CHAPTER V

DISCUSSION

Aim 1: To investigate whether pulpal inflammation leads to increased levels of $Na_V1.8$ and $Na_V1.9$ expression in the dental pulp of human primary teeth

The major findings of this study were that there was an increased expression of both $Na_V1.8$ and $Na_V1.9$ in the inflamed dental pulp of permanent teeth, as in previous studies (Renton et al., 2005; Warren et al., 2008; Wells et al., 2007), whereas only $Na_V1.8$ expression was increased in the inflamed dental pulp of primary teeth, compared to normal teeth. This is the first time that the expression of $Na_V1.8$ and $Na_V1.9$ has been demonstrated in dental pulp of primary teeth. It was also found that the innervation density, indicated by the relative amounts of PGP9.5, in the dental pulp of teeth with pulpitis was not different from that in normal teeth, in spite of the increased levels of inflammation, indicated by the relative amounts of MMP-9, in dental pulp of both inflamed primary and permanent teeth compared to normal teeth.

There has been a report of increased innervation density by the sprouting of sensory nerve fibers during pulpal inflammation in both permanent and primary teeth (Rodd and Boissonade, 2000). However, another study in rats showed that the amounts of nerve fibers are not always increased but there are dynamic changes in pulpal innervation based on the depth of carious lesions (Byers et al., 1990). Those changes are as follows: sprouting of nerve fibers that subsided within a few days in teeth with shallow cavities, or sprouting of nerve fibers that was subsequently substituted with reparative dentin in teeth with deep cavities, or necrosis of pulpal

tissues in teeth with pulpal exposure (Byers et al., 1990). Other factors, such as degree of caries activity and the pathogenicity of the lesions have also been suggested to have influence on the neural density of dental pulp (Rodd and Boissonade, 2001). The physiologic root resorption of primary teeth used in this study was not more than 1/3 of the root length and did not affect the overall neural density nor the ability of nerve fibers to perceive pain or to participate in the defensive response (Monteiro et al., 2009). All the inflamed dental pulp used in this study was taken from teeth with deep carious lesions that almost exposed the pulp, or from carious teeth with pulpal exposure that implied chronic pulpal inflammation, in which the pulp was in a reparative or degenerative rather than a sprouting stage. The findings from those studies may be the reasons behind the finding in this study that there was no change in the overall amounts of nerve fibers despite the significantly increased levels of pulpal inflammation.

The absence of any change in the innervation density of normal and inflamed dental pulp of both primary and permanent teeth in the present study suggests the similarity, between normal and inflamed dental pulp, in the number of potential sites of sodium channel expression, which usually occurs in nociceptive fibers (Amaya et al., 2000). Therefore, the increased expression of $Na_V1.8$ in inflamed primary and permanent tooth pulp and of $Na_V1.9$ in inflamed permanent tooth pulp may not result from nerve sprouting, but may result from 1) the upregulation of $Na_V1.8$ and $Na_V1.9$ itself at the nociceptors, which normally express these sodium channel subtypes, or 2) a *de novo* synthesis of $Na_V1.8$ and $Na_V1.9$ by silent nociceptors that normally do not express these sodium channels.

The demyelination process, which is a common process during inflammation, causes an increase in the number of atypical nodal sites in inflamed dental pulp (Henry et al., 2009). This process results in an altered proportion of myelinated and unmyelinated nerve fibers: a decrease in numbers of myelinated nerve fibers, where $Na_V1.8$ is located, and an increase in numbers of unmyelinated nerve fibers, where both $Na_V1.8$ and $Na_V1.9$ are located (Amaya et al., 2000). Besides, the sodium channels have been found aggregated at the atypical nodal sites in inflamed dental pulp of permanent teeth, whereas no change in the overall amount of sodium channels was detected (Henry et al., 2009). Those previous studies suggested that there may be an upregulation of some sodium channel subtypes at specific sites, coinciding with the remodeling process of nerve fibers from myelinated to unmyelinated nerve fibers, without change in the overall amount of sodium channel expression. Therefore, these studies support the finding that there may be a *de novo* synthesis of $Na_V1.8$ and $Na_V1.9$ at the silent nociceptors during pulpal inflammation in inflamed dental pulp of permanent teeth.

As in previous studies (Renton et al., 2005; Warren et al., 2008; Wells et al., 2007), this study found the upregulation of $Na_V1.8$ and $Na_V1.9$ expression in inflamed dental pulp of permanent teeth. However, there has never been any report of the expression of $Na_V1.8$ and $Na_V1.9$ in primary teeth, either with or without pulpitis. The present study is the first to report the expression of $Na_V1.8$ in the dental pulp of normal and inflamed primary teeth and it was also found that there was the upregulation of $Na_V1.8$ in primary teeth with pulpitis compared to normal teeth. $Na_V1.9$ was also found in dental pulp of normal primary teeth but, unlike $Na_V1.8$, $Na_V1.9$ was not upregulated in primary teeth with pulpitis. Based on the above, that

$Na_V1.8$ exists at the neural membranes of small myelinated and unmyelinated fibers, whereas $Na_V1.9$ can be found at neural membranes of unmyelinated fibers only (Amaya et al., 2000), it can be assumed that the amounts of $Na_V1.9$ expression in the dental pulp of primary teeth may be less than those of $Na_V1.8$. Therefore, the upregulation of $Na_V1.8$, but not $Na_V1.9$, in the inflamed dental pulp of primary teeth in this study does not mean that only $Na_V1.8$ expression was altered during inflammation of primary teeth, but there may be biosynthesis of $Na_V1.9$ at specific sites in nerve fibers, which normally do not express $Na_V1.9$, without a change in overall amounts of $Na_V1.9$ expression. The other explanation for the unchange in the expression of $Na_V1.9$ in inflamed primary teeth may be that the western blot method is not able to detect small changes in $Na_V1.9$ expression, particularly with the small amounts of $Na_V1.9$ in primary teeth. In addition, due to the method used for sample collection by which inflamed primary pulp was collected from teeth diagnosed with reversible and irreversible pulpitis, whereas inflamed permanent pulp was collected from teeth diagnosed with irreversible pulpitis only, another assumption is possible that the levels of inflammation in inflamed primary teeth may not be as high as in inflamed permanent teeth and may not be high enough to activate the upregulation of $Na_V1.9$ expression. However, this assumption contrasts to the finding that there was a significant increase of MMP-9 levels in inflamed dental pulp of primary teeth. This contrast may be result from the limitation of western blot method, which is able to detect the amounts of MMP-9 protein, representing the total protein levels of inflammation, but do not directly represent the activity of inflammation. Moreover, MMP-9 must be activated from latent to active form before function and, because of this need for activation, the latent MMP-9 can be detected from early in the induction

state to the chronic state of inflammation. Thus, other methods for detection of MMP-9 activity, such as enzyme-linked immunosorbent assay (ELISA), may be needed to confirm the severity of chronic pulpal inflammation.

There may be other reasons for the distinct expression of $Na_V1.8$ and $Na_V1.9$ in inflamed primary teeth. The study in dorsal root ganglion of rats has demonstrated that the expression of $Na_V1.8$ and $Na_V1.9$ is controlled by different promoter regions and is differentially expressed during the development of primary sensory neurons (Benn et al., 2001). Thus, there may be distinct distribution and properties of $Na_V1.8$ and $Na_V1.9$. Moreover, the previous study in rats using antisense targeting $Na_V1.8$ and $Na_V1.9$ have shown that the role of $Na_V1.8$, but not $Na_V1.9$, is involved in inflammaroty pain (Yu et al., 2011). That finding emphasizes that both of these sodium channel subtypes have different functions and mechanism of upregulation in the same pain model and also indicates that the expression of $Na_V1.8$ may be more sensitive to inflammatory pain stimuli than that of $Na_V1.9$. Several inflammatory mediators that are secreted after an injury such as prostaglandin E_2 (PGE2), serotonin, and adenosine, are capable to modulate TTX-R currents, contributing to hyperalgesia (Gold, 1999). Due to the fact that inflammatory mediators have found to be increased with age (Brüünsgaard and Pedersen, 2003), the levels of inflammatory mediators tend to be higher in the group with permanent teeth than the group with primary teeth. The differences in function and distribution of $Na_V1.8$ and $Na_V1.9$ and more expression of inflammatory mediators in permanent teeth may be other possible reasons for the absence of $Na_V1.9$ upregulation in inflamed dental pulp of primary teeth.

Aim 2: To investigate whether the levels of $Na_V1.8$ and $Na_V1.9$ expression correlate with the severity of pulpal pain in dental pulp of human primary teeth

This study found that the quantity of proteins other than $Na_V1.8$ was not correlated with pain intensity in permanent teeth and there was no correlation between any protein expression and pain score in primary teeth. The findings that VAS scores were correlated well with WBFPS scores in subjects with primary teeth and subjects with permanent teeth, and in all subjects, signify that VAS and WBFPS have a similar ability to assess pain intensity both in adults and in children of at least five years of age.

There has been an attempt and failure to find a relationship between overall neural density and reported pain (Rodd and Boissonade, 2000). The explanation may be because of dynamic changes in dental innervation after injuries, changes which depend on the degree and duration of the injuries. By the time of maximum pain, the response of the dental pulp to dental caries may be a progression of three responses: an increased neural density to preserve the dental pulp, repair by reparative dentin substitution, and degeneration of pulpal tissue. Whatever changes occurred in the dental pulp during maximum pain might not have coincided with the status of the dental pulp on the day of pulpal tissue collection. As with the overall neural density, a correlation between levels of inflammation and pain intensity was not found. The reason may be that the degree of pulpal inflammation on the day the dental pulp was collected was different from that when the subjects experienced the maximum pain.

Increased expression of $Na_V1.8$ in inflamed permanent teeth was previously found in subjects with moderate or severe dental pain (Warren et al., 2008). However, the correlation between $Na_V1.8$ expression in dental pulp and dental pain

intensity has never been established. $Na_V1.9$ expression was also previously found to be increased in symptomatic pulptitis of permanent teeth without any report of the correlation of $Na_V1.9$ expression and pain intensity (Wells et al., 2007). The present study is the first to demonstrate the correlation between $Na_V1.8$ expression in permanent teeth and pain intensity, measured by VAS and WBFPS. The correlation between $Na_V1.8$ expression and pain intensity in the group with permanent teeth may emphasize the role of $Na_V1.8$ in chronic inflammatory pain, as shown in previous studies (Joshi et al., 2006; Strickland et al., 2008). On the other hand, the finding that $Na_V1.9$ expression was increased in inflamed dental pulp, despite no correlation with dental pain intensity, cannot confirm the role of $Na_V1.9$ in chronic inflammation, which, until now, is still controversial (Amaya et al., 2006; Coggeshall et al., 2004; Porreca et al., 1999; Strickland et al., 2008).

Although VAS and WBFPS have excellent correlation, as shown in this study, and have been proved to be appropriate pain measurement tools in children at least four years old (Newman et al., 2005), they still have limitations in pain measurement. It is extremely hard to assess real levels of pain and to compare pain intensity inter-individually because pain is subjective and the threshold for pain perception in each individual is different, depending on multiple factors, such as anxiety or previous dental experience, that have been found to enhance pain (Okawa et al., 2005; Versloot et al., 2008). Moreover, this study assessed the maximum attitude of pain that subjects had ever experienced for the teeth indicated for extraction on the day of dental pain assessment. This process required an ability to recall pain sensitivity. It is an observation that children have shorter recall memory than adults but, unfortunately, no study of differences in recall memory between children and adults

has ever been reported. However, these limitations may be the reasons behind the findings that there was no correlation between pain intensity and any protein expression in primary teeth.

Conclusions

Athough much evidence confirms the role of $Na_V1.8$ in chronic inflammatory pain (Joshi et al., 2006; Strickland et al., 2008), the role of $Na_V1.9$ in chronic inflammatory pain is still controversial (Amaya et al., 2006; Coggeshall et al., 2004; Porreca et al., 1999; Strickland et al., 2008). In the dental pulp of inflamed permanent teeth, the finding that both $Na_V1.8$ and $Na_V1.9$ expression were upregulated, although only $Na_V1.8$ was correlated with pain intensity, may suggest $Na_V1.8$ and $Na_V1.9$ as participators in dental pain transmission during chronic inflammation and as interesting targets for the development of novel analgesic drugs and novel anesthetic agents for the treatment of dental pain. The finding that there was an increase in $Na_V1.8$ expression in primary teeth with inflamed dental pulp, in spite of a lack of correlation between any protein expression and pain intensity in primary teeth, may also suggest that only $Na_V1.8$ may be involved in dental pain transmission and may be the target for the treatment of pulpal inflammatory pain in primary teeth. However, further investigations to locate the sites of $Na_V1.8$ and $Na_V1.9$ expression in the dental pulp of primary teeth with and without inflammation and studies in the field of definite mechanisms of $Na_V1.8$ and $Na_V1.9$ upregulation are still needed.

Limitations of this study

1. There was an unequal degree of pulpal inflammation among the samples in the inflamed group, particularly in primary teeth.

2. The sample sizes in the groups with normal primary and inflamed permanent teeth were much smaller than those in the groups with inflamed primary and normal permanent teeth which were used as the comparative group.

3. There was as a variation in the age of subjects used in the study. Increasing age may lead to different inflammatory mediators released during inflammatory process.

4. The dental pulp was collected from various positions of teeth: anterior and posterior teeth of both maxilla and mandible. The different positions of teeth were supplied by different branches of nerve, which may have interplexus variation and may contribute to variation in $Na_V1.8$ and $Na_V1.9$ expression.