Roles of Na^+ /Ca^2+ exchanger isoforms NCX1 and NCX2 in motility in mouse ileum

メタデータ

- 言語: eng
- 出版者: 
- 公開日: 2018-02-20
- キーワード (Ja): 
- キーワード (En): 
- 作成者: Nishiyama, Kazuhiro, Azuma, Yasu-Taka, Morioka, Ai, Yoshida, Natsuho, Teramoto, Midori, Tanioka, Kohta, Kita, Satomi, Hayashi, Satomi, Nakajima, Hidemitsu, Iwamoto, Takahiro, Takeuchi, Tadayoshi
- メールアドレス: 
- 所属: 
- URL: http://hdl.handle.net/10466/15733
Roles of Na\(^+\)/Ca\(^{2+}\) exchanger isoforms NCX1 and NCX2 in motility in mouse ileum

Kazuhiro Nishiyama · Yasu-Taka Azuma* · Ai Morioka · Natsuho Yoshida · Midori Teramoto · Kohta Tanioka · Satomi Kita · Satomi Hayashi · Hidemitsu Nakajima · Takahiro Iwamoto · Tadayoshi Takeuchi

K. Nishiyama · Y.T. Azuma · A. Morioka · N. Yoshida · M. Teramoto · K. Tanioka · S. Hayashi · H. Nakajima · T. Takeuchi

Laboratory of Veterinary Pharmacology, Division of Veterinary Science, Osaka Prefecture University Graduate School of Life and Environmental Science, Osaka 598-8531, Japan

S. Kita · T. Iwamoto

Department of Pharmacology, Faculty of Medicine, Fukuoka University, Fukuoka 814-0180, Japan

*Corresponding author at: Laboratory of Veterinary Pharmacology, Division of Veterinary Science, Osaka Prefecture University Graduate School of Life and Environmental Science, 1-58 Rinku-ohraikita, Izumisano, Osaka 598-8531, Japan. TEL/Fax +81-72-463-5264.

E-mail address: azuma@vet.osakafu-u.ac.jp (Y.T. Azuma).
Abstract

The Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (NCX) is a plasma membrane transporter that is involved in regulating intracellular Ca\textsuperscript{2+} concentrations in various tissues. The physiological roles by which NCX influences gastrointestinal motility are incompletely understood, although its role in the heart, brain and kidney has been widely investigated. In this study, we focused on the functions of the NCX isoforms, NCX1 and NCX2, in the motility of the ileum in the gastrointestinal tract. We investigated the response to electric field stimulation (EFS) in the longitudinal smooth muscle of the ileum obtained from wild-type mice (WT), NCX1-heterozygote knockout mice (NCX1 HET), NCX2 HET and smooth muscle-specific NCX1.3 transgenic mice (NCX1.3 Tg). EFS induced a phasic contraction that persisted during EFS and a tonic contraction that occurred after the end of EFS. We found that the amplitudes of the phasic and tonic contractions were significantly smaller in NCX2 HET, but not in NCX1 HET, compared to WT. Moreover, the magnitudes of acetylcholine (ACh)- and substance P (SP)-induced contractions of NCX2 HET, but not of NCX1 HET, were smaller compared to WT. In contrast, the amplitudes of the phasic and tonic contractions were greater in NCX1.3 Tg compared to WT. Similar to EFS, the magnitude of ACh-induced contraction was greater in NCX1.3 Tg than in WT. Taken together, our findings indicated that NCX1 and NCX2 play important roles in ileal motility and suggest that NCX1 and NCX2 regulate the
motility in the ileum by controlling the sensitivity of smooth muscles to ACh and SP.

**Key words**

Na+/Ca2+ exchanger, ileum, longitudinal smooth muscles, contraction, acetylcholine

**Abbreviations**

ACh  Acetylcholine  
EFS  electrical field stimulation  
HET  heterozygous mice  
ICC  interstitial cells of Cajal  
NCX  Na+/Ca2+ exchanger  
SP  substance P  
NCX1.3 Tg  smooth muscle-specific NCX1.3 transgenic mice  
WT  wild-type
Introduction

Contractions of longitudinal smooth muscles in the mouse ileum are classified as phasic or tonic. Upon electrical field stimulation (EFS), a phasic contraction exhibits a transient and rapid contraction, whereas a tonic contraction consists of a sustained contraction. The different patterns of phasic and tonic contractions can be explained by the differences in their excitatory transmitters, such as acetylcholine (ACh) and substance P (SP), respectively, followed by the activation of their G protein-coupled receptors. Activated receptors result in the contraction of smooth muscles via Ca\(^{2+}\) influx. In gastrointestinal smooth muscles, Ca\(^{2+}\) influx triggers Ca\(^{2+}\) release from the sarcoplasmic reticulum. The combination of Ca\(^{2+}\) influx from extra medium and release from sarcoplasmic reticulum increases the free intracellular Ca\(^{2+}\) concentration, thereby allowing Ca\(^{2+}\) to bind to a calcium binding protein, which then activates myosin light chain kinase (Berridge, 2008; Wang et al., 2008). Over time, intracellular Ca\(^{2+}\) is transported out of the cytosol via pathways involving plasma membrane Ca\(^{2+}\)-ATPase, the Na\(^{+}\)/Ca\(^{2+}\) exchanger (NCX), sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPase and mitochondrial Ca\(^{2+}\) transporters (Wray and Burdyga, 2010; Webb, 2003). However, the potential role of NCX in gastrointestinal tissue is still incompletely understood among these Ca\(^{2+}\) transport pathways.
Our group focused on the effect of Ca$^{2+}$ movement via NCX on gastrointestinal motility because Ca$^{2+}$ homeostasis is central to the regulation of gastrointestinal smooth muscle functions. NCX is a plasma membrane transporter that is involved in the regulation of intracellular Ca$^{2+}$ concentrations in tissues, such as the brain, kidney, and smooth muscle. NCX electrogenically exchanges Na$^{+}$ and Ca$^{2+}$ across the plasma membrane, depending on the membrane potential and transmembrane gradients. The mammalian NCX family consists of three isoforms: NCX1 (Nicoll et al., 1990), NCX2 (Li et al., 1994) and NCX3 (Nicoll et al., 1996). To specifically assess the physiological role of NCX isoforms, we used NCX1 heterozygote knockout mice (HET) (Wakimoto et al., 2000) and NCX2 HET (Gotoh et al., 2015). We previously demonstrated that NCX2 HET, but not NCX1 HET, displays altered motility in the distal colon (Azuma et al., 2012). In addition, we recently demonstrated that NCX1 HET and NCX2 HET display altered motility in the gastric fundus (Azuma et al., 2016a). Currently, little is known about the potential role of NCX isoforms in small intestinal motility. This study aimed to determine the effect of NCX isoforms heterozygous deficiency on the motility of the ileum. To further test whether smooth muscle NCX1 plays a critical role in motility in the ileum, we used transgenic mice expressing canine NCX1.3 driven by the smooth muscle-specific $\alpha$–actin promoter. To better understand the action of NCX isoforms, we used an organ-tissue bath system to characterize the motility of longitudinal muscle strips isolated from the ileum.
Materials and Methods

Drugs

ACh and atropine were purchased from Wako Pure Chemical (Osaka, Japan). An Alexa Fluor 488-labeled goat anti-rabbit IgG was purchased from Molecular Probes Inc. (Eugene, OR, USA). A mouse polyclonal antibody against PGP9.5 was purchased from UltraClone Limited (Isle of Wight, UK). SP was purchased from the Peptide Institute (Osaka, Japan). Rabbit polyclonal antibodies against NCX1 and NCX2 were produced as previously described (Iwamoto et al., 1998).

Animals

NCX1 HET, NCX2 HET and smooth muscle-specific NCX1.3 transgenic mice (NCX1.3 Tg) were produced as reported previously (Wakimoto et al., 2000; Gotoh et al., 2015; Iwamoto et al., 2004). These three mice on the C57BL/6 background appeared healthy and were comparable in all analyses to age-matched wild-type mice (WT). All procedures used in this study were performed according to the institutional policies of the Osaka Prefecture University Animal Care and Use Committee.

Immunofluorescent staining
Immunofluorescent staining on frozen sections was performed as previously described (Azuma et al., 2008) with some modifications (Nishiyama et al., 2013). Immunoreactivity of NCX1 and NCX2 was detected using an Alexa Fluor 488-labeled goat anti-rabbit IgG antibody. To detect neurons, a mouse antibody against PGP9.5 was used, and the immunoreactivity of PGP9.5 was detected using an Alexa Fluor 488-labeled goat anti-mouse IgG antibody. Confocal images were obtained using a laser-scanning microscope (C1si; Nikon Corporation, Tokyo).

Quantitative real-time PCR

Quantitative real-time PCR for NCX1 and NCX2 mRNA was performed as described previously (Azuma et al., 2010a) with some modifications (Matsuo et al., 2015). Briefly, the ileum was removed from the mice. Total RNA was extracted. The primers used for the amplification of NCX1 and NCX2 were as follows: 5′-CGCTGGGGAAGATGACGATG -3′ and 5′-TGGGACGAAGGCAAACAGAAC -3′, and 5 ′-ATGGCTCCCTTGGCTTTGATG - 3 ′ and 5 ′ -CAGCGGTAGGAACCTTGGC -3 ’, respectively. The amplification of HPRT mRNA was used as an endogenous control for each experimental sample to account for differences in the amount and quality of total RNA added to each reaction.

Western blot analysis
Western blot analysis was performed using a previously described method (Azuma et al., 2010b) with some modifications (Nishiyama et al., 2014). Briefly, the ileum was harvested with Cell Lysis Buffer (Cell Signaling). Aliquots were electrophoresed on a 10% polyacrylamide gel and subsequently blotted on a PVDF membrane. The PVDF membrane was reacted with antibodies against NCX1 or NCX2 and then reacted with a peroxidase-conjugated secondary antibody. Finally, the proteins that reacted with the antibody were visualized using ECL™ detection reagents (GE Healthcare).

Recording of responses to EFS in longitudinal smooth muscles of the ileum

Responses to EFS were recorded using previously described methods (Tekeuchi et al., 2007; Azuma et al., 2016). Briefly, the ileum was removed from mice (10–15 weeks old). Whole-wall strips were prepared in the orientation of the longitudinal muscle layer. These strips were mounted in organ baths and held at a resting tension of 0.5 g. Responses to EFS were detected using isotonic force transducers (TD-112A; Nihonkohden, Tokyo, Japan). Specifically, the strips were exposed to EFS with trains of 100 pulses of 0.5 ms and 30 V for 10 sec, with a 10-min interval between tests. Atropine (1 μM) was directly added to the bath at least 10 min prior to EFS. Phasic and tonic contractions were analyzed by measuring the extent of the maximal contraction in response to 60 mM KCl.
Statistical analysis

The results were expressed as the mean ± S.E. Statistical significance was determined using one-way ANOVA for non-repeated measures to detect differences among WT, NCX1 HET and NCX2 HET. The differences between groups were determined using the Tukey-Kramer test. The statistical significance of the parametric data was evaluated using a two-tailed Student’s t-test to detect differences between WT and NCX1.3 Tg. A p value less than 0.05 was considered significant.

Results

Localization of NCX1 and NCX2

We initially investigated the expression and localization of NCX1 and NCX2 in the ileum of WT using immunofluorescent staining. As shown in Figure 1A, strong immunoreactivity of NCX1 and NCX2 was observed within the myenteric plexus layers. The expression of NCX1 and NCX2 was also observed in the longitudinal and circular muscle layers. To estimate the histological change of NCX1- and NCX2-HET tissue preparations in the ileum, we dyed myenteric neurons with the PGP9.5 antibody. As shown in Figure 1B, immunoreactivity of PGP9.5 was observed within the myenteric plexus layers among WT, NCX1 HET and NCX2 HET. In addition, there are no marked histological changes in the ileum between WT and NCXs HET.
Decreased expression of NCX1 and NCX2

As shown in Figure 2A, the mRNA expression level of NCX1 was significantly lower in NCX1 HET, whereas NCX2 HET expressed significantly lower levels of NCX2 mRNA. In accordance with these results, the protein expression level of NCX1 was significantly lower in NCX1 HET, whereas NCX2 HET expressed significantly lower levels of NCX2 protein (Fig. 2B).

EFS-induced responses

First, we evaluated the amplitude and spike numbers of spontaneous contractions. Consequently, there were no significant changes among WT, NCX1 HET and NCX2 HET (data not shown). Next, we investigated the frequency-response relationship of EFS at 1, 3 and 10 Hz in the ileum from WT, NCX1 HET and NCX2 HET. Figure 3A shows representative recording traces of responses to EFS. In WT, EFS induced both a phasic contraction that persisted during the stimulus and a tonic contraction that was recorded after the end of the stimulus. To measure the extent of the phasic and tonic contractions, we analyzed the extent of the maximal contractions in response to 60 mM KCl among WT, NCX1 HET and NCX2 HET. The magnitudes of maximal contractions in response to 60 mM KCl were similar among WT, NCX1 HET and NCX2 HET (Fig. 4). Importantly, the magnitude of the phasic
contraction at 1 Hz was smaller in NCX2 HET, but not NCX1 HET, compared to WT (Fig. 3B top). However, NCX1 HET and NCX2 HET showed phasic contractions at 3 Hz and 10 Hz in a manner similar to WT (Fig. 3B top). In contrast, the tonic contractions at 1 and 3 Hz were smaller only in NCX2 HET (Fig. 3B bottom).

We previously demonstrated that ACh is the representative transmitter of phasic contractions and that SP is the main transmitter of tonic contractions in the mouse ileum (Takeuchi et al., 2007). To characterize the tonic contraction, EFS was performed after the tissues were incubated with atropine, a muscarinic ACh receptor antagonist. The magnitude of the phasic contraction was completely suppressed in WT, NCX1 HET and NCX2 HET. Nevertheless, tonic contractions remained. The magnitude of the tonic contraction was smaller in NCX2 HET, but not in NCX1 HET, compared to WT (Fig. 3C). Taken together, these results indicate that NCX2 HET produced decreases in the response to excitatory mediators, such as ACh and SP.

Responses of smooth muscles

Because NCX1 and NCX2 are expressed in longitudinal smooth muscles and in neurons of the myenteric plexus layers (Fig. 1A), we determined the effect of NCX isoforms in heterozygous deficient mice on the contractile response to ACh and SP in smooth muscle cells. We examined the responses to three different concentrations of ACh and two different
concentrations of SP. ACh and SP induced contractions in a dose-dependent manner (Fig. 5 and Fig. 6). The magnitude of the ACh-induced contractions was smaller in NCX2 HET than in WT when ACh concentration was only 0.1 μM (Fig. 5B). Similarly, the magnitudes of the SP-induced contractions were smaller in NCX2 HET than in WT at both concentrations examined (Fig. 6B). However, ACh and SP induced similar magnitudes of contraction in NCX1 HET compared to WT (Fig. 5B and Fig. 6B). Thus, NCX2 heterozygous deficiency in smooth muscles may lower the sensitivity of these cells to ACh and SP.

Overexpression of NCX1

These observations suggested that NCX1 is not likely to affect ileum contractions, although NCX1 was expressed in longitudinal and circular muscle layers and in the myenteric plexus layers. To further test whether NCX1 plays a critical role in ileal contractions, we examined the EFS-induced responses in the longitudinal smooth muscles of the ileum in NCX1.3 Tg in the next series of experiments. We confirmed the overexpression of NCX1 in the ileum of NCX1.3 Tg using western blot analysis. As shown in Figure 7, NCX1 protein was overexpressed in the ileum of NCX1.3 Tg at a two-fold higher level compared to endogenous NCX1.

EFS-induced responses in NCX1.3 Tg
To measure the extent of the phasic and tonic contractions, we analyzed the extent of the maximal contractions in response to 60 mM KCl between WT and NCX1.3 Tg. The magnitudes of the maximal contractions in response to 60 mM KCl were similar between WT and NCX1.3 Tg (Fig. 8). We found that the magnitudes of the phasic and tonic contractions were greater in NCX1.3 Tg than in WT at 10Hz (Fig. 9B). Tonic contractions during atropine administration were also greater in NCX1.3 Tg than in WT at 10Hz (Fig. 9D). The time from full amplitudes to half amplitudes of the phasic contraction were compared in WT and NCX1.3 Tg (Fig. 9A). Interestingly, the time to half amplitudes was approximately 4 seconds longer in NCX1.3 Tg than in WT (Fig. 9C).

Responses of smooth muscle cells in NCX1.3 Tg

Consistent with the results that phasic contraction was greater in NCX1.3 Tg than in WT, the magnitude of ACh-induced contractions was greater in NCX1.3 Tg than in WT (Fig. 10A). In contrast, NCX1.3 Tg exhibited SP-induced contractions that were similar to those of WT (Fig. 10C). The time from full amplitudes to half amplitudes of the ACh and SP-induced contractions were significantly longer in NCX1.3 Tg than in WT (Fig. 10B and 10D).

Discussion

The first aim of this study was to investigate the physiological role of NCX isoforms in the
motility of the ileum. Our immunohistochemical results provide evidence indicating the presence of NCX1- and NCX2-positive cells in the myenteric plexus in addition to the longitudinal and circular muscle layers. Our findings indicate a role for the NCX isoform in regulating murine ileal motility and highlight the importance of NCX isoforms in this regulation. We demonstrated that NCX2 HET show decreased amplitudes of EFS-induced contractions. This decreased contraction is not pronounced in NCX1 HET, which suggests that NCX2 plays a dominant role than NCX1 in ileal motility. How does NCX2 HET decrease the contractions? There are at least two potential explanations. One possibility may be a decreased release of an excitatory transmitter, such as ACh or SP, from myenteric neurons during EFS. The second possibility is an attenuated sensitivity to the excitatory transmitters of smooth muscles. In the present study, the amplitude of the ACh-induced contraction was significantly decreased in NCX2 HET compared to WT, suggesting that decreased phasic contraction in NCX2 HET was associated with the attenuated sensitivity of smooth muscles to ACh. Similar to ACh, the amplitude of SP-induced contraction was significantly decreased in NCX2 HET compared to WT, suggesting that the decreased tonic contraction in NCX2 HET is associated with the attenuated sensitivity of smooth muscles to SP. Consistent with previous findings, we found that there are NCX2-positive cells in the myenteric plexus. What is the role of NCX2 expressed in the myenteric plexus? It is important to demonstrate which cell type contributes to the regulation of motility in the ileum. We hypothesized that the decreased
expression of NCX2 in the smooth muscles of NCX2 HET would result in small contractions. However, to date, the role of NCX2 in the myenteric plexus of the ileum remains unclear. We previously showed that ACh release decreased in the colon of NCX2 HET (Azuma et al., 2012). Thus, the possibility that a decrease in the release of the transmitters may also be responsible for the small EFS-induced contractions in the ileum of NCX2 HET cannot be ruled out. Further experiments are required to investigate the direct role of NCX2 in the myenteric plexus. Further investigation will focus on precisely distinguishing between the contributions of myenteric plexus NCX2 versus smooth muscle NCX2 to the contractile function of the ileum. In smooth muscles, elevated intracellular Ca\(^{2+}\) concentrations are essential for the contraction in response to ACh or SP. In all of the previous and recent findings, NCX, including NCX2, act to increase the efflux of Ca\(^{2+}\) and influx of Na\(^{+}\), resulting in a decrease in smooth muscle tone. Originally, we expected that the decrease in the expression level of NCX2 in NCX2 HET might result in an increase in smooth muscle tone, which is inconsistent with the present results. If NCX2 contributes to Ca\(^{2+}\) efflux, then NCX2 HET may result in large contractions. However, the present results revealed that NCX2 HET showed small contractions. Thus, we hypothesized that NCX2 contributes to Ca\(^{2+}\) influx, but not Ca\(^{2+}\) efflux, during contraction in response to ACh or SP. It is likely that NCX2 HET occurs following weakly elevated intracellular Ca\(^{2+}\) concentrations. In support of this hypothesis, it has been reported that Ca\(^{2+}\) influx via NCX contributes to neurotransmitter release in rat cortical
neurons (Wu et al., 2008). Furthermore, the promotion of Ca\(^{2+}\) influx via NCX has been
suggested to occur during heart failure (Satoh et al., 2000) and ischemia (Imahashi et al.,
2005). Together with previous evidence, these results suggest that NCX2 may contribute to
Ca\(^{2+}\) influx in the ileum.

Furthermore, this study indicated that 60 mM KCl-induced contractions were similar
among WT, NCX1 HET and NCX2 HET, and between WT and NCX1.3 Tg. If NCX1 and
NCX2 can contribute to Ca\(^{2+}\) influx in the ileum, why were 60 mM KCl-induced contractions
in NCXs HET and NCX1.3 Tg not changed? It is likely that the role and contribution of Ca\(^{2+}\)
influx is different between ACh- and SP-induced contractions and KCl-induced contraction.
Another possibility is that the role and contribution of NCX is different between ACh- and
SP-induced contractions and KCl-induced contraction. A further issue that is raised by the
current work is to determine the role of NCX in the contraction machinery of the ileum.

Regarding NCX1 HET, our observations suggested that NCX1 is not likely to affect
ileal contraction, although NCX1 was expressed in both the longitudinal and circular muscle
layers and in the myenteric plexus layers. Then, what is the role of NCX1? Using NCX1.3 Tg,
we demonstrated that NCX1 overexpression enhanced EFS-induced contractions. Importantly,
findings using NCX1.3 Tg indicated a prolonged time period from full amplitudes to half
amplitudes. We found that NCX1 overexpression also enhanced ACh-induced contraction,
which suggests that the enhanced contraction in NCX1.3 Tg is associated with an increased
sensitivity to ACh. Next, we considered the mechanism by which NCX1.3 Tg showed enhanced contraction and prolonged time to half amplitudes. Elevated intracellular Ca$^{2+}$ concentrations are essential for contractions in smooth muscles. In urinary bladder smooth muscles, NCX1.3 Tg showed enhanced contractions and a prolonged duration of Ca$^{2+}$ sparks (Yamamura et al., 2013). In combination with previous findings, NCX1.3 Tg has been hypothesized to experience increased Ca$^{2+}$ influx via NCX, which extends the period of high Ca$^{2+}$ levels and maintains the contraction. Consistent with this hypothesis, NCX1 overexpression also prolonged the time to half amplitudes, though it had no effect on the amplitude of the SP-induced contractions. Taken together, these results suggest that increased Ca$^{2+}$ influx via NCX after the contraction extends the period of high Ca$^{2+}$ levels, followed by a prolonged time to half amplitudes.

Given that NCX1 and NCX2 proteins are normally expressed on all cell types in the gastrointestinal tissues—nerves, glia, interstitial cells of Cajal (ICC) and smooth muscle (Oda et al., 2011; Ota et al., 2013; Yamakawa T et al., 2012), we predicted that disruption of Na$^+$/Ca$^{2+}$ homeostasis in NCX1 HET and NCX2 HET may affect many signaling pathways in all or any of the cells that contribute to gastric motility. ICC, which are also called pacemaker cells (Kito and Suzuki, 2003; Kito et al., 2005), have been suggested to be involved in the neurotransmission in gastrointestinal smooth muscles (Bayguinov et al., 2010; Tanahashi et al., 2014). In addition, ICC-MY have been suggested to express NCX1 and to be involved in
its Ca\textsuperscript{2+} handling (Cho and Daniel, 2005; Lowie et al., 2011). Thus, these results can potentially imply the deficient function of ICC in NCXs HET and the function of ICC in NCX1.3 Tg. A further issue that was raised by the current work was to determine the role of NCX expressed in ICC.

In conclusion, our findings suggest that NCX isoforms play prominent roles in contraction in the ileum. Thus, NCXs HET and NCX1.3 Tg may be valuable tools for delineating the mechanisms of NCX isoforms action in gastrointestinal motility. To this end, the current study may provide useful information for the identification of therapeutic targets.
References


Azuma YT, Samezawa N, Nishiyama K, Nakajima H, Takeuchi T (2016b) Differences in time to peak carbachol-induced contractions between circular and longitudinal smooth muscles of mouse ileum. Naunyn Schmiedebergs Arch Pharmacol 389:63-72


Iwamoto T, Pan Y, Nakamura TY, Wakabayashi S, Shigekawa M (1998) Protein kinase C-dependent regulation of Na\(^+\)/Ca\(^{2+}\) exchanger isoforms NCX1 and NCX3 does not require their direct phosphorylation. Biochemistry 37:17230-17238


Nicoll DA, Quednau BD, Qui Z, Xia YR, Lusis AJ, Philipson KD (1996) Cloning of a third


Na\(^+\)/Ca\(^2+\) exchanger 1 transgenic mice display increased relaxation in the distal colon.
Pharmacology 94:230-238


Fig. 1. Immunohistochemical staining. (A) NCX expression in the tissue sections of the ileum of WT. Tissue sections were stained for NCX1 or NCX2 (green). Images shown are representative of three experiments. CM, circular muscle layer; MP, myenteric plexus layer; LM, longitudinal muscle layer. Scale bar, 50 μm. (B) Immunohistochemical staining of the tissue sections in the ileum of NCX HETs. Tissue sections were stained for PGP9.5 (green) which is a marker of neurons. Images shown are representative of three experiments. MP, myenteric plexus layer. Scale bar, 20 μm.

Fig. 2. Decreased expression of NCX in the ileum. (A) The mRNA expression level of NCX1 in WT (n=7), NCX1 HET (n=7) and NCX2 HET (n=5) and the mRNA expression level of NCX2 in WT (n=7), NCX1 HET (n=6) and NCX2 HET (n=7) were examined using quantitative real-time PCR. The mRNA level of each NCX is shown as a fold increase relative to the level of HPRT mRNA. *P<0.05 compared with WT. (B) Protein expressions of NCX in the ileum. Total cell lysates were prepared from the ileum in WT (n=4) and NCX1 HET (n=4) and blotted with antibody against NCX1, and in WT (n=7) and NCX2 HET (n=7) and blotted with antibody against NCX2. *P<0.05 compared with WT.

Fig. 3. Decreased phasic and tonic contractions in NCX2 HET. EFS-induced contractions in longitudinal smooth muscles isolated from the ileum in WT (n=7), NCX1 HET (n=11) and
NCX2 HET (n=7). The muscle strips were stimulated by EFS at 1, 3 and 10 Hz for 10 s. (A) Representative recording traces of EFS-induced contractions are shown. Bars indicate the duration (10 s) of EFS. After basal tones were recorded, the chart speed was increased to make the EFS-induced contraction clear. (B) Quantitative data on phasic and tonic contractions. Phasic and tonic contractions were expressed as percentages of 60 mM KCl-induced contraction. **P<0.01 for WT vs. NCX2 HET. (C) EFS in the presence of atropine in longitudinal smooth muscles isolated from the ileum in WT (n=5), NCX1 HET (n=5) and NCX2 HET (n=4). Quantitative data on the EFS-induced tonic contraction. Tonic contractions were expressed as percentages of 60 mM KCl-induced contraction. *P<0.05 for WT vs. NCX2 HET.

Fig. 4. KCl-induced contraction. KCl-induced contractions in longitudinal smooth muscles isolated from the ileum in WT (n=21), NCX1 HET (n=10) and NCX2 HET (n=9). Quantitative data on KCl-induced contractions are expressed as mm.

Fig. 5. ACh-induced contraction. ACh-induced contractions in longitudinal smooth muscles isolated from the ileum in WT (n=4), NCX1 HET (n=5) and NCX2 HET (n=4). (A) Representative recording traces of ACh-induced contractions are shown. (B) Quantitative data on ACh-induced contractions. ACh-induced contractions are expressed as percentages of 60
mM KCl-induced contractions. **P<0.01 for WT vs. NCX2 HET.

**Fig. 6.** SP-induced contraction. SP-induced contractions in longitudinal smooth muscles isolated from the ileum in WT (n=4), NCX1 HET (n=5) and NCX2 HET (n=7). (A) Representative recording traces of SP-induced contractions are shown. (B) Quantitative data on SP-induced contractions. SP-induced contractions are expressed as percentages of 60 mM KCl-induced contractions *P<0.05 for WT vs. NCX2 HET.

**Fig. 7.** NCX1 expression in the ileum. Total cell lysates were prepared from the ileum in WT (n=3) and NCX1.3 Tg (n=3) and blotted with antibody against NCX1. *P<0.05 for WT vs. NCX1.3 Tg.

**Fig. 8.** KCl-induced contraction. KCl-induced contractions in longitudinal smooth muscles isolated from the ileum in WT (n=13) and NCX1.3 Tg (n=11). Quantitative data on KCl-induced contractions are expressed as mm.

**Fig. 9.** EFS-induced contraction in NCX1.3 Tg. (A-C) EFS-induced contractions in longitudinal smooth muscles isolated from the ileum in WT (n=6) and NCX1.3 Tg (n=7). The muscle strips were stimulated by EFS at 10 Hz for 10 s. (A) Representative recording traces of
EFS-induced contraction are shown. Bars indicate the duration (10 s) of EFS. (B) Quantitative data on phasic and tonic contractions. Phasic and tonic contractions were expressed as percentages of 60 mM KCl-induced contraction. *P<0.05, **P<0.01 for WT vs. NCX1.3 Tg. (C) t1/2 describes time from full amplitudes to half amplitudes of the phasic contraction. *P<0.05 for WT vs. NCX1.3 Tg. (D) EFS in the presence of atropine in longitudinal smooth muscles isolated from the ileum in WT (n=3) and NCX1.3 Tg (n=4). Quantitative data on the EFS-induced tonic contraction. Tonic contractions were expressed as percentages of 60 mM KCl-induced contraction. *P<0.05 for WT vs. NCX1.3 Tg.

Fig. 10. ACh- and SP-induced contraction in NCX1.3 Tg. (A) ACh-induced contractions in longitudinal smooth muscles isolated from the ileum in WT (n=5) and NCX1.3 Tg (n=6). Quantitative data on ACh-induced contractions. ACh-induced contractions are expressed as percentages of 60 mM KCl-induced contractions. *P<0.05 for WT vs. NCX1.3 Tg. (B) t1/2 describes time from full amplitudes to half amplitudes of ACh-induced contraction. *P<0.05 for WT vs. NCX1.3 Tg. (C) SP-induced contractions in longitudinal smooth muscle strips isolated from the ileum in WT (n=4) and NCX1.3 Tg (n=5). Quantitative data on SP-induced contractions. SP-induced contractions are expressed as percentages of 60 mM KCl-induced contractions (D) t1/2 describes the time from full amplitudes to half amplitudes of SP-induced contraction. *P<0.05 for WT vs. NCX1.3 Tg.