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Expression and Localization of the Orexin-1 Receptor (OX1R) After Traumatic Brain Injury in Mice

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Abstract Orexins are neuropeptides that have a wide range of physiological effects, and recent studies have suggested that the orexin system may be involved in traumatic brain injury. However, the expression and localization of orexin receptors have not been examined yet under brain injury conditions. In the present study, we used immunohistochemical techniques to investigate the expression of orexin-1 receptor (OX1R) and its time-dependent changes in the mouse brain after controlled cortical impact (CCI) injury. OX1R-like immunoreactivity was first detected 6 h after injury in the surrounding penumbra of the injury. The intensity of this immunoreactivity was increased at 12 h, peaked at day 1, and then decreased from day 2 to day 7. To identify the cellular localization of OX1R, we also performed double-immunohistochemical staining with OX1R and several cell marker antibodies. OX1R-like immunopositive cells were clearly co-localized with immunoreactivity for the neuronal marker NeuN at day 7. It was also expressed on the periphery of cells immunopositive for CD11b, a microglial cell marker, at days 1 and 7. These results suggest that orexin and its receptor may play roles in traumatic brain injury, and that OX1R is induced in neurons and microglial cells after traumatic brain injury.

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Introduction

Orexins (orexin-A and orexin-B) are neuropeptides that have various physiological effects, including the regulation of the sleep–wake cycle, feeding behavior, and neuroendocrine functions (de Lecea et al. 1998; Nishino et al. 2000; Dauvilliers et al. 2003). Orexins derive from a single precursor, prepro-orexin, and act via two types of G-protein-coupled receptors, orexin-1 (OX1R) and orexin-2 (OX2R), which are also widely distributed in the brain (Hervieu et al. 2001; Marcus et al. 2001; Suzuki et al. 2002). OX1R mRNA is located in many brain regions including the prefrontal and infralimbic cortex, hippocampus, paraventricular thalamic nucleus, ventromedial hypothalamic nucleus, dorsal raphe nucleus, and locus coeruleus (Marcus et al. 2001). Subsequent studies have revealed that a chronic human sleep disorder, narcolepsy, is specifically associated with a reduced production of these peptides (Peyron et al. 2000; Thannickal et al. 2000). Recent studies have demonstrated that the orexins and their receptors might be involved in neuronal cell death and could play important roles in various acute or subacute neurological disorders such as cerebrovascular diseases, brain tumors, neurotrauma, Parkinson's disease, and Guillain–Barre syndrome (Ripley et al. 2001; Drouot et al. 2003; Dohi et al. 2005; Nishino and Kanbayashi 2005; Dohi et al. 2006). In cerebral ischemia, it has been reported that permanent middle cerebral artery occlusion in the rat induces increased cortical expression of the orexin-1 receptor (OX1R), suggesting an involvement of the hypocretin/orexin system in ischemic insult (Irving et al. 2002). We have already shown that

OX1R immunoreactivity is widely detected in the hippocampus, cortex, hypothalamus, and amygdala in the mouse in response to global ischemia induced by transient common carotid artery occlusion (Nakamachi et al. 2005).

In traumatic brain injury, it is also known that changes in sleep patterns, energy homeostasis, and neuroendocrine functions often occur (Nishino and Kanbayashi 2005). Recent studies have demonstrated that CSF orexin concentrations are decreased in brain trauma patients with sleep disorders (Ripley et al. 2001; Baumann et al. 2005; Baumann et al. 2007). These findings suggest that orexin signaling may be altered during acute and chronic traumatic brain injury and that altered orexin signaling may be related to a range of symptoms associated with traumatic brain injury. However, the dynamics of orexin signaling after traumatic insults, including the expression and localization of OX1R in the brain, have not been investigated.

In the present study, therefore, we have used immunohistochemical techniques to study the expression of OX1R-like immunoreactivity in the mouse brain in response to a controlled cortical impact (CCI) injury. With the aid of double immunofluorescence methods, we also examined the cellular localization of OX1R-like immunoreactivity in the brain.

Materials and Methods

Animals

Male C57BL/6 mice aged 8 weeks were purchased from SLC Japan (Saitama, Japan). All mice were maintained on a 12 h light/dark cycle at 23°C with constant humidity (40± 15%). All experimental procedures involving animals were approved by the Institutional Animal Care and Use Committee of Showa University.

Surgical Procedures

Mice were anesthetized with sodium pentobarbital (20 mg/kg, i.p.), positioned in a stereotaxic frame and subjected to CCI as previously described (Dixon et al. 1991). A craniotomy was performed using a portable drill and trephine over the left parietotemporal cortex, and the bone flap was removed. Mice were then subjected to CCI using a pneumatic cylinder with a velocity of 5.82 m/s, duration of 47 ms, depth of 1.2 mm, and with a driving pressure of 73 psi. The bone flap was replaced and the scalp was sutured closed. The mice were returned to their cages to recover from the procedure and anesthesia, and began moving around within 30 min. Injured mice were then sacrificed at 0, 3, 6, 12 h, 1, 2, 4, or 7 days after injury to determine the time course of change of OXIR expression in the brain.

Histology

Mice were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) at 0, 3, 6, 12 h, 1, 2, 4, or 7 days after CCI injury, and brains were fixed by perfusion with saline followed by 2% paraformaldehyde in 50 mM phosphate buffer. After fixation, brain tissues containing the bregma −1.6 to −2.2 mm were embedded in OCT compound after immersion in 20% sucrose for cytoprotection as frozen sections.

Single Immunostaining for OX1R

For immunoperoxidase labeling, the cryostat sections were incubated in 0.3% hydrogen peroxide (H_2O_2) in methanol (MeOH) for 30 min at room temperature to block endogenous peroxidase activity and incubated in phosphate-buffered saline (PBS) containing 5% normal horse serum (NHS) for 60 min at room temperature to block non-specific reactions. The sections were incubated in rabbit anti-OX1R antibody (1:5,000; CHEMICON, Temecula, CA, USA) at 4°C overnight. Antibody detection was carried out by incubating slices with biotinylated goat anti-rabbit IgG (1:200; Vector, Burlingame, CA, USA) as a secondary antibody for 120 min at room temperature, followed by Vectastain ABC (Vector) for 60 min at room temperature, and then with stable 3,3′-diaminobenzidine complex (DAB; Vector).

Double Immunofluorescence Staining for OX1R and Neurons, Astrocytes, and Microglial Cells

Primary antibodies to detect neurons, astrocytes and glial cells were as follows: mouse anti-NeuN antibody (1:1,000; CHEMICON) as a neuronal cell marker, mouse anti-glial fibrillary acidic protein (GFAP) antibody (1:1,000; Sigma, St Louis, MO, USA) as a marker of astrocytes, and rat anti-CD11b antibody (1:500; Serotec, Oxford, UK) as a microglial cell marker. Sections were blocked with 10% NHS in PBS for 60 min at room temperature. The sections were then incubated overnight at 4°C in anti-OX1R antibody (1:5,000; CHEMICON) along with the different cell marker antibodies. The immunoreactivity of OX1R was detected using Alexa 546-labeled goat anti-rabbit IgG (1:400; Invitrogen, Carlsbad, CA, USA), while that of NeuN, GFAP, and CD11b were detected using Alexa 488-labeled goat anti-mouse or anti-rat IgG antibodies (1:400; Invitrogen) for 120 min at room temperature. Double immunolabeling was detected using a confocal laser microscope (A1si; Nikon, Tokyo, Japan).

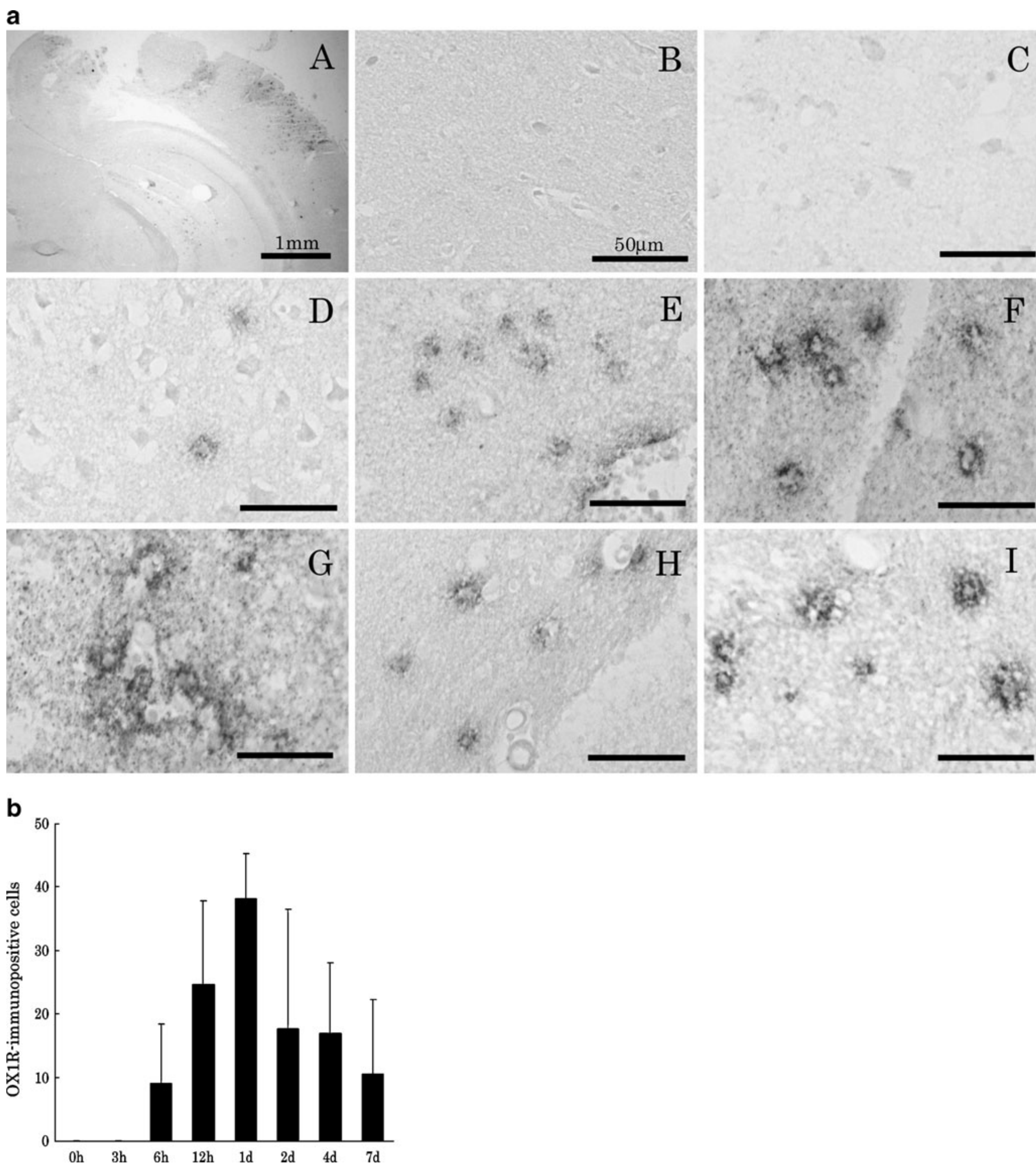

Fig. 1 **a** Distribution of OX1R immunoreactivity in the surrounding penumbra of the trauma at 0 day (**B**), 3 h (**C**), 6 h (**D**), 12 h (**E**), 1 day (**A**, **F**), 2 days (**G**), 4 days (**H**), and 7 days (**I**) after CCI injury. OX1R-like immunopositive cells were clearly observed from 6 h, showing a ring-like staining pattern. **b** The number of OX1R-like immunopositive cells with the ring-like staining pattern per unit area. The OX1R-like immunopositive cells increased at 12 h, peaked at day 1, and then progressively decreased from day 2 to day 7

Single Immunostaining for Orexin A

Sections were incubated overnight at 4°C in goat anti-orexin A antibody (1:2,000; Santa Cruz, CA, USA). Antibody detection was carried out by incubating slices with biotinylated horse anti-goat IgG (1:200; Vector) as a secondary antibody for 120 min at room temperature, followed by Vectastain ABC (Vector) for 60 min at room temperature, and

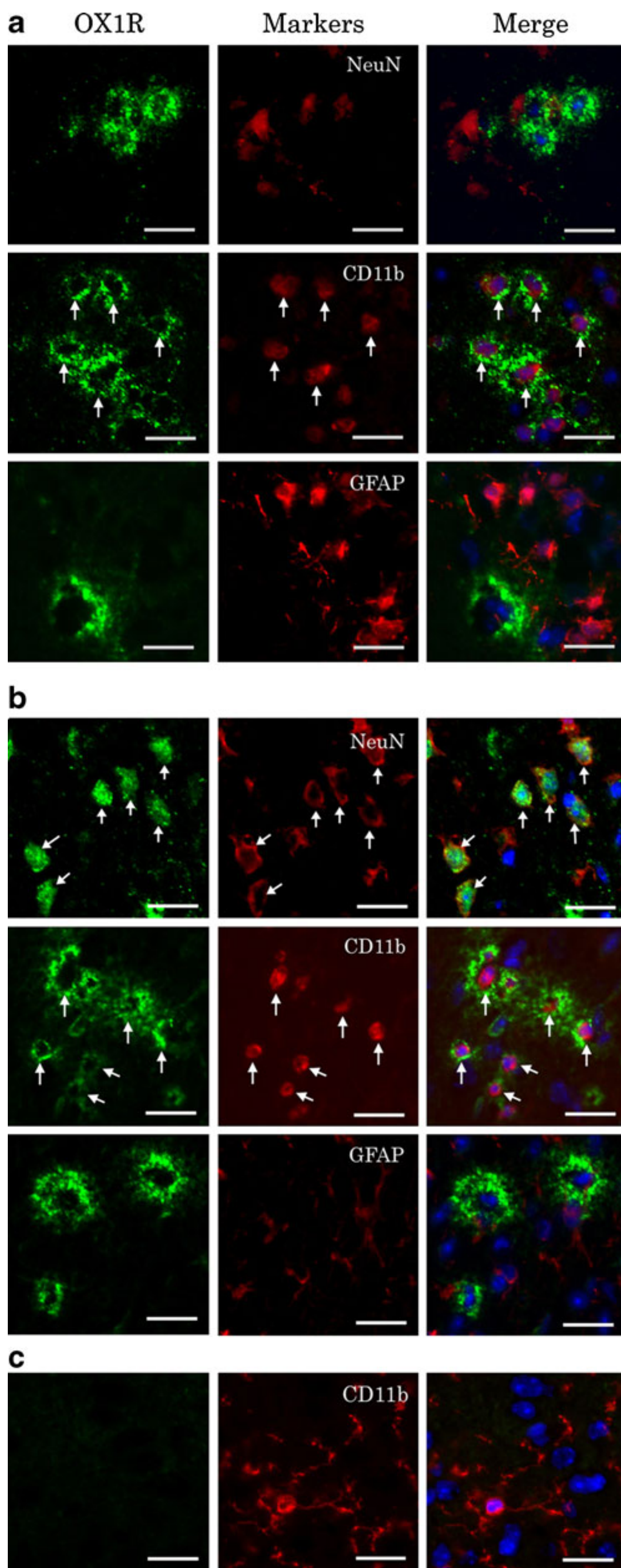

Fig. 2 Double immunofluorescence staining for OX1R and neuron, astrocyte, and microglial cell markers in the peri-contusional cortex at day 1 (**a**), day 7 (**b**), and day 0 (**c**). Immunostaining was carried out using antibodies for OX1R together with NeuN, CD11b, and GFAP as neuron, microglia, and astrocyte markers, respectively. At days 1 and 7, the OX1R immunoreactivity was clearly co-localized on the periphery of the CD11b-immunopositive cells (*arrows*). The OX1R immunoreactivity was also co-localized with NeuN-immunopositive cells at day 7 (*arrows*). At day 0, OX1R immunoreactivity was neither detected nor co-localized with CD11b. Scale bar, 20μm

then with stable 3,3′-diaminobenzidine complex (DAB; Vector).

Double Immunofluorescence Staining for OX1R and Orexin A

Sections were blocked with 5% NHS in PBS for 60 min at room temperature and then incubated overnight at 4°C in goat anti-orexin A antibody (1:1,000; Santa Cruz) along with rabbit anti-OX1R antibody (1:5,000; Chemicon). The immunoreactivity of orexin A was detected using Alexa 546-labeled donkey anti-goat IgG antibody (1:400; Invitrogen), and that of OX1R was detected using Alexa 488-labeled goat anti-rabbit IgG antibody (1:400; Invitrogen) following 120 min incubation at room temperature. Double immunolabeling was detected using a confocal laser microscope (A1si; Nikon).

Results

Single Immunostaining for OX1R

At 0 and 3 h after CCI injury, OX1R immunoreactivity was widely detected throughout the mouse brain, even though the level of expression was very low. It was first detected clearly in the surrounding penumbra of the trauma 6 h after induction of the CCI injury (Fig. 1a). The OX1R-like immunopositive cells appeared stained in a ring-like pattern form. The number of the OX1R-like immunopositive cells with the ring-like staining pattern per unit area increased at 12 h and peaked at day 1, and progressively decreased from day 2 to day 7 (Fig. 1b). The OX1R immunoreactivity was distributed only in the surrounding penumbra of the trauma from 6 h to day 2. It then extended to the thalamus, hippocampus, and a few scattered areas in the non-injured hemisphere across the longitudinal cerebral fissure in the late phase of the injury (days 4 and 7).

Double Immunofluorescence Staining for OX1R in Neurons, Astrocytes, and Microglia

To identify OX1R-expressing cells at days 0, 1, and 7 after CCI injury, we performed double-immunohistochemical

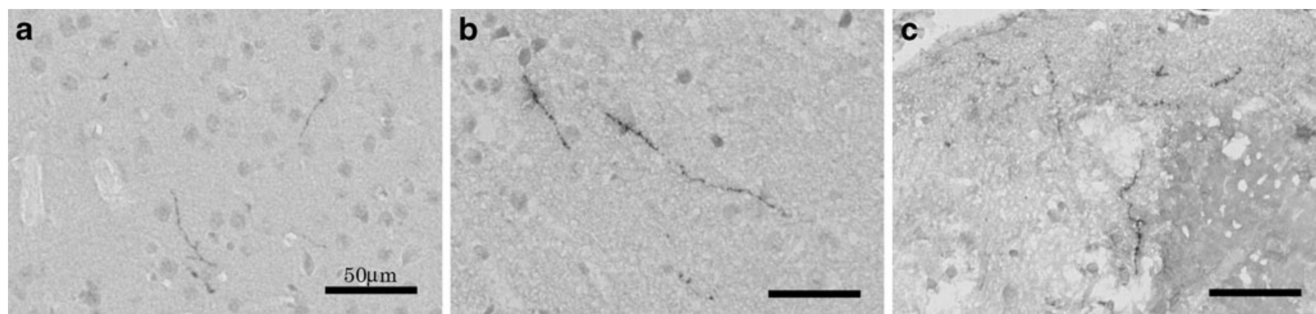

Fig. 3 Distribution of orexin A immunoreactivity in the surrounding penumbra of the injury at day 0 (**a**), day 1 (**b**), and day 7 (**c**). Orexin A-immunoreactive fibers were clearly detected in the surrounding penumbra of the trauma at days 1 and 7, while the expression was relatively low at day 0

staining with antibodies against OX1R and several cell markers. OX1R-like immunoreactivity was clearly co-localized with NeuN, a neuronal marker, at day 7 (Fig. 2b). OX1R-like immunoreactivity was also clearly seen to be expressed on the periphery of cells immunopositive for CD11b, a microglial cell marker, at days 1 and 7 (Fig. 2a, b). OX1R-like immunoreactivity was not co-localized with GFAP, an astrocyte cell marker, in normal or injured areas. OX1R-like immunoreactivity was not detected nor co-localized with CD11b at day 0 (Fig. 2c).

Single Immunostaining for Orexin A

To determine the distribution of orexin A after CCI injury, single immunostaining experiments for detecting orexin A were performed. Orexin A-immunoreactive fibers were clearly detected in the surrounding penumbra of the trauma at days 1 and 7, while the expression level was relatively low at day 0 (Fig. 3).

Double Immunofluorescence Staining for Orexin A and OX1R

To identify the direct interaction between orexin A-like immunoreactive nerve fibers and OX1R, we performed double-immunohistochemical staining with orexin A and OX1R antibodies. Orexin A-like immunoreactive nerve fibers were observed to make contact with OX1R containing neurons in the surrounding penumbra of the trauma (Fig. 4).

Discussion

In the present study, we have shown the time-dependent distribution of OX1R immunoreactivity following CCI injury. OX1R immunoreactivity was detected in the surrounding penumbra of the trauma 6 h following CCI injury, which was increased at 12 h, peaked at day 1, and then progressively decreased from day 2 to day 7. These results suggest that similarly to chronic neurodegenerative conditions or brain ischemia, altered orexin signaling occurred following CCI injury. In traumatic brain injury, it has been reported that orexin neurons were reduced and CSF orexin levels decreased after the injury as a consequence of secondary damage to hypothalamic neurons (Baumann et al. 2005, 2009). The distribution of OX1R following CCI injury was mainly detected in the surrounding penumbra of the injury, although in some mice it gradually spread to the periphery such as the thalamus and hippocampus in the late phase of the injury. These time-dependent changes of the number and the distribution of OX1R might be related with the advance of secondary brain damage.

To estimate the function of orexins and receptor, we also determined cellular localization of OX1R after CCI injury. Double-immunohistochemical staining experiments showed significant co-localization of OX1R on the periphery of microglia, although not in neurons or astrocytes at day 1. It was noteworthy that the single immunostaining of OX1R showed a characteristic expression of ring shape, which was the same pattern as that observed for the double-immunohistochemical staining with CD11b (microglia). At day 7, however, the OX1R immunoreactivity was also co-localized with neurons. These results signify that microglia might be involved in the expression of OX1R in the early phase following the injury, and that the cell

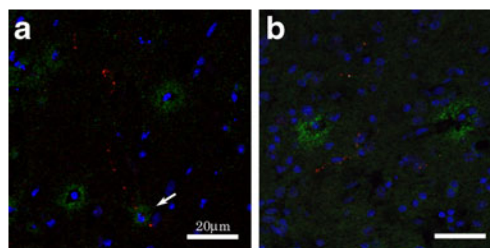

Fig. 4 Double immunofluorescence staining of orexin A and OX1R in the surrounding penumbra of the trauma at day 1 (**a**) and day 7 (**b**). Orexin A-immunoreactive fibers were seen to make contact with OX1R-containing neurons in the surrounding penumbra of the injury (*arrow*)

distribution of OX1R changes as a function of the time post-injury.

Microglia are the resident immune cells of the central nervous system and have been implicated as active contributors to neuronal damage in neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease (Block et al. 2007). In traumatic brain injury, it has been suggested that microglia mediate secondary tissue damage (Heiler 2008). After brain injury, neurons, astrocytes, and oligodendroglia have different responses to the injured microenvironment: neurons and oligodendroglia die via either necrosis or apoptosis, while astrocytes and microglia are activated (Conti et al. 1998; Savitz and Rosenbaum 1998). Activated microglia up-regulate a variety of surface receptors, including the major histocompatibility complex and complement receptors. They also undergo dramatic morphological changes from resting ramified cells to activated amoeboid microglia (Kreutzberg 1996). Besides morphological changes and surface molecule up-regulation, activated microglia secrete a host of soluble factors which are potentially beneficial to the survival of neurons, this being similar to the neuroprotective role played by activated astrocytes (Liu and Hong 2003). Under other circumstances, however, microglia become over-activated and can induce significant and highly detrimental neurotoxic effects due to the excess production of a large array of cytotoxic factors such as superoxide, nitric oxide, and tumor necrosis factor-α (TNF-α) (Block et al. 2007).

In this study, CD11b immunopositive cells, which has been considered as microglial cells, revealed a ramified morphology with a small cell body and highly branched processes at day 0, suggesting that they had been in the resting state before CCI injury. At days 1 and 7, the CD11b immunopositive cells revealed a granular, rounded, and amoeboid appearance, suggesting that they had been activated after the injury. These results suggest that the activated microglia could play an important role in the up-regulation of their expression of OX1R in traumatic brain injury.

In the global ischemia model, however, OX1R immunoreactivity co-localized with neurons, astrocytes, and oligodendrocytes, but not with microglia (Nakamachi et al. 2005). These results suggest that OX1R expression is induced by different mechanisms in the CCI injury model compared with the ischemia model, particularly in the early phase of the injury, which comes as a consequence mainly due to the role of activated microglia. However, the pathophysiology of traumatic brain injury is complex and involves a variety of systemic and intracranial processes including hypotension, hypoxia, ischemia, hyperthermia, as well as intracranial pressures (Bramlett et al. 1999). In addition, various factors, such as hypoglycemia, hypothalamic function, a range of different stresses, and glucocorticoid levels, are likely to alter orexin levels (Ida et al. 2000; Stricker-Krongrad and Beck 2002) and hence physiological responses mediated OX1R.

Recent studies have demonstrated that the orexins and their receptors might participate in the process of neuronal cell death (Ripley et al. 2001; Drouot et al. 2003; Dohi et al. 2005; Nishino and Kanbayashi 2005; Dohi et al. 2006). Furthermore, other studies have revealed that orexins induce apoptosis that results in a massive reduction of cell growth in a set of tumor cell lines including human colon cancer cells (Rouet-Benzineb et al. 2004), human neuroblastoma cells (Rouet-Benzineb et al. 2004), and rat pancreatic tumor cells (Voisin et al. 2006). The cellular mechanism whereby orexins drive apoptosis involves mitochondrial cytochrome *c* release into the cytosol and activation of caspase-3 and caspase-7 (Rouet-Benzineb et al. 2004). In addition, a very recent study revealed that the tissue level of OX1R increased in diabetes mellitus where it co-localized with cleaved caspase-3 in islet cells (Adeghate et al. 2010). In traumatic brain injury, it is not yet clear whether OX1R up-regulation acts in a neuroprotective or neurotoxic manner; however, it was shown that activated microglia and orexins play important roles in the early phase of traumatic brain injury. Further research is needed to demonstrate the function of microglial cell-expressed OX1R and the role of OX1R following CCI injury. This could be achieved by performing double immunohistochemistry for OX1R and apoptotic or neurodegenerative markers.

In conclusion, we have determined the distribution of OX1R immunoreactivity in the surrounding penumbra of the injury. Immunoreactivity, which was first seen 6 h after induction of the CCI injury, peaked at day 1 and then progressively decreased from day 2 to day 7. Double immunofluoresence studies revealed that OX1R co-localized on the periphery of microglia at day 1, then also in neurons at day 7. These results suggest that OX1R was first up-regulated with microglial activation and could play an important role in the physiological response to traumatic brain injury.

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