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CELL-TYPE SPECIFIC DISTRIBUTION OF CHLORIDE TRANSPORTERS IN THE RAT SUPRACHIASMATIC NUCLEUS

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Abstract—The suprachiasmatic nucleus (SCN) is a circadian oscillator and biological clock. Cell-to-cell communication is important for synchronization among SCN neuronal oscillators and the great majority of SCN neurons use GABA as a neurotransmitter, the principal inhibitory neurotransmitter in the adult CNS. Acting via the ionotropic $GABA_A$ receptor, a chloride ion channel, GABA typically evokes inhibitory responses in neurons via Cl^- influx. Within the SCN GABA evokes both inhibitory and excitatory responses although the mechanism underlying GABA-evoked excitation in the SCN is unknown. GABA-evoked depolarization in immature neurons in several regions of the brain is a function of intracellular chloride concentration, regulated largely by the cation-chloride cotransporters NKCC1 (sodium/potassium/chloride cotransporter for chloride entry) and KCC1-4 (potassium/chloride cotransporters for chloride egress). It is well established that changes in the expression of the cation-chloride cotransporters through development determines the polarity of the response to GABA. To understand the mechanisms underlying GABA-evoked excitation in the SCN, we examined the SCN expression of cation-chloride cotransporters. Previously we reported that the K^+/Cl^- cotransporter KCC2, a neuron-specific chloride extruder conferring GABA's more typical inhibitory effects, is expressed exclusively in vasoactive intestinal peptide (VIP) and gastrin-releasing peptide (GRP) neurons in the SCN. Here we report that the K^+/Cl^- cotransporter isoforms KCC4 and KCC3 are expressed solely in vasopressin (VP) neurons in the rat SCN whereas KCC1 is expressed in VIP neurons, similar to KCC2. NKCC1 is expressed in VIP, GRP and VP neurons in the SCN as is WNK3, a chloride-sensitive neuron-specific with no serine–threonine kinase which modulates intracellular chloride concentration via opposing actions on NKCC and KCC cotransporters. The heterogeneous distribution of cation-chloride cotransporters in the SCN suggests that Cl^- levels are differentially regulated within VIP/GRP and VP neurons. We suggest that GABA's excitatory action is more likely to be evoked in VP neurons that express KCC4.

Key words: circadian rhythms, GABA, KCC2, KCC4, NKCC1, WNK3.

The hypothalamic suprachiasmatic nucleus (SCN) is a circadian oscillator which functions as a biological clock (Hastings et al., 2003; Herzog, 2007; Pickard and Sollars, 2008). The SCN generates an endogenous rhythm in neural activity in the absence of external temporal cues with action potential firing rate high during the subjective day and low during the subjective night (Brown and Piggins, 2007). Many SCN neurons function as autonomous oscillators and cell-to-cell communication is responsible for synchronization among neuronal oscillators and between sub-regions within the SCN (Welsh et al., 1995; Yamaguchi et al., 2003; Quintero et al., 2003; Herzog et al., 2004; Albus et al., 2005; Aton and Herzog, 2005). The majority of SCN neurons contain GABA and express glutamate decarboxylase, a key enzyme of GABA synthesis (Moore and Speh, 1993; Belenky et al., 1996, 2008; Castel and Morris, 2000). Most synaptic terminals within the mammalian SCN are GABAergic (van den Pol, 1986), SCN neurons are interconnected by GABAergic synapses (Strecker et al., 1997), and both GABA receptor A subtype ($GABA_A$) and GABA receptor B subtype ($GABA_B$) receptors are widely, although unevenly, distributed throughout the SCN (Gao et al., 1995; Belenky et al., 2003, 2008). GABA appears to play an important role in intra-SCN network activity and synchronization of firing rhythms among SCN neurons (Liu and Reppert, 2000; Shirakawa et al., 2000; Albus et al., 2005) although it has also been reported that GABA signaling is not required for synchronization among SCN neurons (Aton et al., 2006).

Although GABA is typically considered an inhibitory neurotransmitter in the adult nervous system, GABA has been reported to evoke excitatory responses in the SCN. Wagner and colleagues (1997) first reported a day/night difference in GABA's action in the SCN with GABA decreasing firing frequency during the subjective night but increasing firing frequency during the subjective day. Whereas some investigators have reported only inhibitory

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Abbreviations: BSA, bovine serum albumin; $[Cl^-]i$, intracellular chloride concentration; DAB, 3,3′-diaminobenzidine tetrahydrochloride; $GABA_A$, GABA receptor A subtype; $GABA_B$, GABA receptor B subtype; GRP, gastrin releasing peptide; IgG, immunoglobulin G; K^+/Cl^-, potassium/chloride; KCC, potassium/chloride cotransporter; $Na^+/K^+/2Cl^-$, sodium/potassium/chloride; NKCC, sodium/potassium/chloride cotransporter; PBS, phosphate buffered saline; PFA, paraformaldehyde; RT, room temperature; SCN, suprachiasmatic nucleus; TBS, tris buffered saline; VIP, vasoactive intestinal peptide; VP, vasopressin; WNK, with no lysine; ZT, zeitgeber time.

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effects of GABA in the SCN at all phases of the circadian cycle (Gribkoff et al., 1999, 2003), others have reported nocturnal excitatory effects of GABA (De Jeu and Pennartz, 2002). Albus and co-workers (2005) reported excitatory responses to GABA in the SCN but with a much higher incidence of GABA-evoked excitation in the dorsal as compared to the ventral SCN and these responses were more evident during the late day/early night. In a recent study by Choi and colleagues (2008), most GABA-evoked responses observed in the SCN were inhibitory but some GABA-mediated excitation was observed in both the dorsal and ventral SCN irrespective of the time of day. However, GABA-evoked excitatory responses were most commonly observed during the night in the dorsal SCN region (Choi et al., 2008). There is growing consensus that GABA can evoke excitatory responses in mature SCN neurons, however it remains unclear if these responses are restricted to particular phases of the circadian cycle and/or to particular cell types or sub-regions of the nucleus.

The cellular mechanisms underlying GABA-evoked excitation in the SCN are unknown. It is well documented that GABA acts as an excitatory neurotransmitter early in the development of the CNS (Payne et al., 2003; Ben-Ari et al., 2007). The ionotropic $GABA_A$ receptor is a Cl^- channel that opens upon GABA binding. In immature neurons, $GABA_A$ receptor-mediated responses are depolarizing and facilitate the generation of Na^+ and Ca^{2+} currents whereas later during development and in mature neurons, $GABA_A$ receptor-mediated responses are hyperpolarizing. The shift in GABAergic responses during development is related to the expression and function of cation-chloride cotransporters (Rivera et al., 1999).

Electroneutral in nature, cation-chloride cotransporters do not generate any current but rather contribute to the inwardly or outwardly directed net flux of ions generated by gradients that are set by active transporters such as Na^+/K^+-ATPase. Thus the neuronal K^+ gradient is employed by the K^+/Cl^- cotransporter (KCC) to extrude Cl^- from the cell whereas the $Na^+/K^+/2Cl^-$ cotransporters (NKCC) use the neuronal Na^+ gradient to increase intracellular Cl^- concentration ($[Cl^-]_i$). During early postnatal development, expression of NKCC1 is high, raising $[Cl^-]_i$. As development proceeds, KCC2 expression increases, lowering intracellular Cl^- resulting in the characteristic inhibitory responses to GABA (Rivera et al., 1999; Payne et al., 2003).

It has been suggested that $[Cl^-]_i$ may be dynamically regulated within the SCN (Wagner et al., 1997, 2001) and a circadian rhythm in $[Cl^-]_i$ has been reported in acutely dissociated SCN neurons (Shimura et al., 2002). The role of cation-chloride cotransporters in regulating $[Cl^-]_i$ in the mammalian SCN has only recently begun to be examined. Using *in situ* hybridization, mRNA expression for two of the four known isoforms of the K^+/Cl^- cotransporter, KCC1 and KCC2 were localized to the ventrolateral SCN (Kanaka et al., 2001). KCC2 is neuron specific and is thought to be the main chloride extruder in the nervous system whereas KCC1 is widely expressed in different tissues and is considered to be a "housekeeping" transporter (Hebert et al., 2004). KCC2 protein was subsequently shown to be expressed exclusively in vasoactive intestinal peptide (VIP) and gastrin-releasing peptide (GRP) neurons located in the ventral and ventrolateral SCN; no KCC2 was observed in neurons expressing vasopressin (VP) (Belenky et al., 2008). KCC4 mRNA has also been described in the SCN whereas the same study found no evidence for KCC3 expression in the SCN (Le Rouzic et al., 2006).

In the present study using immunocytochemical procedures at both the light and electron microscopic level of examination, we describe the distribution of KCC4, KCC3 and KCC1 in the SCN. In addition we examine the distribution of NKCC1, a chloride cotransporter responsible for Cl^- influx, and with WNK3, a chloride-sensitive with no lysine protein kinase that modulates cellular Cl^- flux by altering the phosphorylation state of cation-chloride cotransporters (Kahle et al., 2008). To define more precisely the cellular localization of these proteins within the SCN, double-label immunocytochemical experiments were conducted to determine whether the cotransporters were expressed in a cell-type specific manner in association with VIP, GRP, or VP neurons.

EXPERIMENTAL PROCEDURES

Animals and tissue preparation

Thirty-two adult male rats of the Sprague–Dawley strain (Harlan Israel, Jerusalem, Israel) were used for light and electron microscopic immunocytochemical analysis of chloride transporters in the SCN. Animals were housed in cages with food and water available *ad libitum* and maintained under a 12 h light:12 h dark cycle with lights on at 7:00 h. To analyze transporter expression at different stages of the light/dark cycle, a group of rats (n=5) was maintained under an inverted day/night cycle with lights on at 19:00 h; all animals were killed between 11:00 and 13:00 h. All procedures used in the study adhered to guidelines approved by the Hebrew University of Jerusalem Animal Care and Use Committee and conform to National Institutes of Health (USA) guidelines. All possible efforts were made to minimize animal suffering and the number of animals used.

Prior to fixation, rats were deeply anaesthetized with pentobarbital (40 mg/kg) ip. After opening the thorax, approximately 100 μl of heparin (5000 IU/ml; Choay, Paris, France) were injected into the left cardiac ventricle, followed by transcardiac perfusion with phosphate buffered saline (PBS) (0.1 M phosphate buffer (PB) containing 0.9% sodium chloride; pH 7.4) and then by perfusion with 300 ml of freshly prepared fixative containing either 4% paraformaldehyde (PFA) or 4% PFA solution enriched with 0.25% glutaraldehyde (both from Electron Microscopy Sciences, Fort Washington, PA, USA) in 0.1 M PB. Brains were dissected, trimmed and stored in the same fixative for an additional 4–5 h at room temperature (RT). The brains were serially sectioned in the coronal plane with a Vibratome (Technical Products International, St. Louis, MO, USA) at 30 or 50 μm for light and electron microscopy, respectively, and sections through the SCN region were collected in ice-cold PBS.

Colchicine injections

To increase accumulation of neuropeptides and chloride cotransporter proteins within SCN cells, rats (n=9) were anaesthetized with sodium pentobarbital (40 mg/kg) ip, placed into a BenchMark Angle One stereotaxic instrument (http://myNeuroLab.com, St.