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THE TIME COURSE OF SEROTONIN 2C RECEPTOR EXPRESSION AFTER SPINAL TRANSECTION OF RATS: AN IMMUNOHISTOCHEMICAL STUDY

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Abstract—In the spinal cord serotonin (5-HT) systems modulate the spinal network via various 5-HT receptors. Serotonin 2A receptor and serotonin 2C receptor (5-HT2A and 2C receptors) are likely the most important 5-HT receptors for enhancing the motoneuron excitability by facilitating the persistent inward current (PIC), and thus play an important role for the pathogenesis of spasticity after spinal cord injury. In conjunction with our 5-HT2A receptor study, using a same sacral spinal transection rat model we have in this study examined 5-HT2C receptor immunoreactivity (5-HT2CR-IR) changes at seven different time intervals after spinal injury. We found that 5-HT2CR-IR was widely distributed in different regions of the spinal gray matter and was predominantly located in the neuronal somata and their dendrites although it seemed also present in axonal fibers in the superficial dorsal horn. 5-HT2CR-IR in different regions of the spinal gray matter was seen to be increased at 14 days after transection (with an average ~1.3-fold higher than in sham-operated group) but did not reach a significant level until at 21 days (~1.4-fold). The increase sustained thereafter and a plateau level was reached at 45 days (~1.7-fold higher), a value similar as that at 60 days. When 5-HT2CR-IR analysis was confined to the ventral horn motoneuron somata (including a proportion of proximal dendrites) a significant increase was not detected until 45 days post-operation. 5-HT2CR upregulation in the spinal gray matter is confirmed with Western blot in the rats 60 days post-operation. The time course of 5-HT2CR upregulation in the spinal gray matter and motoneurons was positively correlated with the development of tail spasticity (clinical scores). This indicates that 5-HT2CR is probably an important factor underlying this pathophysiological development by increasing the excitability of both motoneurons and interneurons.

Key words: spinal cord injury, spasticity, serotonin, serotonin receptor, immunohistochemistry.

INTRODUCTION

In the central nervous system serotonin (5-HT) exerts a large number of functions via different 5-HT receptors, which consist of at least seven different types and 14 subtypes (Nichols and Nichols, 2008). With exception for the 5-HT3 receptors, which belong to ligand-gated ion channels, all other 5-HT receptors belong to the G-protein-coupled receptor (GPCR) family. Among these, 5-HT2 is one of the most extensively investigated receptor type, including 5-HT2A, B and C subtypes (serotonin 2A receptor, serotonin 2B receptor and serotonin 2C receptor (5-HT2AR, 5-HT2BR and 5-HT2CR)), and all of them were found to be expressed in the spinal cord although studies on 5-HT2BR are rare (Marlier et al., 1991; Thor et al., 1993; Helton et al., 1994; Pompeiano et al., 1994; Fonseca et al., 2001; Doly et al., 2004; Holmes, 2005; Murray et al., 2011; MacFarlane et al., 2011). In the spinal cord several 5-HT receptor subtypes, especially the 5-HT1A receptor (5-HT1AR), 5-HT2AR and 5-HT2CR, seem to be among the most important receptors with respect to the regulation of normal spinal motor functions and the functional recovery after spinal cord injury (Kim et al., 2001; Zhou et al., 2001; Antri et al., 2002, 2003, 2005; Fuller et al., 2005; Kao et al., 2006; Lee et al., 2007; Halberstadt et al., 2009; for review see Schmidt and Jordan, 2000). For example, evidence from animal experiments has shown that the effects of long-term stimulation of 5-HT receptors on locomotor recovery following spinal cord transection are mediated, at least partially, by 5-HT1ARs, 5-HT2ARs and 5-HT2CRs (Antri et al., 2002, 2003, 2005; Ung et al., 2008); the induction of respiratory recovery after cervical (C2) spinal cord hemisection involves 5-HT2ARs (Zhou et al., 2001; Fuller et al., 2005); 5-HT2CRs are shown to facilitate a long-lasting spinal reflex (Machacek et al., 2001; Shay et al., 2005) and to promote weight-supported stepping after spinal cord transection (Kim et al., 2001; Kao et al., 2006).

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Abbreviations: 5-HT, serotonin; 5-HT2AR, serotonin 2A receptor; 5-HT2BR, serotonin 2B receptor; 5-HT2CR, serotonin 2C receptor; BSA, bovine serum albumin; ChAT, choline acetyltransferase; DOI, 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane; GPCR, G-protein-coupled receptor; IR, immunoreactivity; LLR, long-latency reflex; MAP2, microtubule-associated protein 2; NeuN, neuronal nuclei; PBS, phosphate-buffered saline; PBS–T, phosphate-buffered saline with Triton X-100; PIC, persistent inward current; TBS–T, Tris-buffered saline with Triton X-100.

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Following complete spinal transection there is a hyporeflexia (spinal shock) which after a few weeks develops into hyperreflexia. This corresponds to an early period of decreased excitability of the motoneurons followed by a later hyperexcitability. The hyperreflexia, in the chronic phase, is characterized by exaggerated cutaneous reflexes and velocity-dependent stretch reflexes, also termed spasticity (Lance, 1980; Dietz, 2000; Nielsen et al., 2007). Experiments in cats (Eken et al., 1989) and rats (Bennett et al., 1999, 2001a,b; Li et al., 2004) have suggested that the appearance of plateau potentials generated by persistent inward currents (PICs) in motoneurons is one of the mechanisms underlying the hyperexcitability observed in the chronic spinal phase. In a rat model with a complete transection at sacral 2 (S2) level Bennett and colleagues have demonstrated that the magnitude of PICs in motoneurons below the lesion is increased and the motoneurons become 30-fold supersensitive to 5-HT or 5-HT2A/C receptor agonist (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-amino-propane (DOI) (Harvey et al., 2006a; Li et al., 2007). Recently they showed that the constitutive isoforms of 5-HT2BR and 5-HT2CR are very important for the PIC enhancement and thus are implicated for the development of spasticity (Murray et al., 2010, 2011).

Using immunohistochemistry we have demonstrated that after spinal transection at S2 level 5-HT2AR begun to upregulate within 24 hours (h) in the motoneurons below the lesion (Kong et al., 2010a, 2011). The upregulation was so dramatic that at 2 days (d) after the lesion a four to fivefold increase of 5-HT2AR expression was observed and this increase is sustained for an extended period. However, this early upregulation of 5-HT2AR after spinal cord injury is not correlated with the development of spasticity. As 5-HT2CR has been demonstrated to be an important factor for the recovery of motoneuron PICs and for the underlying spasticity, we have thus investigated the expression changes of this receptor and its relationship with the development of spasticity. Although a few studies have reported its upregulation following different types of spinal injury (e.g., Kao et al., 2006; Hayashi et al., 2010), the time course of its expression changes is still unknown. In this study we have used the same experimental methods as have been used in our 5-HT2AR studies (Kong et al., 2010a, 2011) to investigate the time course of 5-HT2CR expression changes in the same rat spinal cord lesion model at seven different post-lesion intervals. We found that 5-HT2CR is upregulated throughout the spinal gray matter, including motoneurons, although to a less extent and at a later phase than that of 5-HT2AR. The preliminary results have been reported in abstract form (Kong et al., 2010b; Ren et al., 2011).

EXPERIMENTAL PROCEDURES

Animal operation and tissue preparations

All experiments were conducted in accordance with the guidelines of the *EU Directive 86/609/EEC* and were approved by the Danish Animal Experiments Inspectorate. All efforts were made to minimize the number of animals used and their suffering. In total, 98 adult male Wistar rats were used with a body weight of 150–250 g at the beginning of the experiments. Among these, 12 rats were used for Western blotting and two rats for normal control 5-HT2CR immunolabeling. The other animals were divided into seven different time groups (with a post-operation time of 2 d, 7 d, 14 d, 21 d, 28 d, 45 d and 60 d) and used for time course study of 5-HT2CR-immunoreactivity (IR), in which the spinal cords of 2 d, 7 d, 28 d and 60 d rats were obtained from our previous experiments (Kong et al., 2010a, 2011). The rats in each group were further divided into two subgroups: a spinal transection and a sham-operation subgroup with six pairs of animals in each subgroup. The spinalized and sham-operated animals in each time group were operated pair-wise over two consecutive days. For the sham-operation, only the skin and muscles at the lumbar vertebral level were cut open and the second lumbar vertebra was removed but the dura was kept intact. For the spinal transection operation the dura was opened and a 1–2-mm piece of the spinal cord tissue at the S2 segment was gently removed. The operation procedure has been described in detail elsewhere (Wienecke et al., 2010; Kong et al., 2010a, 2011).

Upon the desired post-operative survival time, immediately prior to being sacrificed, the spinalized animals in each group underwent a clinical assessment of tail spasticity as described by Bennett et al. (1999, 2004). A score with 0–5 was assigned to each rat according to the degree of the tail spasticity. After this clinical evaluation the animals were anaesthetized with Mebumal (50 mg/kg, i.p; Sygehus Apotekerne, Copenhagen, Denmark) and transcardially perfused with 4% paraformaldehyde in 0.1 M cold phosphate buffer. The whole spinal cord was removed immediately and post-fixed in the same fixative for 20–24 h at 4 °C. While removing the spinal cord the completeness of the spinal transection was inspected under a stereo-microscope and for a majority of the spinalized rats it was easy to confirm that their spinal cords were completely transected at the S2–S3 level. However, for some rats that survived for a shorter time (2 d and 7 d) it was difficult to judge using the stereo-microscope. In such cases a histochemical stain with Fast Blue and Thionin was performed on horizontal sections across the lesion site to verify the completeness of the spinalization (see Kong et al., 2011). If there were clinical signs of a lesion rostral to the S2 level (lack of urinary bladder control or hindlimb paresis) the animal was sacrificed early in the post-operation period. Likewise, if the lesion site was found below S3 when the spinal cord was removed the spinal tissue would not be processed further. In the end 4–6 rats in each spinalized/sham-operated group were left for further processing (Table 1). After post-fixation the spinal cords were cryoprotected in 0.01 M phosphate-buffered saline (PBS) with 30% sucrose for up to 48 h at 4 °C. The spinal segments from S4 to caudal 1 (Ca1) spinal cord were cut transversely into 40-μm-thick sections with a sliding microtome.

Western blots

The 5-HT2CR antibody used was a polyclonal rabbit affinity-purified serum against a synthetic peptide derived from within residues 400 to the C-terminus of rat 5-HT2CR (No. ab32172, Abcam, UK). To verify the specificity of the antibody we have performed Western blot on extracts of the rat spinal cord. The spinal cord tissues were sampled from cervical and lumbar segments of two rats. In addition, we also performed Western blot on sacrocaudal spinal tissues from 10 rats with a post-operation time being 60 d (5 spinalized and 5 sham-operated) to compare 5-HT2CR protein changes in the entire spinal cord. The samples were homogenized in a 2× Laemmli buffer containing 73% 155 mM Tris buffer (pH 8.3), 9% sodium dodecyl sulfate, 16 mM bromophenol blue, 18% glycerol with 10% 2-mercaptoethanol (0.1 g tissue/ml buffer). Samples were