in a network structure, the Na^+/Ca^{2+} exchangers may modulate neurotransmission by shaping the Ca^{2+} responses.

2. Results

2.1. *NCX and NCKX are differentially expressed in cultured cortical neurons*

To examine the expression of the various *ncx* and *nckx* isoforms in cultured E14.5 cortical neurons, specific primer sets (Table 1) against each isoform were used to amplify cDNA from the mRNA isolated from freshly isolated cortical neurons or neurons at 4, 6, 8 and 14 D.I.V (days *in vitro*)(Fig. 1). The results showed that *ncx1*, *ncx3*, *nckx2*, *nckx3* and *nckx4* genes were constitutively expressed in freshly isolated and cultured cortical

Table 1 – Primers for RT-PCR

Gene	Primer[1]	GI number	Expected size (bp)
NCX1	F: GGGAGGACTTTGAGGACACCTG	78214330	461
	R: GAGGGCCAGGTTCGTCTTCTTA	288229	352
NCX 2	F: TGGTGGTGTGCACTACGAGGAT	17530966	382
	R: AAACTGCTCCCTCCATGAGTGG		
NCX 3	F: ACAGTAGAAGGAACAGCCAAGGGT	17530968	435
	R: TCCTGCTGCACTAACAGTGATGG		
NCKX1	F: TCTGTCCTTGGAATGGCCTGA	9910551	265
	R: AATTGAAGTGCCTGCCGCTAG		
NCKX2	F: ATCTTGGCAGCTGGAACCTCTATC	13994178	313
	R: ACGCGAAGTAGAGGCCAAACAT		
NCKX3	F: CCAGCCTCATTGTAGCCAGACA	17160891	370
	R: GCAGGTGACCTGGTCAATCATTAG		
NCKX4	F: TGGCAGTCTCTAACACCATCGG	26024350	428
	R: AAGAGTTGACAGTGGCTGCCAA		

Primers are in 5′ to 3′ direction; F indicates the forward primer and R indicates the reverse primer.

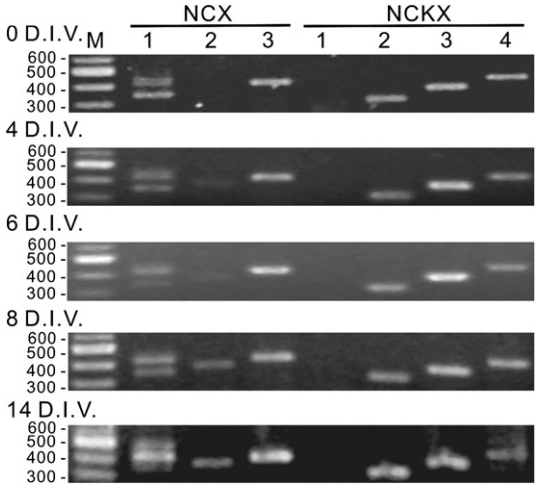

Fig. 1 – Expression of *ncx* and *nckx* transcripts during maturation of cortical neurons *in vitro*. Specific primers against each NCX and NCKX isoform were used to amplify the cDNA synthesized from mRNA isolated from cortical neurons freshly isolated (0 D.I.V.), at 4, 6, 8 and 14 D.I.V. as indicated. M: 100 bp ladder marker.

neurons. However, the expression of *ncx2* was barely detectable in cortical neurons before 6 D.I.V., but became comparable to other transcripts after 8 D.I.V. The primer set for *ncx1* was designed to include the alternative splicing region and two dominant bands with predicted sizes of 461 and 352 bp were identified. These results suggest that the isoforms of *ncx* and *nckx* are expressed differentially during the maturation of cultured neurons.

2.2. K^+-dependence of reverse mode exchange activity changes with time in culture

To activate the reverse mode Na^+/Ca^{2+} exchange activity, neurons were first treated with ouabain, an inhibitor of Na^+/K^+-ATPase, to elevate the intracellular Na^+ concentration and then the extracellular Na^+ was replaced with *N*-methyl-D-glucamine (NMG) to reverse the Na^+ gradient (Pan and Kao, 1997). To examine the K^+ dependence of exchanger activities in cultured cortical neurons, neurons were locally perfused with NMG solution without K^+ for 60 s; the neurons were then allowed to rest for 180 s before perfusion again with NMG solution containing K^+ for another 60 s (Fig. 2). The results showed that for neurons at 6 D.I.V., the $[Ca^{2+}]_i$ elevation was small in the absence of extracellular K^+; however, the elevation was greatly enhanced by the presence of extracellular K^+ when recorded from the same neuron with there being an average increase from 27±9 nM to 318±103 nM (Fig. 2B). This was followed by a slow recovery to the basal level in about 4 min. In contrast, for neurons at 10 D.I.V., the elevation in $[Ca^{2+}]_i$ was similar for the two situations and averaged 140±73 and 175±73 nM, respectively, in the absence or presence of K^+. These results indicate that both K^+-dependent and K^+-independent exchange activity can be identified in the same neurons and that the K^+-dependence of the exchanger activity become more significant in neurons after 10 D.I.V.

2.3. Intracellular Ca^{2+} stores are involved in the $[Ca^{2+}]_i$ elevation elicited by rNC(K)X

Our previous results in bovine chromaffin cells have shown that $[Ca^{2+}]_i$ elevation elicited by rNC(K)X is mostly due to the release of Ca^{2+} from intracellular Ca^{2+} stores (Pan et al., 2006). To understand whether a similar mechanism also exists in cultured neurons, neurons at 10 D.I.V. were pretreated with ryanodine (Ry) to block Ca^{2+} release through the Ry receptors (RyR) and thapsigargin (Tg) to deplete the intracellular Ca^{2+} stores (Fig. 3). The results showed that the elevation in $[Ca^{2+}]_i$ elicited by NMG perfusion was decreased by Ry and Tg pretreatment from an average of 601±59 to 323±33 and 184±31 nM, respectively. There was no difference in basal $[Ca^{2+}]_i$ before stimulation. Cyclopiazonic acid (CPA, 10 μM), another inhibitor of the sarcoplasmic reticulum Ca^{2+}-pump, inhibited the $[Ca^{2+}]_i$ response by 55±8%. These results suggest that most of the elevation in $[Ca^{2+}]_i$ elicited by rNC(K)X is contributed by the release of Ca^{2+} from intracellular Ca^{2+} stores.

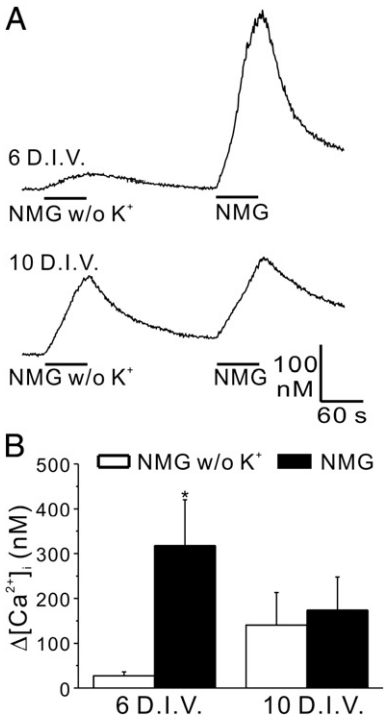

Fig. 2 – Changes in the K^+-dependence of reverse mode exchange activities. Neurons were treated with ouabain (100 μM) for 1 h and $[Ca^{2+}]_i$ was monitored by fura-2 fluorescence. The single neuron was first stimulated for 60 s by K^+-free NMG buffer (NMG w/o K^+); after resting for 180 s, the cell was stimulated by K^+-containing NMG buffer (NMG) for another 60 s as indicated. A. Representative traces of $[Ca^{2+}]_i$ recorded from the neurons at 6 D.I.V. (upper trace) or at 10 D.I.V. (lower trace). B. Averaged changes in $[Ca^{2+}]_i$. The $[Ca^{2+}]_i$ elevations from each neuron after the basal subtraction were averaged and normalized. Data are mean±SEM from 12 and 16 neurons for 6 D.I.V. and 10 D.I.V, respectively and analyzed by Student's *t*-test. *indicates $p<0.05$ vs. NMG without K^+ treatment.

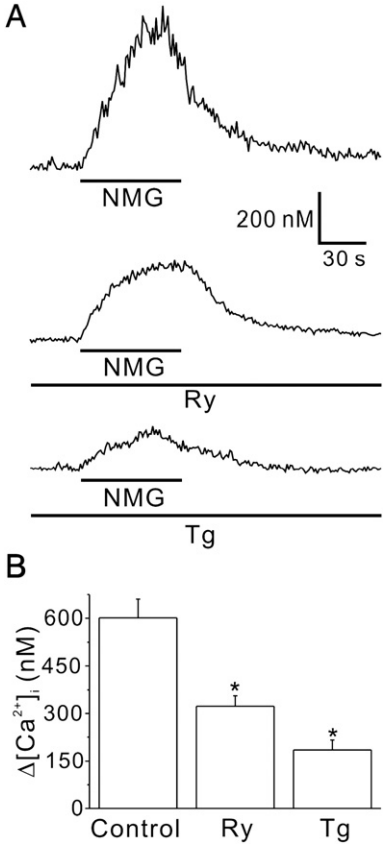

Fig. 3 – $[Ca^{2+}]_i$ elevation is suppressed by blocking Ca^{2+} release from intracellular Ca^{2+} stores. Neurons at 10 D.I.V. were treated with ouabain (100 μM) and loaded with fura-2 for 1 h. Neurons were treated with Tg (Tg, 0.5 μM) or Ry (Ry, 10 μM) and then extracellular Na^+ was replaced by NMG for 60 s as indicated to initiate the reverse mode exchanger. A. Representative $[Ca^{2+}]_i$ responses from neurons without treatment (Top trace) and treated with Ry (middle trace) or Tg (bottom trace). B. Averaged changes in $[Ca^{2+}]_i$. The changes in $[Ca^{2+}]_i$ after Na^+ replacement in each single neuron were averaged and are presented as mean ± SEM from 55, 23 and 60 neurons for the control, Ry and Tg, respectively. The resting $[Ca^{2+}]_i$ was 166 ± 18, 179 ± 18 and 147 ± 15 nM for the control, Ry and Tg, respectively. The data were analyzed by Student's t-test and * indicates $p<0.05$ when comparing to the control group.

2.4. *Both rNCX and rNCKX are present in cortical neurons*

Results from Fig. 2 show that NCKX activity became more significant in neurons 10 D.I.V. We further examined the relative contribution of rNCX and rNCKX to the NMG-induced $[Ca^{2+}]_i$ increase in these neurons. It is possible that the first stimulation by K^+-free NMG buffer might consume part of the Na^+ accumulated in the cytosol and lower the $[Ca^{2+}]_i$ rise elicited by a second stimulation. The activity of rNCX and rNCKX were therefore measured in different sets of neurons. Neurons at 10 D.I.V. pretreated with or without Tg were individually stimulated in the presence of KB-R7943, an rNCX inhibitor (Iwamoto and Shigekawa, 1998) or in the absence of extracellular K^+ (Fig. 4). The $[Ca^{2+}]_i$ was greatly elevated when stimulated by normal NMG buffer to an average of 589 ± 94 nM, while in the absence of extracellular K^+, the $[Ca^{2+}]_i$ increase was lower, 313 ± 44 nM (Fig. 4A and C). In the presence of KB-R7943, the elevation in $[Ca^{2+}]_i$

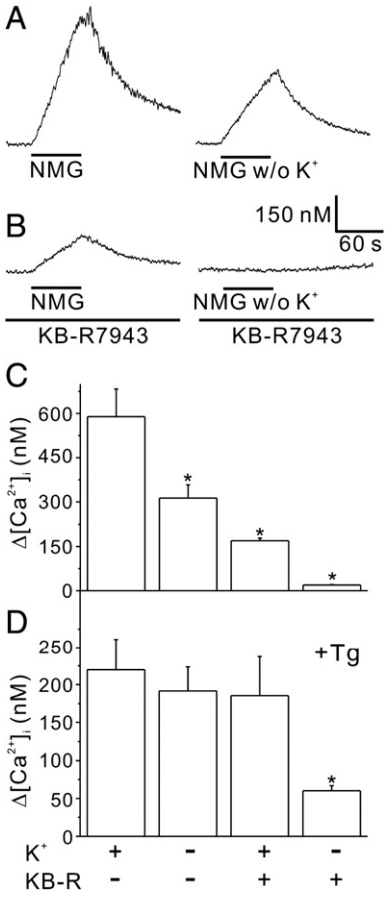

Fig. 4 – $[Ca^{2+}]_i$ elevations in cultured cortical neurons are elicited by both rNCX and rNCKX. Neurons at 10 D.I.V. were treated with ouabain (100 μM) for 1 h and $[Ca^{2+}]_i$ was monitored by fura-2 fluorescence. A. Representative $[Ca^{2+}]_i$ responses from single neurons stimulated by K^+-containing (NMG) or K^+-free (NMG w/o K^+) NMG buffer for 60 s as indicated. B. Representative $[Ca^{2+}]_i$ traces from neurons stimulated by the same protocol as in A in the presence of KB-R7943 (10 μM). For the cells stimulated with buffers without K^+, there was no K^+ in the bath buffer. C. Changes in $[Ca^{2+}]_i$ elicited by different buffers. The changes in $[Ca^{2+}]_i$ after Na^+ replacement in single neurons were averaged and are presented as mean ± SEM from 31, 20, 37 and 11 neurons treated with NMG, NMG w/o K^+, NMG + KB-R7943 (KB-R) and NMG w/o K^+ + KB-R buffers, respectively. The averaged basal $[Ca^{2+}]_i$ before stimulation for NMG, NMG w/o K^+, NMG + KB-R and NMG w/o K^+ + KB-R were 145 ± 15, 90 ± 9, 130 ± 12 and 82 ± 15 nM, respectively. D. Changes in $[Ca^{2+}]_i$ elicited by different buffers after Tg treatment. The changes in $[Ca^{2+}]_i$ after Tg treatment were averaged and presented as mean ± SEM from 10, 7, 7 and 8 neurons stimulated by NMG, NMG w/o K^+, NMG + KB-R and NMG w/o K^+ + KB-R buffers; the averaged basal $[Ca^{2+}]_i$ before stimulation were 128 ± 10, 154 ± 9, 147 ± 16 and 121 ± 8 nM, respectively. The data were analyzed by Student's t-test and * indicates $p<0.05$ vs. NMG group. The scales are different in C and D.

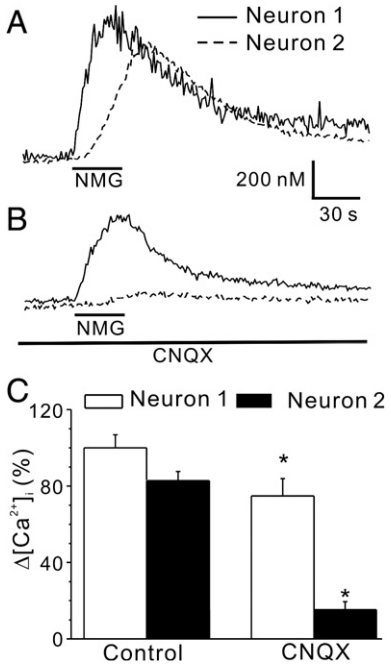

Fig. 5 – Activation of rNC(K)X triggers neurotransmitter release. Neurons at 10 D.I.V. were treated with ouabain (100 μM) for 1 h and the $[Ca^{2+}]_i$ was measured by fura-2 fluorescence. NMG buffer was puffed onto the soma of a neuron (neuron 1) for 60 s as indicated; the $[Ca^{2+}]_i$ response in the soma of neuron 1 and a nearby neuron (neuron 2), which was located at least 200 μM away on the opposite direction of perfusion, was recorded. A and B. Representative $[Ca^{2+}]_i$ responses from two pairs of neuron 1 (solid line) and neuron 2 (dashed line) in the absence (A) or presence (B) of CNQX (10 μM). C. Normalized changes in $[Ca^{2+}]_i$. The averaged changes in $[Ca^{2+}]_i$ after NMG application in each single neuron were normalized to the averaged value of neuron 1. Data are mean ± SEM from 20, 10, 50 and 29 neurons for neuron 1, neuron 1 + CNQX, neuron 2 and neuron 2 + CNQX, respectively and analyzed by Student's t-test. *indicates $p < 0.05$ when comparing to neurons without CNQX treatment.

induced by NMG buffer was decreased to 169 ± 9 nM and there was no significant elevation in the absence of extracellular K^+ (Fig. 4B and C). To ensure that there was no interference by K^+ from the bath solution among the neurons stimulated with NMG without K^+, the neurons were incubated in a bath solution containing no K^+. The basal $[Ca^{2+}]_i$ in buffer containing K^+ was higher than that in the absence of K^+. However, there was no significant effect of the resting $[Ca^{2+}]_i$ level on the transient $[Ca^{2+}]_i$ rise ($t = 1.58$, at 90% confidence level under ordinary least square method).

In neurons that have been pretreated with Tg, the averaged $[Ca^{2+}]_i$ elevations stimulated with NMG, which include both rNCX and rNCKX activity, were significantly lower than that without Tg pretreatment as that shown in Fig. 3. The effects of Tg treatment on rNCX and rNCKX activity were then examined separately (Fig. 4D). In the absence of K^+, approximately 40% of the NMG-induced $[Ca^{2+}]_i$ elevation was inhibited by Tg; while in the presence of KB-R7943, the NMG-induced $[Ca^{2+}]_i$ elevation was not significantly affected by Tg treatment (Fig. 4C vs. D). Furthermore, after Tg treatment, the NMG-induced $[Ca^{2+}]_i$ rise was similar at about 220 nM, except when both rNCX and rNCKX activities were inhibited in the absence of K^+ and in the presence of KB-R7943. The time required to reach the maximum became longer after NMG perfusion in the absence of K^+ or in the presence of KB-R7943; this is presumably because only rNCX or rNCKX, respectively, was functional (data not shown). These results suggest that NCX interacts with the ER more closely than NCKX.

2.5. *Reverse Na^+/Ca^{2+} exchange causes neurotransmitter release*

The cultured neurons contact each other to form functional synapses (Lesuisse and Martin, 2002), which can be verified by paired patch-clamp recording on two nearby neurons in our system (data not shown). To characterize the roles of rNC(K)X in neurotransmitter release at the axon terminal, the exchangers in the soma of one neuron (10 D.I.V.) were activated and changes in $[Ca^{2+}]_i$ in nearby neurons were recorded. Fig. 5A shows the $[Ca^{2+}]_i$ response recorded from a pair of ouabain-treated neurons; when the target neuron (neuron 1) was puffed with NMG solution, the $[Ca^{2+}]_i$ was elevated by activation of rNC(K)X. After a time lag, the $[Ca^{2+}]_i$ in the nearby neuron (neuron 2) was also elevated. The $[Ca^{2+}]_i$ elevations in nearby neurons were significantly reduced by 72% in the presence of 6-cyano-7-nitroquinoxaline-2, 3-dione (CNQX), an antagonist of the AMPA receptor. These results suggest that the $[Ca^{2+}]_i$ elevation elicited by rNC(K)X in the first neuron is able to propagate to the terminal of that neuron and evokes a release of a neurotransmitter to activate the first neuron's postsynaptic neurons.

2.6. *An action potential is not involved in transmitting the rNC(K)X-evoked Ca^{2+} signaling*

To further characterize how the Ca^{2+} signaling evoked by rNC(K)X was transmitted to the nearby neurons, the neurons at 10 D.I.V.

Table 2 – Normalized changes in $[Ca^{2+}]_i$ in connected neurons evoked by NMG solution

	Control		Treatment	
	Neuron1	Neuron2	Neuron1	Neuron2
AP5	100.0 ± 19.9 (n = 13)	80.3 ± 10.5 (n = 52)	79.7 ± 27.5 (n = 8)	88.2 ± 12.4 (n = 29)
TTX	100.0 ± 19.9 (n = 13)	80.3 ± 10.5 (n = 52)	73.7 ± 22.7 (n = 8)	78.4 ± 15.7 (n = 24)
TBOA	100.0 ± 16.6 (n = 10)	84.7 ± 7.3 (n = 54)	105.2 ± 14.9 (n = 8)	96.2 ± 7.1* (n = 45)
Tetanus toxin	100.0 ± 14.8 (n = 15)	67.1 ± 6.8 (n = 59)	111.5 ± 3.6 (n = 16)	30.1 ± 5.1* (n = 85)

Neurons at 10 D.I.V. were treated with ouabain (100 μM) for 1 h and the $[Ca^{2+}]_i$ response elicited by Na^+ replacement was measured by fura-2 fluorescence. AP5, TTX and TBOA were added 15 min before the start of the experiments; tetanus toxin was added 3 h in advance. Neurons treated with AP5 or TTX shared the same control group. The datasets were normalized against the averaged $[Ca^{2+}]_i$ changes in neuron 1 of the control group and are presented as mean ± SEM. *$p < 0.05$ compared with the control cells by Student's t-test.

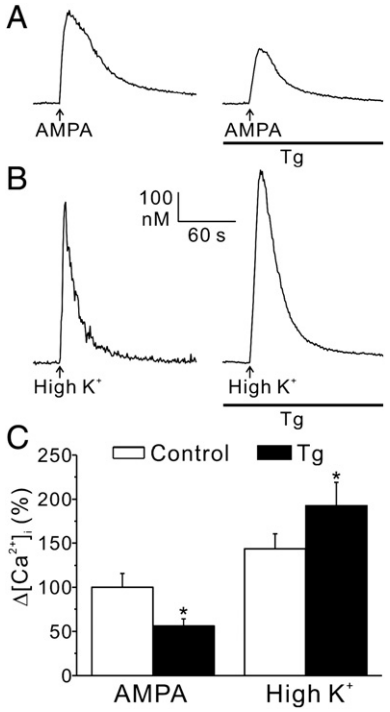

Fig. 6 – Tg inhibits AMPA-elicited elevation in $[Ca^{2+}]_i$. Neurons at 10 D.I.V. were incubated in normal bath buffer and locally perfused with AMPA (10 μM) or high-K^+ buffer for 3 s as indicated by the arrows. A. Representative $[Ca^{2+}]_i$ traces from neurons stimulated by AMPA pretreated without (left trace) and with (right trace) Tg (0.5 μM). B. Representative $[Ca^{2+}]_i$ traces from neurons stimulated by high-K^+ buffer pretreated without (left trace) and with (right trace) Tg (0.5 μM). C. Averaged changes in $[Ca^{2+}]_i$. The changes in $[Ca^{2+}]_i$ after baseline subtraction of each treatment were averaged and normalized to cells stimulated with AMPA. Data are mean ± SEM from 23 and 19; 28 and 21 neurons for AMPA stimulation without and with Tg and high-K^+ buffer stimulation without and with Tg, respectively. The results were analyzed by Student's *t*-test and * indicates $p < 0.05$.

were pretreated with D-2-amino-5-phosphonopentanoate (AP5), tetrodotoxin (TTX), DL-threo-β-benzyloxyaspartate (TBOA) and tetanus toxin to inhibit the cell's NMDA receptors (Levy et al., 2006), Na^+ channels (Jaekel et al., 2006), Na^+/glutamate cotransporters (Lalo et al., 2006) and synaptic vesicle fusion (Verderio et al., 1999), respectively. The results (Table 2) show that in control pairs of neurons, when the rNC(K)X activities in neuron 1 were activated, the elevation in $[Ca^{2+}]_i$ in neuron 2 were about 70–80% of that in neuron 1. On treatment with AP5 and TTX, the $[Ca^{2+}]_i$ elevations in neuron 1 were slightly inhibited by about 25%; however, the $[Ca^{2+}]_i$ elevation in neuron 2 was not significantly affected and this was elevated to a level comparable to that of the control neuron 2. These results suggest that the transmitted Ca^{2+} signaling evoked by rNC(K)X in the nearby neurons is not dependent on the firing of an action potential and the activation of NMDA receptors. When treated with TBOA, the $[Ca^{2+}]_i$ responses of neuron 1 were similar to that of control neuron 1; furthermore, the elevation in neuron 2 was significantly higher than that of control neuron 2. This is what would be expected from TBOA inhibition of glutamate reuptake. In contrast, tetanus toxin greatly inhibited the $[Ca^{2+}]_i$ elevation in neuron 2 and the $[Ca^{2+}]_i$ rise was less than half that of the control neuron 2. These results indicate that glutamate is released at the synaptic terminal by the activation of rNC(K)X at the soma.

2.7. *Intracellular Ca^{2+} stores contribute to AMPA-stimulated $[Ca^{2+}]_i$ elevation*

To examine whether the influx of Na^+ through AMPA receptor activates rNC(K)X, neurons at 10 D.I.V. were stimulated by AMPA in normal bath buffer to allow Na^+ influx through the AMPA receptor; this depolarizes the cell and then opens the voltage-gated Ca^{2+} channels. The $[Ca^{2+}]_i$ was elevated quickly to an average of 923 ± 143 nM by AMPA stimulation (Fig. 6); this elevation in $[Ca^{2+}]_i$ elicited by AMPA needs both extracellular Na^+ and Ca^{2+} (data not shown). However, if the neurons were pretreated with Tg, 44% of the elevation was inhibited. For comparison, neurons were depolarized by high-K^+ buffer to open the voltage-gated Ca^{2+} channels without an influx of Na^+

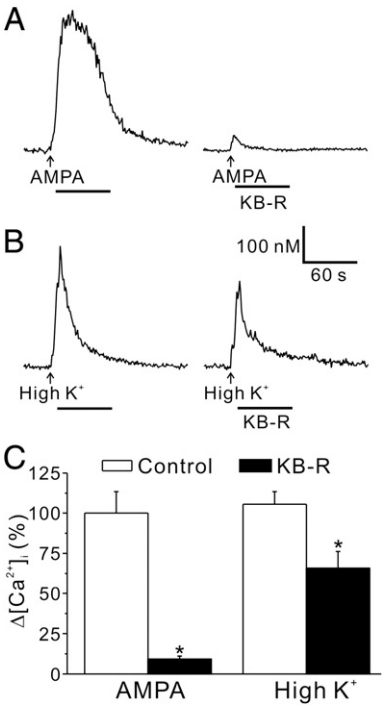

Fig. 7 – KB-R7943 inhibits AMPA-elicited elevation in $[Ca^{2+}]_i$. Neurons at 10 D.I.V. were incubated in normal Ca^{2+}-containing bath buffer and locally perfused with 10 μM AMPA in Ca^{2+}-free buffer (A) or Ca^{2+}-free high-K^+ buffer (B) for 3 s (arrow), then this was immediately followed by a normal Ca^{2+}-containing bath buffer with (right trace) or without (left trace) 10 μM KB-R7943 for 60 s (black bar). C. Normalized changes in $[Ca^{2+}]_i$. The increase in $[Ca^{2+}]_i$ of the neurons was averaged from 24 and 16 neurons for AMPA stimulation without and with KB-R7943; and 38 and 29 neurons for high-K^+ stimulation without and with KB-R7943, respectively. Data shown are mean ± SEM and normalized to that stimulated with AMPA with KB-R7943 treatment. *$p < 0.05$ by Student's *t*-test.

through the AMPA receptor. The $[Ca^{2+}]_i$ elevation by high-K^+ buffer was 43% higher than that of AMPA stimulation and Tg pretreatment significantly enhanced this elevation by 34% (Fig. 6C). These results suggest that intracellular Ca^{2+} stores are involved in enhancing $[Ca^{2+}]_i$ elevation when a Na^+ flux enters the neuron and this is due to the opening of the ionotropic AMPA receptors.

2.8. KB-R7943 inhibits $[Ca^{2+}]_i$ elevation after AMPA stimulation

To further investigate whether rNC(K)X is activated by the Na^+ influx through the ionotropic glutamate receptors, KB-R7943 was puffed onto the neurons after AMPA stimulation (Fig. 7). Neurons at 10 D.I.V. were first stimulated by AMPA or high-K^+ in a Ca^{2+}-free bath buffer (to minimize Ca^{2+} influx during stimulation) for 3 s and this was followed by normal Ca^{2+}-containing buffer with or without KB-R7943 for another 60 s (to minimize the side effects of KB-R7943 on the AMPA receptors). In the presence of KB-R7943, the $[Ca^{2+}]_i$ elevation after AMPA stimulation was decreased by 91%; while the high-K^+ stimulated $[Ca^{2+}]_i$ elevation was only inhibited by 34%. The small inhibitory effect of KB-R7943 on the high-K^+-induced responses may be explained by the non-specific effects of KB-R7943 on the Ca^{2+} channels (Birinyi et al., 2005). Furthermore, the $[Ca^{2+}]_i$ elevation elicited by AMPA in neuron 1 (100 ± 20%) was able to propagate to the axon terminal to activate $[Ca^{2+}]_i$ elevation in neuron 2 (67 ± 16%). However, this propagation was greatly suppressed by the addition of KB-R7943 down to 12% ± 2% and 11% ± 1% in neuron 1 and neuron 2, respectively. These results support the hypothesis that rNCX is involved in the AMPA-stimulated $[Ca^{2+}]_i$ rise.

3. Discussion

This report shows that the *ncx* and *nckx* isoforms are differentially expressed in cultured rat E14.5 cortical neurons. The exchange activity shifts from K^+-dependent to both K^+-dependent and K^+-independent as the neurons aged from 6 D.I.V. to 10 D.I.V. We have also demonstrated that the $[Ca^{2+}]_i$ elevation elicited by rNC(K)X is enhanced by Ca^{2+} released from the intracellular Ca^{2+} stores and this can propagate to the terminal and trigger neurotransmitter release. Furthermore, $[Ca^{2+}]_i$ elevation elicited by the Na^+ influx through the AMPA receptors is mediated by activation of rNC(K)X. These results indicate the importance of cooperation between rNC(K)X and the intracellular Ca^{2+} stores when modulating Ca^{2+} signaling and neurotransmitter release.

We found that both the expression and activity of the exchanger isoforms found in the cortical neurons changed in the cultured neurons. *Ncx2* appeared in neurons after 6 D.I.V. while the expression of the other *ncx* and *nckx* isoforms did not show a significant change. For neurons at 6 D.I.V., the increase in $[Ca^{2+}]_i$ induced in the presence of extracellular K^+ (~291 nM, Fig. 2) is comparable to that inhibited by K^+-free NMG buffer in neurons at 10 D.I.V. (~277 nM, Fig. 4). This hints that rNCKXs' activity and expression level do not change during neuron maturation. In contrast, KB-R7943 inhibited the $[Ca^{2+}]_i$ elevation elicited by NMG solution containing K^+ by 36% (data not shown, no statistical significance compared to control cells) and 71% (Fig. 4) in neurons at 6 and 10 D.I.V., respectively. This further demonstrates that the K^+ dependence of the exchange activities changed during culture. The increase in rNCX activities may be explained by the expression of *ncx2*. Further experiments are required to specifically characterize the contribution of each individual exchanger isoform to Ca^{2+} signaling.

Differential expression of the exchangers in different tissues and at different developmental stages has been reported previously. For example, in cultured rat E18 hippocampal neurons, *ncx2* and *nckx4* are not detectable until 4 D.I.V. (Kip et al., 2006) and in new-born rat hippocampus, all three *ncx* genes can be detected at the transcription level (Li et al., 2002; Yu and Colvin, 1997). In the adult rat brain, the various different isoforms of NCX are selectively expressed in different CNS areas, which may underlie their different functions (Canitano et al., 2002). Furthermore, NCX2, but not NCX1, is upregulated during cerebellum development and downregulated in partially depolarized cultured granule neurons (Li et al., 2000); however, during ischemia, only NCX1 is significantly decreased (Li et al., 2006). Taken together, different NC(K)X isoforms including the alternative splicing subtypes would seem to be differentially expressed during brain development and the functions of these exchangers in modulating Ca^{2+} signaling may thus change accordingly. Our results suggest the differential expression of the exchanger isoforms in neurons after different time in culture; however, the correlation between the changes in K^+-dependence and the changes in expression profile needs to be further elucidated.

Our results demonstrate that the $[Ca^{2+}]_i$ elevation elicited by rNC(K)X was inhibited by blocking the release of Ca^{2+} from intracellular Ca^{2+} stores. It is possible, due to the close proximity of the exchangers and Ca^{2+} stores, that the influx of Ca^{2+} activates the RyR on the stores to evoke Ca^{2+}-induced Ca^{2+} release. The presence of NCX in the plasma membrane has been reported to be in the form of a lace-like pattern that is close to the ER in neurons and astrocyte cells (Blaustein et al., 2002). Indeed, it has been shown that activation of the reverse mode exchange activity induces the release of Ca^{2+} from intracellular Ca^{2+} stores in cardiac and chromaffin cells (Levi et al., 1994; Pan et al., 2006; Ritter et al., 2003) and that NC(K)X, ER and mitochondria work cooperatively to modulate $[Ca^{2+}]_i$ and exocytosis in chromaffin cells (Yang and Kao, 2001). In the mouse neocortical preplate, the activation of the Na^+ channels with veratridine leads to the activation of rNC(K)X and an elevation of $[Ca^{2+}]_i$. This elevation can be attenuated by Tg treatment (Platel et al., 2005). Therefore, it is conceivable that the Ca^{2+} released from the Tg-sensitive Ca^{2+} stores enhances the $[Ca^{2+}]_i$ rise elicited by rNC(K)X in cultured neurons.

The $[Ca^{2+}]_i$ rise stimulated by AMPA is reduced in the absence of the ER Ca^{2+} store, while the high-K^+-induced $[Ca^{2+}]_i$ response was significantly elevated (Fig. 6). The opening of the AMPA receptors allow the influx of Na^+, which then depolarizes the cell to activate the voltage-gated Ca^{2+} channels; high-K^+ buffer stimulation leads directly to the opening of voltage-gated Ca^{2+} channels. It appears that the ER acts as a Ca^{2+} sink or source depending on the pathway of Ca^{2+} entry; specifically, it acts as a Ca^{2+} source for the $[Ca^{2+}]_i$ response induced by rNC(K)X, but as a sink when induced by high-K^+. These results also exclude the possibility that the Ca^{2+} taken up by the intracellular Ca^{2+} stores overloads the capacity of the stores and activates Ca^{2+}-releasing channels from lumen (Bezprozvanny and Ehrlich, 1994). However, the

possibility that high $[Ca^{2+}]_i$ inhibits the opening probability of the Ca^{2+}-releasing channels in the ER (Fabiato, 1992) cannot be excluded.

If the involvement of the ER Ca^{2+} stores in the rNC(K)X-induced $[Ca^{2+}]_i$ elevation is considered, the relatively low level of rNCX activity in neurons at 6 D.I.V. may be accounted for by the absence of an interaction between NCX and the ER. It is possible that both types of exchangers are already expressed in both young and old neurons (Fig. 1), but the ability of rNCX to trigger the release of Ca^{2+} from intracellular Ca^{2+} stores is not established until 10 D.I.V. After Tg treatment, rNCX and rNCKX then contribute similarly to the elevation of $[Ca^{2+}]_i$ in neurons at 10 D.I.V. (Fig. 4D); however, without Tg treatment, the activation of rNCX caused a higher increase in $[Ca^{2+}]_i$ than rNC(K)X (Fig. 4C and D). It is possible that rNCX has a stronger linkage to the ER Ca^{2+} stores than rNCKX.

Our results show that when $[Ca^{2+}]_i$ in the soma of a neuron was elevated by the activation of rNC(K)X, the $[Ca^{2+}]_i$ in nearby neurons were also elevated after a time lag. This elevation in nearby neurons could be almost completely suppressed by CNQX but not by AP5, which indicates that glutamate is released from the axon terminal to activate the AMPA receptors on postsynaptic neurons (Table 2 and Fig. 5). The inhibitory effect of CNQX makes it unlikely that the $[Ca^{2+}]_i$ elevation in the nearby neurons is due to diffusion of NMG buffer; this is because neuron 2 was selected to be located at least 200 μM from the target neuron and in the opposite direction to the perfusion pipette. The contribution of action potential firing in the target neuron is also excluded because TTX had no significant effect on the transmission of the signals between the two neurons. Although the elevation in $[Ca^{2+}]_i$ in neuron 1 was slightly inhibited by the various drugs used and this may be due to the self innervation (Fig. 5 and Table 2), the $[Ca^{2+}]_i$ level was still high enough to trigger the $[Ca^{2+}]_i$ elevation in nearby neurons.

The protocol used in this study to establish the Na^+ gradient may also reverse Na^+/glutamate cotransporters and this will release glutamate into the synaptic cleft and induce the $[Ca^{2+}]_i$ elevation in the nearby neurons. By blocking the Na^+/glutamate cotransporters with TBOA (Table 2), the elevation in $[Ca^{2+}]_i$ in neuron 2 was found not to be inhibited but was significantly enhanced. Thus, the signal transmission between the two neurons is not due to the glutamate released through cotransporters. In the presence of TBOA, the released glutamate stays at the synaptic cleft for a longer time and prolonged the activation of the glutamate receptors (Tsukada et al., 2005). In contrast, tetanus toxin pretreatment significantly inhibited the elevation in postsynaptic neurons and this strongly supports the occurrence of synaptic vesicle fusion to the plasma membrane at the nerve terminal that had been evoked by rNC(K)X activity. Applied under the same conditions, tetanus toxin was able to inhibit the elevations in $[Ca^{2+}]_i$ in postsynaptic neuron 2 when neuron 1 was depolarized by high-K^+ buffer (data not shown). This propagation of $[Ca^{2+}]_i$ elevation elicited by rNC(K)X may be another pathway for Ca^{2+} signaling to facilitate the neuron network.

Our results support the hypothesis that stimulation of the AMPA receptors allows the influx of Na^+ and causes membrane depolarization and the activation of rNC(K)X (Kim et al., 2005). The elevation in $[Ca^{2+}]_i$ elicited by AMPA was almost completely inhibited by a subsequent application of KB-R7943, which suggests that this inhibition was primarily on rNCX activity. Furthermore, Tg treatment decreased the elevation in $[Ca^{2+}]_i$ stimulated by AMPA but increased the elevation by high-K^+ buffer, which suggests that there is a requirement for the intracellular Ca^{2+} stores to support the AMPA-elicited $[Ca^{2+}]_i$ elevation. It has also been suggested recently that the elevation in $[Ca^{2+}]_i$ induced by Na^+ influx through the glutamate receptors may be partly mediated by rNC(K)X in CA1 (Kiedrowski, 2007). These results indicate that the activation of rNC(K)X activity after the opening of the AMPA receptors and the release of Ca^{2+} from intracellular Ca^{2+} stores is needed to maintain the Ca^{2+} signaling.

The results show that the basal $[Ca^{2+}]_i$ in the neurons treated with Tg or Ry for 1 h were not significantly different from that of the control cells (Fig. 4), which suggests that store-operated Ca^{2+} entry may not be involved at this time point. However, when applied 5 min after CPA treatment, KB-R7943 has been shown to inhibit the sustaining phase of the $[Ca^{2+}]_i$ response due to the activation of store-operated Ca^{2+} entry in neurons (Arakawa et al., 2000). Similarly, TRPC currents have also been reported to be inhibited by KB-R7943 (Kraft, 2007). In neurons at 6 D.I.V., the rNC(K)X-elicited $[Ca^{2+}]_i$ elevation was slightly inhibited by KB-R7943 by 22±14% and 36±16% when applied at 1 and 10 μM, respectively, but this difference is not statistical significant (data not shown). Therefore, it is unlikely that the inhibitory effect of KB-R7943 on the rNC(K)X-elicited $[Ca^{2+}]_i$ elevation shown in this study is due to non-specific effects on store-operated Ca^{2+} entry. We have also used another inhibitor of NCX, SEA0400, and the results showed that at 10 μM, there is a 50% inhibition of the rNCX activity in neurons 10 D.I.V. (data not shown). It is known that SEA0400 inhibits mainly NCX1 but not the other NCX isoforms at concentrations lower than 10 μM (Iwamoto, 2004). Earlier, we have shown that there are more than one isoform of NCX expressed in the cultured neurons used in this study (Fig. 1) and, based on this, KB-R7943 was chosen for this study.

This interaction between the exchangers and the ER Ca^{2+} stores may be important for the physiological response of the AMPA receptors. The Na^+ influx induced by AMPA triggers rNCX and causes a $[Ca^{2+}]_i$ elevation that is greatly decreased in the absence of the ER Ca^{2+} stores. Similar results have been obtained

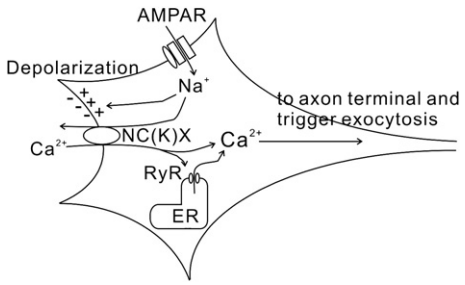

Fig. 8 – Possible roles of rNC(K)X in the neuron. Possible roles of rNC(K)X in the neuron. The binding of glutamate to AMPA receptor (AMPAR) allows the influx of Na^+ causing membrane depolarization. These will activate rNC(K)X and then trigger the release of Ca^{2+} from the ER Ca^{2+} stores through RyR to enhance the $[Ca^{2+}]_i$ elevation. The Ca^{2+} signaling will propagate to the axon terminal and evoke neurotransmitter release.

in cultured forebrain neurons (Hoyt et al., 1998). Here we have further demonstrated that this rNC(K)X-evoked Ca^{2+} signaling, with help from the intracellular Ca^{2+} stores, can propagate toward an axon terminal in parallel with action potential firing to induce neurotransmitter release (Rizzuto and Pozzan, 2006). The reversal potential for NC(K)X is very positive relative to the resting membrane potential; the action potential or changes in the ionic balance that occurs on the opening of the receptor-gated ion channels may cause a transient shift in the reversal potential to 0 mV (Kiedrowski et al., 2004). Therefore, AMPA stimulation activates rNC(K)X when Na^+ fluxes into the cell through the ionotropic glutamate receptors (Fig. 8). The Ca^{2+} transported into the cytosol by rNC(K)X will then trigger the release of Ca^{2+} from the intracellular Ca^{2+} stores to maintain the $[Ca^{2+}]_i$ elevation and this will modulate subsequent neuronal activities. As an addition to electric excitability, rNC(K)X seems to provide another pathway to modulate synaptic transmission through the activation of intracellular Ca^{2+} stores.

4. Experimental procedures

4.1. Chemicals

Tg, CPA, CNQX, AP5, AMPA, Ry and tetanus toxin were obtained from CalBiochem (EMD Biosciences Inc., San Diego, CA, USA). Fura-2 acetomethoxylmethyl ester (Fura-2 AM) was purchased from TefLabs (Austin, TX, USA). KB-R7943 and TBOA were imported from Tocris Cookson Ltd. (Avon, UK). Ca^{2+}, Mg^{2+}-free Hank's Balanced Salt Solution, Neurobasal Medium, B27 and other chemicals for cell culture were bought from Invitrogen Inc. (Carlsbad, CA). All other chemicals were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA) unless otherwise indicated.

4.2. Isolation and culture of rat E14.5 cortical neurons

E14.5 embryos were obtained from pregnant Sprague–Dawley rat by Caesarian section in a procedure that complied with the regulations of the Animal Welfare Regulation of the National Taiwan University. The forebrain was isolated under a dissecting microscope and digested with papain (1 mg/ml in Ca^{2+}, Mg^{2+}-free HBSS) at 37 °C with gentle shaking for 20 min. The tissue was triturated using a 1 ml glass pipette for 20 strokes and centrifuged at 300 ×*g* for 1 min. The pellet was resuspended in Ca^{2+}, Mg^{2+}-free HBSS and adjusted to 10^6 cells/ml. The isolated cells were cultured according to the published protocol (Brewer, 1995). To inhibit the growth of glial cells, cytosine arabinoside (1 μg/ml) was added to the culture medium.

4.3. RT-PCR

Specific primer sets against the various exchanger genes were designed according to the published sequences of the rat *ncx* and *nckx* genes (Table 1). Total RNA of cultured rat embryonic cortical neurons was collected using the Trizol reagent (Life Technology, USA) according to the manufacture's manual. Single strand cDNA was synthesized from the total RNA using oligo-dT as the primer and Superscriptase III (Life Technology, USA). The PCR reactions were performed using a programmed cycle of 94 °C, 30 s, 53 °C, 30 s and 72 °C, 30 s, for 30 cycles.

4.4. $[Ca^{2+}]_i$ imaging

To measure $[Ca^{2+}]_i$, cells were incubated in bath buffer (150 mM NaCl, 5 mM glucose, 10 mM HEPES, 1 mM $MgCl_2$, 5 mM KCl, and 2.2 mM $CaCl_2$; pH 7.3) containing 5 μM fura-2 AM for 1 h at 37 °C. The neurons were then washed 3 times with bath buffer and used for the measurements. For fura-2 excitation, a Lamda DG4 system (Sutter Instrument Co., Novato, CA, USA) was used, which was controlled by Metafluor software (Molecular Devices Corp., Downingtown, PA, USA). Ratiometric calcium estimates were made using 10-nm-wide filters centered on 340 and 380 nM (Chroma Technology Corp., Rockingham, VT, USA), capturing the emitted light (485–540 nm) at each excitation wavelength for 300 ms through a 20× objective (Zeiss Axiovert 200 microscope, Carl Zeiss AG, Germany) and directing it to a cooled CCD camera (CoolsnapFx, Roper Scientific, Tucson, AZ, USA). The ratio within each cell was computed from images obtained at the excitation wavelengths of 340 and 380 nM and by subtracting the appropriate background fluorescence at each wavelength. Ratios were computed every second. A calcium calibration buffer kit (Molecular Probes, Carlsbad, CA, USA) was used to transform the ratio into Ca^{2+} concentration according to manufacturer's manual.

4.5. Measurement of reverse mode exchanger activity

Cells were incubated in bath buffer containing 0.1 mM of ouabain, an inhibitor of the Na^+/K^+-ATPase, to increase the intracellular Na^+ concentration. To elicit rNC(K)X activity, a glass micropipette with an opening of about 3 μM containing NMG buffer (135 mM NMG, 5 mM glucose, 1 mM $MgCl_2$, 5 mM KCl, 2.2 mM $CaCl_2$, and 10 mM HEPES; pH 7.3) was positioned 20 μM from the recorded cell. The buffer was puffed at 5 psi onto the cell when required for 60 s under the control of a Picospritzer III (Parker Instrument, Fairfield, NJ, USA). To measure the responses from nearby neurons, neurons (usually 4–6) located at least 200 μM from the stimulated neuron in the opposite direction to the perfusion were selected.

4.6. Drug treatment

Tg (0.5 μM) and CPA (10 μM) were added into the bath 1 h before the start of an experiment; Ry (10 μM) was added 15 min before the recording. AMPA (10 μM) was dissolved in Ca^{2+}-free bath buffer (bath buffer with no $CaCl_2$ added). To inhibit rNCX, 10 μM of KB-R7943 was added. To depolarize the cell, Ca^{2+}-free high-K^+ buffer (the NaCl in bath buffer was substituted with an equimolar amount of KCl and without adding $CaCl_2$) was used. CNQX (10 μM), AP5 (30 μM), TBOA (100 μM) and TTX (1 μM), were added 15 min before the start of recording and tetanus toxin (30 nM) was added 3 h in advance. The inhibitors were also present during the experiments.

4.7. Data analysis

A calcium calibration buffer kit (Molecular Probes, Carlsbad, CA, USA) was used to transform the ratio of the fura-2 fluorescence into a Ca^{2+} concentration according to manufacture's manual. All data presented are mean ± SEM from at least three experiments and analyzed by Student's *t*-test.

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