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The Role of NKCC2A on the Nervous System Activity

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The Role of NKCC2A on the nervous system activity

A Thesis submitted in partial fulfillment of the requirements for the degree of
Master of Science

By
Annu Kavya
Bachelor of Pharmacy, Osmania University, 2013

2015
Wright State University
I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Annu Kavya ENTITLED The Role of NKCC2A on the nervous system activity BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

Annu Kavya, M.S. Department of Pharmacology and Toxicology, Wright State University, 2015. The Role of NKCC2A co-transporter in the nervous system activity.

The NKCC2A is a splice variant of the Na⁺K⁺2Cl⁻ co-transporter 2 (Slc12a1), which is abundantly expressed in macula densa and in the apical membrane of the tubular cells in the kidney. Most of our knowledge regarding NKCC2 function is limited to the kidney, the organ where NKCC2 is abundantly expressed. However, recent studies have demonstrated that NKCC2 is also expressed in extra-renal tissues. This study was designed to determine if NKCC2A, a splice variant of NKCC2 has an impact on behavior and nervous system activity of mice. To these ends, we used mice (wild type, WT), lacking a single or both alleles of NKCC2A (heterozygous, HE and homozygous, HO, respectively) which were subjected to battery of standardized behavioral tests. NKCC2A-HO mice exhibited a significant gating impairment when compared to the WT or HE genotypes. Interestingly, NKCC2A-HO mice also exhibited a significantly lower immobility time compared to the WT and HE mice in the forced swim test (FST), a finding that could not be reproduced in the tail suspension test (TST). Nevertheless, the caudate nucleus of NKCC2A-HO mice exhibited low content of serotonin (5-HT) and dopamine metabolites, which might account for the reduced immobility time assayed by the FST. NKCC2A-HO mice had significantly lower levels of epinephrine (EPI) and norepinephrine (NE) in the adrenals, with the HE mice having intermediate levels suggesting a role of NKCC2A
in sympathetic activity, a conclusion suggested by the finding that these mice excreted low urinary EPI and NE. Together, the results of this study indicate that NKCC2A impacts behavior and neurochemistry in specific regions of the brain and the periphery.
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1. Introduction

1.1 SLC12A Gene family

The electroneutral inorganic cation-chloride co-transporters (CCCs) belong to the solute carrier 12 gene family (SLC12) which consist of nine homologous members: SLC12A1-9. They play several important roles in human physiology, including cell volume regulation, hydro electrolytic balance, fluid secretion, neurotransmission and hormone secretion. This family of genes encode plasma membrane proteins that mediate the movement of inorganic sodium (Na\(^+\)) and/or potassium (K\(^+\)) cations coupled to the movement of chloride (Cl\(^-\)) anions. For instance, the SLC12A1 and SLC12A2 genes encode Na-dependent K-Cl co-transporters widely known as NKCCs, whereas SLC12A3 encode several Na-Cl co-transporters. These proteins upload Cl\(^-\) into the cells by using the thermodynamic energy produced by the inward movement of Na\(^+\) (Gagnon and Delpire. 2013). All members of the SLC12A family are structurally closely related but differ in functional properties, transcriptional regulation and post-transcriptional modulation of activity which may contribute to their relative role in different functions (Hubner et al. 2001).

1.2 NKCC1

The SLC12A2 gene encodes the Na\(^+\)/K\(^+\)/2Cl\(^-\) co-transporters-1 (NKCC1) which are ubiquitous and secretory in nature (Gamba et al. 1994). Multiple studies on NKCC1 knock out mice confirmed that it is not essential for the survival. Head bobbing and circling behavior is the most confounding phenotype of NKCC1 knockout mice (Delpire et al. 1999). They had defects in hearing and balance suggesting both cochlear and vestibular defects (Gagnon and Delpire. 2013; Hubner et al. 2001). NKCC1 was found to be involved in a variety of adult organ functions including the
central nervous system (CNS). Active Cl\(^-\) accumulation via NKCC1 activated the GABA\(_A\) receptor mediated depolarization in immature neurons (Clayton et al. 1998). As the neurons mature, the intracellular Cl\(^-\) concentration decreases which result in a shift towards GABA\(_A\)–mediated inhibition. It was also shown that GABA was excitatory in arginine vasopressin (AVP) neurons which are located in the paraventricular nucleus of the hypothalamus that produce and release oxytocin and vasopressin into the blood circulation and so, this effect was increased by chronic hyperosmotic stress of salt loading. In this study, these effects were attributed to NKCC1. However, NKCC2 may also be important in this regard. Indeed, it has been recently demonstrated that NKCC2 is expressed in AVP neurons and that its expression is increased in response to chronic dehydration (Konopacka et al. 2015).

Figure 1 - Diagrammatic representation of NKCC2 functional role in the kidney
1.3 NKCC2

The SLC12A1 gene encodes several Na⁺/K⁺/2Cl⁻ co-transporters-2 (NKCC2) which are expressed in the apical membrane of the tubular cells of the thick ascending limb (TAL) of Henle and in the macula densa (Gamba et al. 1994; Gagnon and Delpire. 2013; Hubner et al. 2001). NKCC2s are considered absorptive isoforms of the NKCCs. They are responsible for 25-30% of body’s total renal salt retrieval and therefore, they participate in the concentration capacity of the kidney (Castagné et al. 2001). Indeed, approximately 80% of the NaCl uptake across the apical membrane of the TAL cells is mediated by NKCC2 (Castrop and Schnermann. 2008). NKCC2s as well as NKCC1 are sensitive to furosemide and bumetanide the most commonly prescribed diuretics. Inactivating mutations in the SLC12A1 gene cause Bartter’s syndrome type 1, an autosomal recessive salt-wasting disorder characterized by polyuria, renal tubular hypokalemic alkalosis, and hypercalciuria (Simon et al. 1996; Gagnon and Delpire. 2013).

1.4 NKCC2 Isoforms

The SLC12A2 gene produce at least three variants named NKCC2A, NKCC2B and NKCC2F. They are the result of alternative selection of three mutually exclusive exons (Gamba et al. 1994; Carota et al., 2010; Payne and Forbush, 1994; Vargas-Poussou et al., 1998). These isoforms differ in their localization and transport characteristics along the TAL. The renal localization of the NKCC2 isoforms follows a general pattern despite a few interspecies differences. They are found in the apical membrane and in subapical vesicles along the TAL and in the macula densa segment. NKCC2A is expressed in the outer stripe of the outer medulla and in the cortical portions of the TAL in rodents (Oppermann et al., 2006; Oppermann et al., 2007). NKCC2A is the dominant isoform in the human cells and is found along the entire
kidney. The expression of different isoforms in humans is NKCC2A>>NKCC2F>NKCC2B whereas in the mice it is NKCC2F>>NKCC2A>NKCC2B (Castrop and Schnermann. 2008).

1.5 NKCC2A

NKCC2A exhibits a markedly higher Cl⁻ affinity than NKCC2F whereas NKCC2B has highest Cl⁻ affinity but lower transport capacity (Plata, Meade, Vazquez, Hebert, and Gamba, 2002). At low tubular Cl⁻ concentrations, Cl⁻ reabsorption was significantly reduced in NKCC2B-deficient mice when compared to WT mice, whereas no differences were observed for NKCC2A-HO mice (Oppermann et al., 2007). However, the lack of NKCC2A in mice resulted in reduced Cl⁻ absorption at high Cl⁻ concentrations. Under low Cl⁻ load, TAL reabsorption becomes dependent on the activity of the high- Cl⁻-affinity NKCC2B isoform. Conversely, absence of NKCC2A function becomes obvious in mice subjected to a high load of NaCl including that high tubular Cl⁻ concentrations modulate the transport activity NKCC2A. This suggests that NKCC2A transport activity exceeds that of NKCC2B, which might be related to both the higher transport capacity of NKCC2A essentially and its higher expression levels (Castrop and Schnermann, 2008).

1.6 Expression and role of NKCC2 in extra-renal tissues

NKCC2 has been typically regarded as a kidney-specific ion transporter. However, recent findings suggest that it is also expressed in the endolymphatic sac (ES) epithelium (Nishimura, Kakigi, Takeda, Takeda, and Doi, 2009). There was also evidence for NKCC2 mRNA transcripts, and protein expression in the rat gastrointestinal tract (Xue et al. 2009). In fact, NKCC2 appears to be widely distributed and expressed in the enteric neurons in the myenteric plexus and it is
considered to play a role in the ion transport and neuronal excitability in the gut. Its expression in other nerve tissues has never been reported previously but there is a recent study showing the expression of NKCC2 in the brain hypothalamo-neurohypophyseal system (HNS), in particular the supraoptic and paraventricular nuclei (Konopacka et al. 2015). HNS is a specialized brain region which contain the neurons that produce peptide hormones, arginine vasopressin (AVP) and oxytocin (OXT). AVP modulates the ability of the kidney to retain water free of solutes whereas OXT has an important role in sexual reproduction, social behavior, lactation or parturition (Konopacka et al. 2015).

The hypothalamus is a fundamental structure involved in the integration of the nervous and endocrine systems, and a key regulator in the whole body homeostasis including glucose homeostasis (Lam et al. 2009). Although the impact of NKCC2 in glucose homeostasis remains undefined, a potential role of NKCC2 in insulin secretion has been suggested. Indeed, NKCC1 and NKCC2 are co-expressed in insulin-secreting beta-cells (Alshahrani and Di Fulvio. 2012a; Alshahrani and Di Fulvio. 2012b) and elimination of NKCC1 does not preclude secretion of the hormone in response to bumetanide.

If a mutation or deletion of a gene is thought to modify a physiological response or a single function, there could be other unexpected alterations in the whole animal. However, this reasoning cannot exclude the possibility that the gene of interest may produce its proteins in previously undescribed locations exhibiting different functions. This may be the case of mice lacking a single variant of NKCC2, where its “kidney-specific” location and very well-known function cannot represent its potential function in extra-renal tissues including brain, where our preliminary data suggested NKCC2 expression (unpublished data) and a recent report confirmed (Konopacka et
al. 2015). The influence of a given gene on a specific behavior can be evaluated by conducting behavioral studies in mice lacking expression of such gene (Komada et al. 2008). NKCC1 knockout mice had behavioral alterations relating this effects to its presence in the central and peripheral neurons. So, a quick way to evaluate neural functions and activity alterations is behavior. Behavioral effects reflect the changes in nerve cell communication and integration as well as morphological alterations (Kulig et al. 1996). Our preliminary studies indicating presence of NKCC2 in some brain areas which lead us to investigate effects of deletion of NKCC2A in the mice.

1.7 Behavior

The work of the present thesis sprouted upon casual noticing of uncommon behavior in mice with functional deficiency in a single or in both alleles of the SLC12A1 gene. As mentioned, this gene is responsible for one of the three main splice variants of the Na⁺/K⁺/2Cl⁻ co-transporter-2 (NKCC2), named NKCC2A. These observations were originally made in the laboratory of Dr. Mauricio Di Fulvio and included: unusual aggressiveness and defensive postures toward the corner of cages, absence of withdrawal reflexes, head bobbing, seizure-like behavior or abnormal weight gain in some but not all of these mice. Hence, a battery of standardized behavioral tests was requested and thoroughly conducted by Teresa Garrett in the laboratory of Dr. James Lucot in the Department of Pharmacology and Toxicology at Wright State University, Boonshoft School of Medicine.

Several tests to analyze behavior in mice have been developed. Some of them are more specific than others and represent the functional interaction of particular regions within the brain. Several of these tests are summarized in Table 1.
Visually observed deficits in the gait of NKCC2A-HO mice led us to design experiments to evaluate the behavioral abnormalities of this animal model according to the established tests (Table 1). Their unusual overall aggressiveness (unpublished observation) suggests there might be increased sympathetic activity which can be

Table 1 - Summary of Behavioral tests

<table>
<thead>
<tr>
<th>Behavioral test</th>
<th>Implicated brain regions</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Functional operational battery (FOB)</td>
<td>CNS</td>
<td>To assess and quantify the behavioral and physiologic state of the mouse</td>
</tr>
<tr>
<td>Open field test (OFT)</td>
<td>Amygdala, Hippocampus</td>
<td>To assess locomotor activity and anxiety in rodents</td>
</tr>
<tr>
<td>Elevated plus maze (EPM)</td>
<td>Amygdala, Right prefrontal cortex</td>
<td>To assess anxiety in rodents</td>
</tr>
<tr>
<td>Y maze test</td>
<td>Hippocampus</td>
<td>To assess exploratory activity</td>
</tr>
<tr>
<td>Prepulse inhibition (PPI)</td>
<td>Pons, Prefrontal cortex, Forebrain</td>
<td>Measure of sensory motor gating</td>
</tr>
<tr>
<td>Rotarod</td>
<td>Cerebellum</td>
<td>To evaluate motor coordination and balance</td>
</tr>
<tr>
<td>Forced swim test (FST)</td>
<td>Rostral anterior cingulate cortex (amygdala, nucleus accumbens, hypothalamus)</td>
<td>To evaluate anti-depressant-like effect</td>
</tr>
<tr>
<td>Tail suspension test (TST)</td>
<td>Anterior cingulate cortex (prefrontal cortex, amygdala, nucleus accumbens, hypothalamus)</td>
<td>To evaluate anti-depressant-like effects</td>
</tr>
</tbody>
</table>
evaluated by testing urinary catecholamines or by analyzing changes in urine catecholamine output rate.

The behavior of mice is usually assessed by subjecting them to a battery of standardized tests. Collectively known as the functional observational battery (FOB), this systematic observational method is applied to comprehensively assess and quantify the behavioral and physiologic state of the mouse. For instance, locomotor activity and thigmotactic (tolerance to less stressful environment) behavior of mice can be assessed by using the Open field test (OFT) (Castagné, Moser, Roux, and Porsolt, 2001). It can also be used as a surrogate to assess anxiety in rodents. The elevated plus maze (EPM), however, exploits the natural aversion of mice for open and elevated areas, as well as on their normal exploratory behavior towards novel environments (Komada et al. 2008). The EPM is sensitive to anxiolytic effects in neurotoxic lesions of serotonergic neurons and other effects caused by anxiolytic drugs. The prepulse inhibition of startle (PPI) test is an operational measure of sensory gating, usually disrupted in schizophrenia and some other mental disorders and neurodegenerative diseases. The rotarod test is used to evaluate motor coordination and balance. It assesses the animal’s ability to maintain balance and walk on a rotating rod. The forced swim test (FST) is a rodent behavioral test used for evaluation of the antidepressant efficacy of new compounds and experimental manipulations that are aimed at generating or preventing depressive-like states (Castagné et al. 2001). The tail suspension test (TST) is used to screen potential antidepressant drugs, and to assess other manipulations expected to affect depression-related behaviors (50 Can, Adem 2012).

Forced swimming or exposure to high intensity acoustic stimuli or any other stress activates the hypothalamo-pituitary axis and has an anxiogenic effect on behavior.
This activation is dependent on hypothalamic centers including the paraventricular and supraoptic nuclei and the release of hormones which regulate the secretion of adrenocorticotropic hormone from the pituitary as well as sympatho-adrenal outflow. Stressors that disturb physiological homeostasis activates pituitary-adrenal and sympatho-adrenal axis via extended amygdala inputs to the hypothalamus and brain stem, and exert anxiogenic-like behavioral consequences via actions on a variety of mid and forebrain nuclei. Therefore, changes in the mice behavior as analyzed by using FOB may indicate a disturbance in the physiological homeostasis and involves many brain areas such as the amygdala, hypothalamus, brain stem, mid and forebrain.

1.8 Neuronal systems

The neuronal systems involved in the mice behavior can be evaluated by measuring the neurotransmitter concentrations while correlating them to a particular behavior change. As many major theories suggest, neurobehavioral disorders may result from changes in the dopamine (DA) usage in specific brain areas such as frontal cortex, caudate, brain stem, nucleus accumbens, and hypothalamus (Howes and Kapur. 2009; Mehler-Wex et al. 2006). Therefore these mouse brain areas are usually selected to analyze changes in DA or the turnover of the neurotransmitter serotonin/5-hydroxytryptamine (5-HT).
Figure 2 - Dopaminergic cell bodies (A8, A9, and A10) projects to the ventral tegmental area (VTA), substantia nigra (SNC) and the retrorubral field (RRF).

The dopaminergic and noradrenergic cell groups are designated with letter A, where A8-A15 are dopaminergic groups. The serotonergic cell groups are designated with letter B and adrenergic with letter C. Fig 2 illustrates different dopaminergic cell groups such as olfactory bulb (OB) dentritic periglomerular (A16) neurons, the hypothalamic (Hyp; A12, A14 and A15) cell groups. Among this A12 provides
tuberoinfundibular and tuberohypophysial projections involved in the neuroendocrine regulation. A8-A10 are the mesodiencephalic tegmental cell groups comprising SNc (A9 neurons), the VTA (A10 neurons) and the retrorubral fields (RRF; A8 neurons).

Brain areas receive DA neuronal projections from distinct dopamine pathways which have different physiological and pathophysiological significance e.g. mesolimbic-mesocortical and nigrostriatal projections (Fuxe et al. 1974; Inglis and Moghaddam. 1999; Oades and Halliday. 1987; Feldman. 1997). Figure 2 illustrates different DA neuron bodies as mentioned above.

Ventral tegmental area (VTA; A10 cell groups) is the origin for the dopaminergic cell bodies of mesocortical system and mainly innervated frontal cortex of the brain (Fuxe et al. 1974; Oades and Halliday. 1987; Feldman. 1997). It is mainly involved in cognition and behavior. It contains neurons projecting from prefrontal cortex to the caudate and brain stem (Bjorklund et al. 1975; Bjorklund and Dunnett. 2007).

In the nigrostriatal pathway, as illustrated in figures 2 and 3, dopaminergic pathways run from the zona compacta of the substantia nigra (A9), and nigrostriatal fibers which produce very dense innervation project into the caudate nucleus, putamen and the globus pallidus (Fig 3). It is especially involved in the production of movement which is a part of basal ganglia motor loop. Loss of dopamine neurons in this pathway is implicated in several disorders like Parkinson’s disease, tardiv dyskinesia (Fahn and Mayeux. 1980; Feldman. 1997).

In the mesolimbic pathway, the dopamine is transmitted from the VTA (A10) to the limbic system along nucleus accumbens. Mesolimbic and nigrostriatal dopamine axons arising from midbrain nuclear groups retrorubral nucleus (A8), substantia nigra (A9) and VTA (A10) forms a bundle lateral to hypothalamus and projects into
amygdala, hippocampus and some brain regions of the medial prefrontal cortex (Feldman. 1997, Bjorklund and Dunnett. 2007). Different brain regions receiving dopaminergic neurons are involved in different functions.

The amygdala, a component of mesolimbic DA system, participates in conditioned fear (Fadok et al. 2009). Hippocampus is a primary component of the mesolimbic dopaminergic system involved in cognitive functions and dopaminergic projections to the hypothalamus regulates neuroendocrine functions (Bjorklund et al. 1975).
5-HT is also a monoamine neurotransmitter with an important role in the regulation of sleep, mood, appetite and cognitive functions. 5-HT pathways are named from B1-B9 where B1, B2, and B3 are called the caudal group and as illustrated in figure 3, caudal group gives rise to descending pathways into the dorsal, intermediate and ventral horns of the spinal cord. B4-B9 makes up the ascending 5-HT pathways where B5
and B6 pathways innervate the limbic structures, especially from raphe medianus, hypothalamus and the preoptic area. B7, B8 and B9 gives rise to lateral pathway which innervates cingulate cortex. There is another pathway that originates from B7-B9 and primarily innervates the extrapyramidal motor pathways which arise from the caudate nucleus. The cerebellum is also innervated by 5-HT. Very fine 5-HT neurons were observed in septum, hippocampus and into the neo- and mesocortex (Figure 4: Feldman. 1997).

Figure 4 - 5-HT pathways in the brain
1.9 Brain – monoamines

Behavioral deficits are usually correlated with neurochemical changes in specific regions of the brain. The monoamine neurotransmitters norepinephrine (NE), dopamine (DA), and serotonin (5-HT) are important to normal functioning of the brain and periphery. Current theories of the basis for major neurobehavioral disorders involve abnormalities in the use/metabolism of NE, 5-HT and DA in specific brain regions. To correlate potential changes in these monoamines with observed behavior, the concentration of NE, DA and their metabolites homovanillic acid (HVA) and dihydroxyphenylacetic acid (DOPAC) as well as, 5-HT and its metabolite 5-hydroxyindole acetic acid (5-HIAA) are usually measured in the frontal cortex, amygdala, hippocampus, hypothalamus, brainstem, accumbens, caudate and hypothalamus by using reverse phase high performance liquid chromatography.

1.10 Role and Metabolism of DA and 5-HT

DA is the precursor to norepinephrine (NE) and epinephrine (EPI). Apart from this, it has many other important functions in the brain.

Dopaminergic neurotransmission in different brain regions such as the frontal cortex (FC), amygdala and the caudate plays an essential role in cognition, attention, motivation and reward, learning, creativity and motor activity. Catechol-O-methyltransferase (COMT) and monoamine oxidase (MAO) metabolizes DA to HVA and DOPAC. These are the most significant DA metabolites. The neurotransmitter rate of usage can be determined from the ratio of the metabolite to the parent compound e.g. HVA/DA or DOPAC/DA. HVA is the extra-neuronal (metabolized outside the neurons) metabolite whereas DOPAC is the intra-neuronal (metabolized in the neurons) metabolite of DA (Figure 5: (Feldman. 1997)).
Figure 5 - DA metabolism in the dopaminergic nerve terminal and synapse.

In addition to nervous tissue, adrenal glands produce a NE and E as sympathetic nervous system transmitters. Thus to further extend behavioral studies, it is convenient to determine the levels of these catecholamines in adrenal glands as well as in the kidney, the major organ involved in excretion.

1.11 Kidney Dopaminergic Systems

The kidney’s intrarenal dopaminergic system is distinct from other neural dopaminergic input. Dopamine acts as an intrarenal natriuretic hormone (Jose et al. 1992). Kidney dopamine levels can reach high nano molar concentrations whereas circulating dopamine levels are in the pico molar range (Zeng and Jose, 2011). The dopamine precursor L-DOPA (L-dihydroxyphenylalanine) is taken up from the circulation by the proximal tubule or through filtration via the glomerulus. It is then converted to dopamine by aromatic amino acid decarboxylase. This dopamine produced in the proximal tubule serves as an autocrine/paracrine agent that inhibits
the Na\textsuperscript{+}/K\textsuperscript{+}-ATPase activity in the medullary TAL and in the cortical collecting duct mediated by D\textsubscript{1} receptors. Renal proximal tubules produce dopamine which serves as an intrarenal natriuretic factor by direct tubular action independent of hemodynamic mechanisms. Approximately 60\% of the sodium excretion is mediated by D\textsubscript{1} receptors which in turn is mediated by increased renal dopamine production and increased sensitivity of sodium transporters to dopaminergic inhibition.

1.12 NKCC2 and Dopamine in the Kidney

In the kidney, 25-30\% of the body’s total renal salt retrieval is through the reabsorption of NaCl in the TAL, a phenomenon mediated by NKCC2. Dietary salt modulates intrarenal dopamine production (Nishimura et al., 2009). In the mammalian kidney, dopamine receptor activation due to increased dopamine release decreases NaCl and water reabsorption through inhibition of specific tubule transporter activity along the nephron including the Na\textsuperscript{+}/K\textsuperscript{+}-ATPase in the proximal tubule, and NKCC2 in the TAL (Corrigenda for vol. 283, p. F221.2003; Bacic et al., 2005; Grider, 2003). Therefore, dopamine receptor activation indirectly inhibits Na/K-ATPase and NKCC2 function. This is due to increased renal dopamine release and increased sensitivity of sodium transporters to dopaminergic inhibition. Therefore, absence of NKCC2A in mice might impact dopamine utilization and this can be tested by analyzing dopamine concentrations in the urine samples.

In the TAL, NaCl reabsorption is stimulated by vasopressin, parathyroid, glucagon, epinephrine and norepinephrine. This mechanism is mediated, either directly or indirectly, by altering NKCC2 activity. Sodium transport via NKCC2A or NKCC2B at the macula densa initiates the tuberoglomerular feedback mechanism. Dopamine in the kidney is known to stimulate NKCC2 activity, but controversially, dopamine
inhibits the tubuloglomerular pathway. Interestingly NKCC2A-HO mice exhibited decreased maximum tubuloglomerular feedback responses (Oppermann et al. 2007). Therefore, this study is designed to determine the effect of NKCC2A on the E, NE and DA levels in the urine a reflex of the basal sympathetic activity of the animal.

2. Development of Hypothesis

The hypothesis is that elimination of NKCC2A in vivo alters behavior and in neurochemical usage in the central and peripheral nervous systems.

NKCC2A has different level of expression in WT, HE and HO mice tissues. HE mice have NKCC2A expression intermediate to HO and WT. WT had much higher expression than HE whereas the HO did not have any expression (unpublished data). Therefore, to find the effect of NKCC2A, we have included HE mice group along with WT and HO in our studies.

2.1 Specific Aims

Aim 1 -

To test the hypothesis that NKCC2A-HO mice have neurobehavioral deficits.

NKCC2A-HO mice were used as a model to evaluate the behavioral phenotypes. NKCC2A-HO, HE and WT mice were screened for gross functional deficits, locomotion, psychosis, working memory, anxiety, and depression.

Aim 2 -

To test the hypothesis that NKCC2A-HO mice have altered DA, 5-HT metabolism and changes in NE levels in selective brain regions.
Brain regions such as frontal cortex, amygdala, hippocampus, brainstem, accumbens, caudate and hypothalamus were evaluated for DA, 5-HT usage. Reverse-phase high performance liquid chromatography (RP-HPLC) was used to measure concentrations of NE, DA and its metabolites DOPAC and HVA, 5-HT and its metabolite 5-HIAA in selected brain regions with DA and 5-HT neuronal projections.

**Aim 3 -**

To evaluate the impact of NKCC2A-HO on kidney DA levels as well as on basal sympathetic activity by measuring urinary DA, NE and EPI.

**Aim 4 -**

To test the hypothesis that NKCC2A-HO might show changes in total NE and EPI in the adrenal glands. HPLC was used to measure them in the adrenal glands.
3. Experimental Design

20

3 mice groups
1. Homozygous
2. Heterozygous
3. Wildtype

1. Spot Urine Collection (RP-HPLC 0.55 V)
2. Neurochemistry (RP-HPLC 0.55 V)

Brain regions
- Frontal cortex
- Brain stem
- Hippocampus
- Amygdala and Hypothalamus
- Cauda

Brain blocker

Brain sectioning

Isolated brain

Homogenization

Isolated adrenals

2. Neurobehavioral Studies
a. Gross functional deficits: Functional Operant Battery
b. Motor deficits: Open field test
c. Working memory: Y maze test
d. Anxiety: Elevated plus maze test
e. Psychosis: Prepulse inhibition
f. Motor coordination: Rotarod test
g. Depression: Forced swim test and Tail suspension test

3. Neurochemistry (RP-HPLC 0.75 V)

Brain: NE, DOPAC, 5-HIAA, HVA, DA, 5-HI
Adrenals: NE, EPI

Figure 6 - Experimental design
4. Materials and Methods

4.1 Animals

Mice subjected to behavioral studies were generated from mice lacking a single functional allele of NKCC2A (heterozygous) kindly provided by Dr. Hayo Castrop (University of Regensburg) to Dr. Di Fulvio. Mice were used according to approved animal protocols and the material transfer agreement signed by both parties. Mice were used at the age of 8 weeks and both female and male mice were used in all experiments.

4.2 NKCC2A-HO mice generation

NKCC2A-HO mice were generated by Dr. Hayo Castrop at the University of Regensburg and all the procedures were approved by the Animal Use and Care Committee of the National Institute of Diabetes and Digestive and Kidney Diseases and by the Animal Care Committee of the University of Regensburg. NKCC2A-HO mice were generated according to a similar strategy which was used to generate NKCC2B-HO mice earlier (Oppermann et al. 2006). They were generated by altering the exon 4A of the SLC12A1 gene by the introduction of in-frame stop codons which result in the premature termination of the translation. Homologous arms were generated by long-distance PCR (Roche, Indianapolis, IN). Progeny lacking the neo gene was intercrossed to generate NKCC2A-HO, HE and WT littermates (Oppermann et al. 2007).

4.3 NKCC2A-HO, HE and WT genetic analysis

To identify WT, HE or HO mice, we took advantage of the genetic strategy followed to create these mice to design primer sets for genotyping. We used the polymerase
chain reaction (PCR) to determine them. The genotyping primers recognizing flanking intronic sequences 5’ and 3’ exon A were: 5’-TGC AGG AGT GGG TAG TCC AA-3’ AND 5’-CCG TAG CAT GTG ACG TG G AT-3’. This primer set amplifies a single DNA fragment of 206 bp or 302 bp from genomic DNA obtained from WT or HO mice, respectively, to the two fragments simultaneously in the case of HE mice. The procedure for genotyping mice was developed and set up in our laboratory by Victor Otano-Rivera (Undergraduate STREAMS student) and Amma Boayke (Undergraduate HONORS student) and was based on the original protocol written by Glenn Travis in Laboratory of Molecular Systematics, Smithsonian Institution. Washington, DC 20560, and the protocols of Walsh and collaborators and the one described by Morin and Woodruff in Paternity exclusion using multiple hypervariable microsatellite loci amplified from nuclear DNA of hair cells. Pages 63-81 of (Martin R.D. (Zürich) Dixson A.F. (Franceville) Wickings E.J. (Franceville). 1992). When genotypes were not clearly defined by the PCR, we troubleshooted this by blindly testing the animals and then regenotyping them from the tissues. All animals were fed on standard chow diet and water and were housed under 12h light: 12h darkness cycles.

4.4 Behavioral Testing

a. General Handling Procedures:

Animal handling was performed first. The animals were removed from home cages, placed in clean clothes and handled approximately 30 seconds each. All animals were handled before individual tests in order to minimize handling-related stress. All animals were acclimatized for at least 30 minutes in the testing room before each test. The behavioral tests were spaced by a week to minimize interference by previous
procedures. Tests were administered in ascending order of novelty in order to reduce
carry over effects. All observational scoring was checked for inter-rater reliability.
The behavioral studies are described below in the order in which they were
conducted.

b. Functional Observational Battery:

The functional observational battery is a noninvasive method used to detect gross
functional deficits prior to more specific neurobehavioral evaluation. It is used to
comprehensively assess and quantify the behavioral and physiological state of the
mouse. Due to the visually observed deficits in ambulation, a functional observational
battery was developed from a published industry version (Irwin, 1968) for use with
this group of animals. The body position, locomotor activity, bizarre behavior,
tremors, finger-withdrawal, touch escape, ataxic gait, and hypotonic gait are observed
and scored in these tests (Irwin, 1968).

First the animals were subjected to the least provoking stimuli which involved an
initial phase of undisturbed observation and a later manipulative phase. The
assessment in this phase and the undisturbed behavior i.e., body position, locomotor
activity, bizarre behavior, exophthalmos, respiration, tremors, twitches and
convulsions were assessed. The animals were then transferred and were briskly
dropped onto the floor of the viewing arena for testing the transfer arousal and spatial
locomotion. Observations were then made if tremors were present. Observations for
gait and limb rotation were also made. The categories measured included general
arousal, gait, reflexes, and motor behavior. Scoring of 0 to 8 range was used
throughout FOB, with values 0, 2, 4, 6, and 8 which represents none, slight, moderate,
marked and extreme magnitudes of behavior. The scores were summed for each category and then for over all categories (Irwin, 1968).

c. Open field test (OFT):
The locomotor activity and thigmotactic behavior can be evaluated using an open field chamber in which infrared photo-beams (Hamilton Kinder, Motor Monitor Version 3.11; Poway, CA, USA) are employed to measure locomotor activity in real time. The open field arena has dimensions of 40 X 40 cm and is divided into central (20X20 cm) and peripheral (10 cm wide) zones. The IR beams are interrupted by movements and sorted into different measures based on algorithms in the Motor Monitor Software (Lad et al. 2010; Tatem et al. 2014). Each mouse was placed in the center of the open field arena under the red light and allowed to explore it for 10 minutes. Measurements such as horizontal beam interruptions, vertical beam interruptions (rearing), distance traveled, time ambulating, spatial distribution of behaviors, and fine movements (repeated activation of one horizontal beam only) were measured. If mice spend more time in the area close to the wall then it is an indicator of increased stress level, whereas if they explore the center of the arena then it indicates that they can tolerate a mildly stressful environment. The open field arena was cleaned with 70% ethanol solution and let dry after testing each mouse.

d. Elevated Plus Maze (EPM):
This is a behavioral paradigm that takes advantage of the conflict behavior of rodents between exploration of a novel area and aversion to open and elevated spaces (Hata et al. 2001). The expression of scores as percentage of time and distance in the open arms allows for the correction of overall changes due to exploration of the maze, reducing the activity-induced artifacts (Hogg. 1996).
The device has the shape of a cross with each arm being of 5 X 35 cm. Two arms are open and two of them have walls of 15 cm high, with the arms connected by a 5X5 cm central square. The arms are 76 cm above the floor.

Mice were tested on the plus maze in a room with low, indirect incandescent red lighting and very low noise levels. The mouse was placed at the center of the maze with head facing the open arm and allowed to explore for 5 minutes. The number of entries into open and closed arms, as well as the time spent and distance travelled in open and closed arms was recorded with the help of an automated EPM (Hamilton Kinder, Version 3.11; Poway, CA). Animals sensitive to stressful environments avoid the open arms of the maze. The mazes are cleaned with 70% ethanol and permitted to dry between sessions. Automated software (Kinder Scientific Motor Monitor Software Package, Built 08356-14) kept a record of the number of entries, distance travelled (inches), and time spent (seconds) in the closed and open arms. Entry was determined when all the four paws of the mouse were on the arm.

e. Y maze activity:

The Y-maze measures a mouse’s working memory status and exploits the innate tendency to explore novel areas. The apparatus used for this test is an acrylic maze with 3 arms at 120 degrees to each other, each arm 3.5 cm wide and 20 cm long. The acclimatization period to the room was 1 hour. Then the animal is placed in the center of the apparatus. The sequence of arm entries (entry defined as all four paws within the arm), alternation sequence (entering three different arms in succession e.g. ABC or BCA) are video recorded for 8 minutes for each sequence. The percentage of alternation was determined by dividing the total number of alterations by the total number of choices minus 2, multiplied by 100.
f. Pre-pulse Inhibition (PPI):

This is a test to evaluate psychosis-like behavior. Mice were tested in automated startle chambers (SM100 Startle Monitor System Version 6.12; Hamilton Kinder, Poway, CA, USA) for PPI. They were calibrated prior to the day of testing. Mice were placed in the startle chamber and allowed to acclimate for 5 min. A background noise of 60 dB was on in between trials. The animals were then presented with 30 trials randomly mixed of startle stimuli (loud white noise) and pre-pulse stimuli (lower dB white noise preceded by loud). The startle stimuli consist of a 20 ms white noise stimulus presented at 2 different intensities (85 or 100 dB). The pre pulse stimulus consists of 20ms, 70 dB white noise pulse, presented 100ms prior to the startle (85 or 100 dB) stimulus (70dB + 100 ms + 85 dB and 70 dB + 100 dB). The inter trial intervals range from 9-16 s. The startle response was recorded on a pressure plate (Sharma, Elased, and Lucot, 2012).

g. Rotarod test:

The rotarod test is used to assess motor coordination by measuring the latency to fall off the rotating drum. The rod is 3cm in diameter and designed so that the mouse only drops 25cm onto a plate that stops a timer. The animals were moved to the experiment room from their home cages 30 minutes prior to testing. There were two protocols followed, one that used a constant speed (25rpm) for 2min and a second that used accelerating speeds for 5min (up to 30rpm). Both tests were conducted with all subjects. Learning sessions were conducted for 3 days in which the animals were placed on a rotarod (24rpm) for a maximum of 60s and the latency to fall off was recorded. The animals were then returned to their home cage for 5-10 minutes and then the session was repeated for 3 times each day of training. For the learning sessions (3 consecutive days): Each mouse was placed on a rotating rod (24 rpm) for a
maximum of 60s and the latency to fall off was recorded. In test session, two trails were conducted at a fixed speed with a maximum time of 60s, and an hour later the animal was tested on an accelerating rod. The animal was placed on a rotating rod and then the speed was increased every minute up to 44rpm for a maximum of 5 minutes. The latency was recorded when the animal falls. The same test was performed again 10 minutes later. This procedure was adapted from Current Protocols in Neuroscience, 2001 and Pallier, Drew, and Morton, 2008.

h. Forced Swim Test:

The Forced Swim Test (FST) is used to evaluate depression-like behavior. In this test, mice were placed in a transparent glass cylinder (diameter 10 cm, height 35 cm) which was filled up to 30 cm with water (23-25°C). The mice were tested separately in different cylinders with an opaque white plexi glass separating them so that the mice cannot see each other during the test. This testing is video recorded and scored for durations of immobility. After the test the mice were dried with a towel and returned to their home cage and placed on a thermal blanket heated to 37°C. A mouse was considered as immobile when floating motionless or making only those movements necessary to keep its head above the water (Castagné et al., 2001; Sharma et al., 2010).

Scoring

A new time-scoring technique was used to score several behaviors during a single viewing session which has been shown to be both reliable and valid for detecting antidepressant effects of both desipramine and fluoxetine in the FST (Detke et al., 1995). The scoring had three different categories scored based on definitions, “floating in the water without struggling and making only those movements necessary
to keep the head above water” which is considered immobility; “making active swimming motions, more than necessary to merely maintain the head above water” (i.e., moving around in the cylinder) called swimming; “making active movements with forepaws in and out of the water, usually directed against the walls” which is climbing; and “having entire body submerged” called diving. In these tests, diving occurred and was not reliably altered by the compounds tested, data for diving are not reported. All of the behavior scoring was done by a single rater, who was unaware of treatment condition. This new procedure to score many active behaviors in the FST was used to re-evaluate the effects of different types of anti-depressant drugs (Detke, Rickels, and Lucki, 1995). Selective norepinephrine (NE) uptake inhibitors, such as desipramine and maprotiline, reduced immobility and selectively increased climbing without affecting swimming. Previously effects of serotonin (5 hydroxytryptamine; 5-HT) uptake inhibitors in the FST were considered as false negatives in this test, but this scoring system revealed the effects of serotonin (5 hydroxytryptamine; 5-HT) uptake inhibitors in the FST, such as fluoxetine, sertraline, and paroxetine also reduced immobility but increased swimming instead of climbing. Thus, an analysis of behavioral patterns in the FST distinguished drugs that have a common therapeutic outcome and those that act on distinct neurotransmitter systems. To avoid these false-positives, tail suspension test (TST) can be used to test the depressive-like behavior which has different false positives. A literature search revealed monoaminergics (e.g., amphetamine, paroxetine)-induced decreases in immobility time which might be false positives in the forced swim test (Zhao et al. 2008).

i. Tail Suspension Test:

Mice were suspended by their tails for 6 minutes (50 Can, Adem 2012). The subject’s behavior was recorded with a video camera and scored afterwards. A suspension bar
(1 cm. height, 1 cm. width, 60 cm. length) and tape (Time Med Labelling Systems, Inc., Burr Ridge IL, 1.9 cm width were used. The mice were suspended by their tails by attaching them to the suspension bar with tape in a way that they cannot escape or hold on to nearby surfaces. The distance between mouse nose and floor was approximately 20-25 cm. The mice were separated from each other using opaque plastic to prevent interaction or observance between each other. A polycarbonate tray was placed at the bottom of each mouse suspension to collect feces and urine.

Some strains of mice (mainly C57s) have a behavior called tail climbing and can thus hold on to their tails during the test and negate it. We suspect that this strain of mouse will have that response because their background strain is C57Bl/6. In order to prevent this from happening a clear hollow cylinder approximately 4 cm long, 1.6 cm outside diameter, 1.3 cm inside diameter, and weighing 1.5 grams made from polycarbonate tubing was placed around the tails of the mice to prevent tail climbing behavior of the mice.

The animals were brought to the testing room an hour before testing. The climb stoppers were placed around the tail of the mouse and the tape was applied at the very end of the tail leaving 2-3 mm outside the tape. The mouse was suspended by placing the free end of the tape on the suspension bar. At the end of 6 minutes the animals were returned to their cages and the tape was removed by gently pulling it off.

4.5 Brain dissection

Animals were sacrificed by decapitation. The brains were removed and the frontal cortex was hand dissected. The remainder of the brain was placed in a brain matrix (Ted Pella, Inc) and sliced into 1mm or 2 mm slabs rostral to the intraneural line, (Caudate : 5-4, Amygdala/Hypothalamus: 3-2, Hippocampus: 2-1, Brain stem1: 1-0,
Brain stem2: 0- -1) which were then stored at -80°C on plastic squares until further dissection. Amygdala and the caudate nucleus were dissected using landmarks in The Mouse Brain Stereotaxic Coordinates (Paxinos and Franklin, 2008) as a guide. Accumbens was taken using a tissue punch (-20°C ice blocks) of 1mm thickness. Other sections were dissected using landmarks in The Mouse Brain Stereotaxic Coordinates (2008) as a guide.

Figure 7 - Brain blocker – used to dissect the brain into 1 mm slabs.
4.6 Neurochemical studies

a. Preparation of tissue samples

All the brain tissues were homogenized in 0.2N HCl using a homogenizer followed by centrifugation. The supernatants were collected into 2 aliquots, which were stored at -80°C until they were analyzed by HPLC.
b. Determination of catecholamines by High Performance Liquid Chromatography (HPLC)

HPLC is high performance liquid chromatography which is used to separate, identify and quantify the components in a mixture. Its function relies on a high-pressure pump which passes a pressurized liquid solvent containing the sample through a short, narrow column which is filled with a solid adsorbent material. Each component of the sample interacts differently with the adsorbent material causing different retention times of each component which helps in separation as they flow through the column. The electrochemical detector attached to the machine generates a signal proportional to the amount of the sample component eluting from the column. Here we used BAS LC-4B and BAS LC-4C detector with a potential of 0.75V and maintained across a glossy carbon working electrode with a positive radial flow. This electrochemical (amperometric) detector is attached to the system to identify the separated components of the sample as they get eluted from the column. In this amperometric cell the column effluent passes electrodes to which a voltage is applied. As the molecules in the column effluent pass the electrodes they become oxidized when the potential is sufficiently large. The amount of current drawn to drive the electrochemical reaction is quantified and concentration determined by reference to an external standard.

The supernatant (sample) in the aliquot is injected directly into the chromatographic system. Chromatographic separation was conducted using a BAS 100 X 3 mm C18 column (BAS model MF-8954), 80 X 4.6mm C18 column (ESA model 68-0100) optimized to 0.75V. The mobile phase consisted of soap (1-octanesulfonic acid), acetonitrile and dimethyl acetamide. The flow rate was kept at 0.3ml/min.
c. Brain monoamine analysis

To identify monoamines, a series of standard solutions containing known amounts of monoamines including norepinephrine (NE), 3,4-dihydroxyphenylacetic acid (DOPAC), 5-Hydroxy Indole acetic acid (5-HIAA), Homovanillic acid (HVA), Dopamine (DA) and serotonin (5-HT) were injected into the chromatography system before and after each sample analysis. The concentrations of monoamines in the samples were determined by comparing the peak heights from the samples relative to that of the standards. The concentrations of NE, DA, HVA, DOPAC, 5-HIAA and 5-HT were expressed as ng/mg of wet tissue. The ratio between the metabolite and the parent compounds (e.g. HVA/DA, DOPAC/DA, 5-HIAA/5-HT) was used as a surrogate index of neurotransmitter usage.

4.7 Spot Urine Collection

Urine was collected from each animal by gently scruffing the animal and placed it on clean surface until it urinated. The urine was collected using a pipette and placed in a micro tube which was placed in ice until storage at -80°C until analysis. Subjects that did not urinate after 30s were placed in a clean empty cage until they did.

4.8 Urine catecholamine extraction

Catecholamines in spotted urine were extracted over neutral alumina, activity grade 1, from Sigma Chemical Co. product A-9003 supplemented with an internal standard consisting of 2,3-dihydroxybenzoic acid (DHBA). This preliminary extraction over alumina is necessary for down-stream electrochemical detection of urine catecholamines. This procedure removes electroactive species in the urine that cannot be separated by HPLC (Smith et al. 2013). Extraction was done as follows: alumina was placed in 1.5mL tubes and supplemented with HCl containing sodium
metabisulfite. To assay samples spot urine, DHBA internal standard and tris buffer were added. The pH was then adjusted to 8.5±0.05 by adding Tris buffer. Then the tube was vortexed, centrifuged and then the supernatants were removed and diluted with deionized water. This was done for 2 times. Then the supernatants were removed and further diluted with HClO₄ containing sodium metabisulfite. The tube was then vortexed; centrifuged and the supernatants were aliquoted into different 0.5 mL tubes and stored at -80°C until analysis (Smith et al. 2013).

4.9 Determination of catecholamines by High Performance Liquid Chromatography (HPLC)

To determine catecholamines in these processed samples, 20µL of them were directly injected into the chromatographic system. Chromatographic separation was conducted using 80 X 4.6mm C18 column (ESA model 68-0100) optimized to 0.55V. The mobile phase consisted of soap (1-octanesulfonic acid), methanol and acetonitrile. The flow rate was kept at 0.3ml/min.

4.10 Adrenal isolation and preparation of the tissue sample

Animals were sacrificed by decapitation and then the adrenal glands were dissected, weighed onto empty 1.5ml tubes and labelled with the animal identification number. A single weighed adrenal gland was submerged in PBS buffer and DHBA stock (as an internal standard) and homogenized in the buffer using a homogenizer keeping the samples on ice in between homogenizing cycles. The homogenates were added to a tube containing HClO4 to be filtered and centrifuged at. Supernatants were then collected into 3 aliquots and stored at -80°C until HPLC analysis.
5. Data Analysis

A DataQ device attached to the detector records the waveforms and using Windaq software.

The data were analyzed by using STATISTICA data analysis software (Stat soft, Tulsa, OK). The results are presented as group means±S.E.M. and analyzed using STATISTICA data analysis software. Data were analyzed by 1-way ANOVA test. It was used to evaluate the overall significance among groups. Tukey-HSD post hoc test was used to identify individual group differences. We also used Kruskal Wallis one way analysis of variance in the FOB and rotarod test.

6. Results

6.1 Behavioral tests

a. Functional Observational Battery

The NKCC2A-HO had significant differences in its walking behavior which is gait. The HO mice group had higher scores compared to the HE and WT mice. As shown in Fig.9, there were significant differences in the gait category (Fig 9) with NKCC2A-HO having higher gait score compared to NKCC2A-HE and WT [factor ‘genotype’ F (2, 28) = 3.524, p < 0.05 vs WT and HE].

b. Forced swim test

As shown in Fig.10, NKCC2A-HO mice were immobile for significantly less time compared to WT and HE mice during the 6 minute forced swim test [factor ‘genotype’ F (2, 28) = 31.044, p < 0.001 vs WT and HE].
c. Tail suspension test
As shown in Fig.11 there were no significant differences between mice of the three genotypes during the 6 minute lasting tail suspension test [factor ‘genotype’, F (2, 22) = 0.59, p >0.05 vs WT and HE].

d. Peripheral Rest time
As shown in Fig.12, during the 10 min of the test session in the open field NKCC2A-HE mice spent significantly more time in the periphery than in the central zone [factor ‘genotype’ F (2, 28) = 5.7466, p < 0.05 vs WT and HO].

e. Rearing
Fig.13 shows a significant differences between NKCC2A-WT, NKCC2A-HE and NKCC2A-HO mice in the rearing phenomena in the open field test [factor ‘genotype’ F (2, 28) = 4.03, p < 0.05].

6.2 Neurochemical Analysis

a. The caudate of NKCC-HO mice exhibit low 5-HIAA/5-HT ratios
The 5-HIAA/5-HT ratio in the caudate of NKCC2A-HO was significantly low (Fig. 14a, p<0.05) when compared to NKCC2A WT (Fig. 14a) [factor ‘genotype’ F (2, 28) = 4.514, p < 0.05 vs WT].

b. The caudate of NKCC2A-HO mice exhibit low DOPAC/DA ratios
The DOPAC/DA ratio in the caudate of NKCC2A-HO was significantly low (Fig. 14b, p<0.05) when compared to NKCC2A-HE and WT (Fig. 14b) [factor ‘genotype’ F (2, 28) = 5.04, p < 0.05 vs WT and HE].
c. The septum of NKCC2A-HE mice exhibit high DOPAC/DA ratios

The DOPAC/DA ratio in the septum of NKCC2A-HE mice was significantly higher than that of NKCC2A-HO and WT mice (Fig. 14c, p<0.05). However, there were no significant differences in individual neurotransmitter levels [factor ‘genotype’ F (2, 28) = 4.07, p < 0.05 vs WT and HO].

6.3 Urine

As shown in Fig 15a, 15b, 15c, there were no significant differences in urine NE, EPI and DA levels between the mice of three genotypes.

6.4 Adrenals

There was a significant decrease in the NE (Fig 16a) levels in NKCC2A-HO mice when compared to NKCC2A-HE and WT [factor ‘genotype’ F (2, 23) = 4.12, p < 0.05 vs WT].

There was a significant decrease in the EPI (Fig. 16b) levels in NKCC2A-HO mice when compared to NKCC2A-HE and WT [factor ‘genotype’ F (2, 23) = 4.744, p < 0.05 vs WT].
7. Results

7.1 Behavior Results

Figure 9 - Functional Observational Battery
Sum of the arousal category and the gait categories in WT, HE and HO. The HO group had significantly higher gait scores [factor ‘genotype’ F (2, 28) = 3.524, p < 0.05 vs WT and HE].

Figure 10 - Forced swim test
Immobility time. Immobility of the HO group was lower compared to the WT and HE [factor ‘genotype’ F (2, 28) = 31.044, p < 0.001 vs WT and HE].
Figure 11 - Tail suspension test

Immobility time. No significant changes in the Immobility of the HO group compared to the WT and HE [factor ‘genotype’, F (2, 22) = 0.59, p >0.05 vs WT and HE].

Figure 12 - Open field test – Peripheral resting time

Time (sec) spent resting in the periphery during a 10 min test. HE mice were different from WT and HO mice. No other differences were significant [factor ‘genotype’ F (2, 28) = 5.7466, p < 0.05 vs WT and HO].
Figure 13 - Open field test - Rearing

Total number of rearing events in the ten min test. The WT were lower than either HE or HO [factor ‘genotype’ F (2, 28) = 4.03, p < 0.05].

7.2 Neurochemistry Results

Figure 14a - 5-HIAA/5-HT ratios in caudate

5-HIAA/5-HT ratios observed in the caudate area of WT, HE and HO mice [factor ‘genotype’ F (2, 28) = 4.514, p < 0.05 – significant difference from WT].
Figure 14b - DOPAC/DA ratios in caudate

DOPAC/DA ratios in caudate area of WT, HE and HO mice [factor ‘genotype’ F (2, 28) = 5.04, p < 0.05 – significant difference from WT and HE].

Figure 14c - DOPAC/DA ratios in septum

DA usage levels in Septum area of WT, HE and HO mice [factor ‘genotype’ F (2, 28) = 4.07, p < 0.05 – significant difference from WT and HO].
7.3 Spot Urine Results

Figure 15a - Norepinephrine levels in spot urine

Figure 15b - Epinephrine levels in spot urine
Figure 15c - Dopamine levels in spot urine

7.4 Adrenal Results

Figure 16a - Norepinephrine levels in adrenals

Norepinephrine levels observed in adrenals of WT, HE and HO mice [factor ‘genotype’ F (2, 23) = 4.12, p < 0.05 – significant difference from WT].
Figure 16b - Epinephrine levels in adrenals

Epinephrine levels observed in adrenals of WT, HE and HO mice [factor ‘genotype’ F (2, 23) = 4.744, p < 0.05 – significant difference from WT].
<table>
<thead>
<tr>
<th>Behavioral test</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Functional operational battery (FOB)</td>
<td>Increased gait in NKCC2-HO compared to the WT and HE</td>
</tr>
<tr>
<td>Open field test (OFT)</td>
<td>Higher rearing in NKCC2A-HO mice compared to the WT and HE</td>
</tr>
<tr>
<td>Elevated plus maze (EPM)</td>
<td>No differences in anxiety across the three groups</td>
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<tr>
<td>Y maze test</td>
<td>No significant spontaneous alterations across the 3 groups</td>
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<tr>
<td>Prepulse inhibition (PPI)</td>
<td>No significant disruption in PPI</td>
</tr>
<tr>
<td>Rotarod</td>
<td>No differences in motor coordination and balance among the 3 groups</td>
</tr>
<tr>
<td>Forced swim test (FST)</td>
<td>Increased immobility time of the NKCC2A-HO mice compared to the WT and HE</td>
</tr>
<tr>
<td>Tail suspension test (TST)</td>
<td>No significant change in immobility time of the NKCC2A-HO mice compared to the WT and HE</td>
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</table>

**Table 2 - Summary of Behavioral results**

<table>
<thead>
<tr>
<th></th>
<th>Accumbens</th>
<th>Amygdala</th>
<th>Brain stem</th>
<th>Caudate</th>
<th>Hypothalamus</th>
<th>Septum</th>
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<td>Higher in HE</td>
<td>Higher in HE</td>
<td>Higher in HE</td>
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<tr>
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<td>Lower in HO</td>
<td>Higher in HE</td>
<td>Lower in HO</td>
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<td>Lower in HO</td>
<td>Higher in HE</td>
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<tr>
<td></td>
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<td>HVA/DA</td>
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<td>5-HIAA/5-HT</td>
<td>Lower in HO</td>
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Table 3 - Summary of Neurochemistry results
8. Discussion

We performed a preliminary characterization of the behavioral and neurochemical phenotypes of mice lacking a single (HE) or both (HO) alleles of NKCC2A and compared the results against WT. The HE tissues exhibit intermediate level of NKCC2A expression, whereas HO does not show any expression with high expression levels in the WT indicating all the three groups have different levels of NKCC2A expression and its effect might be different across the groups. Based on anecdotal behavior observations noted in NKCC2A-HO mice, we evaluated them by using a functional observational battery followed by specific behavioral tests including open field test, y maze activity, elevated plus maze, pre-pulse inhibition, rotarod test, forced swim test, and tail suspension test.

There were few visually observed deficits in ambulation of the NKCC2A-HO mice. A functional observational battery developed from a published industry version (Irwin, 1968) was used with this group of animals which measured the categories of general arousal, gait, reflexes, and motor behavior. The pattern of movement of the limbs during locomotion is gait and abnormal gait is observed in several neurological and musculoskeletal disorders. There were significant differences in the gait category with the NKCC2A-HO group having higher impairment but no significant differences were observed in rotarod test which suggest that NKCC2A might be involved in areas affecting gait rather than balance. Abnormality in gait generally indicate some gross functional deficits in the CNS which were later assessed using specific behavioral tests. Therefore, this suggests that the NKCC2 might affect the CNS in areas involved in locomotor activity like walking.
In FST, which is used to detect the anti-depressant like effect, the NKCC2A-HO mice displayed significantly lower immobility time compared to the HE and the WT mice (Fig 2) which was not reproduced by TST. Motor stimulants (Betin et al. 1982, De Pablo et al. 1989) generally produce false positives in the FST (Panconi et al. 1993). Nicotine (1mg/kg) has a weak but significant anti-depressant like effect consistent with the excitatory substances which constitute false positives for the FST (Porsolt et al. 1978) (Panconi et al. 1993). However, in this study, there were no significant differences in the motor activity (basic movement and fine movement) of NKCC2A-HO mice. Thus, the decrease in immobility time in FST in NKCC2A-HO was independent of changes in locomotor activity suggesting that NKCC2A might have an effect related to the swimming challenge in the FST rather than behavioral despair challenge in the TST.

There might be the effect of thermogenesis in the FST that is producing decreased immobility in NKCC2A-HO mice. Sympathetic nervous system and thyroid hormone play a major role in generation and regulation of heat (Silva. 2006). Interestingly, preliminary results from our laboratory indicate that NKCC2 mRNAs are expressed in the thyroid at very low levels. Therefore, NKCC2A-HO mice might experience hypothermia in FST condition leading to decreased immobile behavior which cannot be observed in temperature-insensitive TST.

There were lower 5-HT and intraneural DA usage in the caudate nucleus which is mainly associated with motor activity, suggesting its role in the FST effect. Caudate nucleus receives projections from the A9 group of dopaminergic cell bodies which are located in the substantia nigra (Feldman. 1997). The significant neurochemical change in the caudate dopaminergic system was not supported by the results of the locomotor activity in the open field test indicating that NKCC2A is not effecting the overall
motor activity. But, there is a study showing significant elevation in prodynorphin (pDyn) mRNA levels following FST in the caudate putamen but not in the nucleus accumbens, hypothalamus, amygdala, frontal cortex or hippocampus indicating that caudate might be solely involved in this process (Reed et al. 2012). No changes in the DA and 5-HT usage in frontal cortex, amygdala, nucleus accumbens and hypothalamus which are common areas involved in both FST and TST explain that these areas might not be responsible for the immobile behavior in the tests. We suggest that NKCC2A might have an effect on DA system in the caudate nucleus but not in the other areas involved in motor activity.

In the open field test, there were no significant differences in locomotor activity which measures the fine movements (usually grooming), distance traveled in total or in zones or total time spent in either zone. The NKCC2A-HO exhibited a higher level of rearing compared to the WT mice in the open field test which may relate to more exploratory behavior in these mice. There was also a difference in the time spent resting in the periphery exhibiting thigmotactic behavior. However, this has uncertain significance in the absence of any difference in total time in the periphery, distance traveled in the periphery or any measurement in the central zone.

The elevated plus maze revealed no significant differences among groups in the time spent either in the open arm or in the closed arm. This is usually interpreted as a lack of difference in their ability to tolerate a stressful environment. This test is strongly responsive to benzodiazepine type anti-anxiety treatments and not to other classes of anxiolytics. No significant differences among the groups suggest that the GABA and benzodiazepine receptors are functionally normal which generally effect anxiety and performance in the elevated plus maze.
Y maze testing revealed no differences among mice of the three genotypes. Frontal cortex is the anatomical site for working memory and dopamine neurotransmission in the frontal cortex plays a major role in y-maze testing. The working memory was not different across the three groups. This observation is consistent with absence of significant changes in the DA usage in frontal cortex.

We used prepulse inhibition (PPI) of startle to evaluate genotype dependent effect on psychosis-like symptoms in NKCC2A-HO mice. There were no significant differences among groups in PPI of startle which measures the inhibitory response to a loud white noise stimulus (85 or 100 dB) when it is immediately preceded by a softer (70 dB) white noise. This is a basic circuit present in all normal species but deficient in schizophrenics. It is used as a screen for antipsychotic drugs by measuring their ability to reverse impairment of the PPI produced by either a dopamine antagonist or dopaminergic neurotransmission in frontal cortex (Zavitsanou et al. 1999) or phencyclidine (Yee et al. 2004; Bakshi and Geyer. 1997). There was no disruption in the PPI behavior which indicates absence of psychosis-like symptoms in the NKCC2A-HO mice and also indicating that NKCC2A-HO did not affect the hearing or sound perception. Interestingly, NKCC2 is expressed in the endolymphatic sac (Nishimura et al. 2009). Together, these results suggest the possibility that either NKCC2B or NKCC2F are expressed in ES or that NKCC2A has a minimal if any role in audition. Dopaminergic neurotransmission in the frontal cortex plays a major role in the modulation of startle reflex measured by PPI (Zavitsanou et al. 1999; Yee et al. 2004; Bakshi and Geyer. 1997; Mehler-Wex et al. 2006). Absence of changes in PPI correlates with the insignificant changes in the frontal cortex and amygdala dopamine usage suggesting that NKCC2A is not affecting the dopamine projections innervating these areas.
There was a significant decrease in the adrenal NE and EPI levels in the NKCC2A-HO mice compared to the wild type and heterozygous mice (Fig 16A, 16B). SLC12A1 gene was found to be expressed in supraoptic and paraventricular neurons of the hypothalamo-neurohypophyseal system during salt load condition. The interactions among the hypothalamus, pituitary and adrenal gland forms the major part of the neuroendocrine system that controls stress related reactions. Expression of NKCC2 in the hypothalamo-neurohypophyseal system, a specialized brain region involved in the elaboration of peptide hormones that directly control the kidney function explains both the central and peripheral role of NKCC2 in the crucial mechanisms to regulate salt and water balance. In the kidney NKCC2 is regulated by arginine vasopressin (AVP). AVP released from an endocrine source or paracrine/autocrine source might provoke NKCC2 synthesis and activity (Konopacka et al. 2015). Thus we speculate that the NKCC2A-HO mice which had low NE and EPI adrenal levels but not significantly lower urinary catecholamine levels, compromise their ability to respond to the sympathetic stimulation.

**Further studies**

There is preliminary evidence of the presence of NKCC2A in some sections of the brain such as the frontal cortex, somatosensory cortex and in the cerebral cortex. Its expression was much higher in the WT mice compared to the HE mice and with no expression in the HO mice (unpublished observation). We did not perform tests to evaluate learning and cognitive behavior, there can be further investigations focused on the attention and cognitive abnormalities of the NKCC2A-HO mice as well as studies related to these brain areas showing NKCC2A expression, to determine the effect of its presence in these areas and to pursue the FST and TST discrepancy which is an interesting finding. Because of greater variability in urinary catecholamine
levels, we could not conclude there was a significant effect of NKCC2A on sympathetic activity. High salt-loaded rats were observed to have elevated NKCC2 expression (Haque et al. 2011), therefore there is a need to look at the urinary catecholamine levels in stress (salt-load) induced mice. Further studies can be focused on stress induced urinary catecholamine levels to see the effect of NKCC2A and also with a greater ‘N’ to increase the statistical significance of the study.
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*Note:* The results of this Master’s Thesis and their interpretations have been presented in part or in whole in Scientific Meetings or have been submitted for publication. The data presented are the result of a collaborative arrangement between the laboratories of Dr Lucot and Dr Di Fulvio, which were responsible for the generation of data, analysis and interpretation, and the maintenance, genotyping and characterization of the NKCC2A colony of mice, respectively.

Finally I would like to thank my parents, my siblings, and my friends for being with me and for their moral support.
10. References


