

# Developmental Expression of Sodium-Potassium-Chloride Symporter in the Rat Enteric Nervous System

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## ABSTRACT

Intracellular chloride concentration is determined by the activity of cation-chloride cotransporters such as sodium-potassium-chloride symporters (NKCCs) and potassium-chloride symporters (KCCs). In the central nervous system, NKCC1 dominates in immature neurons. As development progresses, the expression of NKCC1 decreases and the expression of KCC2 increases. In the enteric nervous system, the intrinsic nervous system in the wall of the gut, the expression of NKCC1 and NKCC2 has been reported. Less is known about the presence and distribution of KCCs in the neurons of the gastrointestinal tract. The purpose of this study was to investigate the developmental expression of NKCC1, NKCC2, and KCC2 in the enteric nervous system of rats. Segments of the rat colon were removed at postnatal day 1 (P<sub>1</sub>) through P<sub>7</sub>, P<sub>14</sub>, P<sub>21</sub>, and 2-month. Myenteric plexus preparations were used for immunohistochemical staining for NKCC1, NKCC2, and KCC2. NKCC2 was expressed at P<sub>1</sub> through P<sub>7</sub>, P<sub>14</sub>, P<sub>21</sub>, and adult. NKCC1 was not present from P<sub>1</sub>-P<sub>14</sub>, but was weakly expressed in adult. There was no expression of KCC2 in the myenteric plexus of the colon at any stage of development. In contrast to the central nervous system, NKCC2 is the predominant cation-chloride cotransporter expressed in the enteric nervous system. There is no developmental change in NKCC2 expression in the enteric nervous system.

## INTRODUCTION

Intracellular chloride concentration plays an important role in cellular functions including the control of membrane potential and excitability in neurons. Cation-chloride transporters including sodium-potassium-chloride symporters (NKCCs) and potassium-chloride symporters (KCCs) determine the intracellular concentration of chloride (Blaesse et al. 2009). Two isoforms of NKCC (NKCC1 and NKCC2) and four isoforms of KCC (KCC1-4) have been identified. Electroneutral in nature, NKCCs or KCCs do not generate any electrical current but rather contribute to the inwardly or outwardly directed net Cl<sup>-</sup> flux using the Na<sup>+</sup> or K<sup>+</sup> concentration gradient created by the Na<sup>+</sup>/K<sup>+</sup>-ATPase. K<sup>+</sup> concentration gradient is employed by the KCCs to extrude Cl<sup>-</sup> from the cell whereas the NKCCs use Na<sup>+</sup> concentration gradient to accumulate Cl<sup>-</sup> in the cytoplasm (Payne et al., 2003).

### *Role of GABA*

$\gamma$ -aminobutyric acid (GABA) is typically considered an inhibitory neurotransmitter in the adult central nervous system (CNS). However, GABA has been reported to evoke excitatory responses in developing immature CNS neurons (Chen et al. 1996; Owens et al. 1996; Ben-Ari 2002). Numerous observations suggest that developmental changes in NKCC1 and KCC2 expression in the CNS contribute to the switch of GABA actions. NKCC1 is predominant in early developmental stages in the CNS, which helps to accumulate Cl<sup>-</sup> in the cytoplasm (Alvarez-Leefmans et al. 1988; Kakazu et al. 1999), resulting in a relatively high intracellular Cl<sup>-</sup> concentration. Because of this property, the opening of Cl<sup>-</sup> conducting channels (e.g. by activating the GABA<sub>A</sub> receptors) causes Cl<sup>-</sup> efflux and depolarization in these neurons. As development progresses, the levels of NKCC1 expression decrease (Plotkin et al. 1997) and the levels of KCC2 expression increase (Rivera et al. 1999; Payne et al. 2003). Expression of KCC2 lowers [Cl<sup>-</sup>]<sub>i</sub>, resulting in the characteristic inhibitory responses to GABA.

The enteric nervous system is the intrinsic nervous system within the wall of the gut. The expression of the Cl<sup>-</sup> accumulators NKCC1 and NKCC2 in the enteric nervous system has been reported recently (Wouters et al. 2006; Xue et al. 2009). There is no information about the distribution of KCCs in the enteric nervous system. The purpose of the study was to investigate the expression of KCC2 in the enteric nervous system and observe the developmental changes in NKCC1, NKCC2, and KCC2 expression in the enteric nervous system of the rats.

## METHODS

### *Tissue harvest*

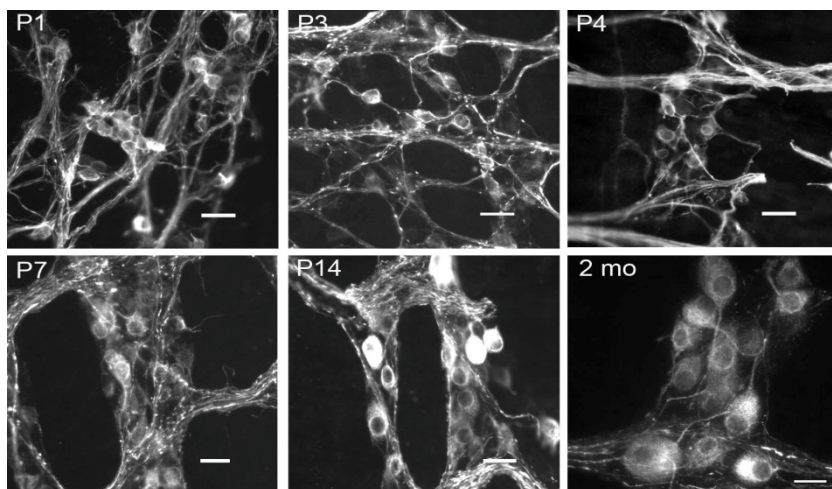
Rats from postnatal day 1 (P<sub>1</sub>) through P<sub>7</sub>, P<sub>14</sub>, P<sub>21</sub>, and 2-month were euthanized using CO<sub>2</sub> inhalation. Segments of the colon were removed and fixed in Zamboni's fixative. Whole mount myenteric plexuses were dissected and stored in phosphate-buffered saline (PBS) containing NaNO<sub>3</sub>.

### *Immunohistochemical staining of NKCCs and KCCs*

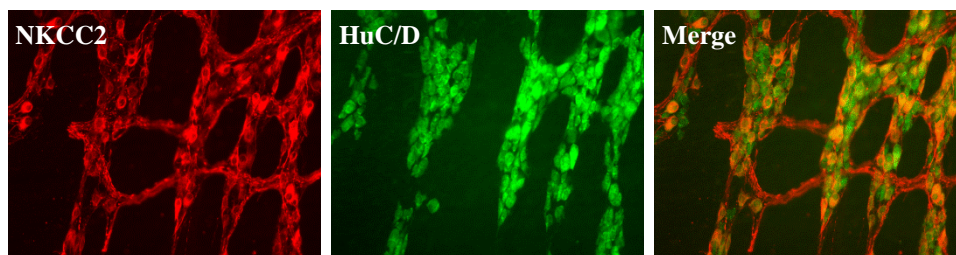
Tissue preparations were incubated in a solution containing anti-NKCC1, anti-NKCC2, or anti-KCC2 antibody for 24 hours. The preparations were washed with PBS and were then incubated in solutions containing the Cy3-labeled donkey anti-rabbit IgG for 1 hour. Tissues were rinsed again with PBS, mounted on slides, and coverslipped with mounting medium. Slides were examined under a Nikon 80i fluorescence microscope for expression of NKCC1, NKCC2, and KCC2.

## RESULTS

NKCC2 was the predominant cation-chloride cotransporter present in the enteric nervous system of the rats. NKCC2 immunoreactivity was observed at P1 and continued to exhibit expression through adulthood (Fig. 1). NKCC2 immunoreactivity was co-localized with a neuronal marker, anti-human neuronal protein C/D (HuC/D) (Fig. 2). No developmental changes in NKCC2 expression were observed in the enteric nervous system. NKCC1 immunoreactivity was not present in the enteric nervous system during P1-P14, but was weakly expressed at 2-month. No KCC2 immunoreactivity was found in the enteric nervous system at any stages of development.



**Figure 1.** NKCC2 expression in the enteric nervous system of the rat colon at different postnatal developing stages.



**Figure 2.** Colocalization of NKCC2 with neuronal marker human neuronal protein C/D (HuC/D) in the enteric nervous system of P<sub>14</sub> rat colon.

## DISCUSSION

Mounting evidence suggests that the activity of the chloride cotransporter, NKCC, is the mechanism responsible for accumulation of Cl<sup>-</sup> in multiple kinds of neurons. In immature CNS neurons, NKCC activity accounts for elevation of intraneuronal Cl<sup>-</sup> and GABA<sub>A</sub>-evoked depolarizing responses (Chen *et al.* 1996; Owens *et al.* 1996; Ben-Ari 2002). Neurons in the enteric nervous system also sustain elevated intracellular Cl<sup>-</sup> (Cherubini and North, 1984; Bertrand and Galligan, 1992). However, what contributes to the elevated intracellular Cl<sup>-</sup> concentration in enteric neurons is still unclear. We found that NKCC2 was continuously expressed in the enteric nervous system from neonates throughout the adulthood. NKCC1 was not present in the neonatal period and was only faintly expressed in the adult enteric nervous system. KCC2 was not present in the enteric nervous system at any developmental stage. The evidence suggests that NKCC2 is the predominant cation-chloride transporter in the enteric nervous system. Expression of NKCC2 in the enteric nervous system during the neonatal period and throughout adulthood may contribute to GABA<sub>A</sub>-evoked depolarizing responses in enteric neurons (Cherubini and North, 1984; Bertrand and Galligan, 1992).

The expression of cation-chloride cotransporters in the enteric nervous system does not appear to coincide with the developmental changes that occur in the central nervous system. In contrast to the central nervous system, NKCC2 was found to be expressed in the enteric nervous system of neonates continuing throughout adulthood.

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