Chapter 9
SAK3-Induced Neuroprotection Is Mediated by Nicotinic Acetylcholine Receptors

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Abstract   Cholinergic neurotransmission plays a critical role in neuronal plasticity and cell survival in the central nervous system (CNS). Two types of acetylcholine receptors (AChRs), muscarinic AChRs (mAChRs) and nicotinic AChRs (nAChRs), trigger intracellular signaling through G protein activity and ion influx, respectively. To assess mechanisms underlying neuroprotection through nAChRs, we developed SAK3, a novel modulator of nAChR activity. Recently, we found that SAK3 enhances T-type calcium channel activity, promoting ACh release in the hippocampal CA1 region of olfactory-bulbectomized mice. Here, we observed potent SAK3 neuroprotective activity in mice with 20-min bilateral common carotid artery occlusion (BCCAO) or hypothyroidism. Treatment of mice with the α7 nAChR-selective inhibitor methyllycaconitine (0.5 mg/kg/day, p.o.) antagonized SAK3-mediated neuroprotection and memory improvement in BCCAO mice. Single administration of the anti-Graves' disease therapeutic methimazole (MMI) to female mice disrupted olfactory bulb (OB) glomerular structure, and cholinergic neurons largely disappeared in the medial septum followed by memory loss. Chronic SAK3 (0.5–1 mg/kg, p.o.) administration significantly rescued the number of cholinergic medial septum neurons in MMI-treated mice and improved cognitive deficits seen in those mice. Overall, our study suggests that, in mice, the novel nAChR modulator SAK3 can rescue neurons impaired by transient ischemia and hypothyroidism. We also address mechanisms common to SAK3-induced neuroprotection in both conditions.

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Abbreviations

Aβ	Amyloid-β
ACh	Acetylcholine
Akt	Protein kinase B
BCCAO	Bilateral common carotid artery occlusion
CNS	Central nervous system
DhβE	Dihydro-β-erythroidine
ERK	Extracellular signal-regulated kinase
HO-1	Heme-oxygenase 1
JAK2	Janus-activated kinase 2
MEC	Mecamylamine
MMI	Methimazole
MLA	Methyllycaconitine
mAChR	Muscarinic ACh receptor
nAChR	Nicotinic ACh receptor
OBX	Olfactory-bulbectomized
PI3K	Phosphatidylinositol 3 kinase
PKC	Protein kinase C
RGC	Retinal ganglion cell
SAK3	Ethyl 8'-methyl-2',4-dioxo-2- (piperidin-1-yl)-2'H-spiro[cyclopentane-1,3'-imidazo[1,2-a]pyridin]-2-ene-3-carboxylate
ST101	Spiro[imidazo[1,2-a] pyridine-3,2-indan]-2(3H)-one

9.1 Introduction

Acetylcholine (ACh) is a major neurotransmitter in the central nervous system (CNS) and transduces signals via two types of ACh receptors (AChRs): muscarinic (mAChRs) and nicotinic (nAChRs). While mAChRs are G-protein-coupled, nAChRs are ligand-gated cation channels consisting of five subunits (Zdanowski et al. 2015). Both AChR pathways function in learning and memory (Melancon et al. 2013; Pandya and Yakel 2013) and play a critical role in cell survival in in vitro and in vivo models (Akaike et al. 2010; Tan et al. 2014; Zdanowski et al. 2015). Drugs that enhance ACh concentration in the CNS, including the acetylcholine esterase (AChE) inhibitors donepezil, galantamine and rivastigmine, are among widely used therapeutics used to treat early stage Alzheimer's Disease (AD). However, it remains unclear whether the effects of AChE inhibitors are mediated by nAChRs or mAChRs in human brain. We recently developed the lead compound of the AD therapeutic SAK3 (ethyl 8′-methyl-2′,4-dioxo-2-(piperidin-1-yl)-2′H-spiro[cyclopentane-1,3′-imidazo[1,2 a]pyridin] -2-ene-3-carboxylate) (Yabuki et al. 2017a, b). SAK3 primarily stimulates T-type voltage gated Ca^{2+} channels in brain, and importantly it enhances ACh release in hippocampus, thereby improving

memory in olfactory-bulbectomized (OBX) mice. We found that SAK3 effects on ACh release and memory improvement were antagonized by nAChR inhibitors, suggesting that SAK3 modulates nAChR. This review focuses primarily on SAK3 neuroprotective activity mediated by nicotinic cholinergic pathways.

9.2   Neuroprotection Mediated by mAChRs

Subchronic treatment with the acetylcholinesterase inhibitor galantamine (3.5 mg/kg, i.p.) prevents cell death and axonal injury after ocular hypertension surgery in rat retinal ganglion cells (RGCs), an effect blocked by the non-selective mAChR antagonist scopolamine, the M1-type mAChR antagonist pirenzepine, or the M4-type mAChR antagonist tropicamide, but not by nAChR inhibitors (Almasieh et al. 2010). In agreement with these results, the M1-type mAChR agonist pilocarpine protects RGCs from glutamate-induced neurotoxicity and ischemia/reperfusion injury in rat primary retinal cultures and in rat retina (Tan et al. 2014). M1-type mAChR activation in PC12 cells promotes protein kinase C (PKC) activity and inhibits glycogen synthase kinase-3β (GSK-3β) activity, thereby increasing levels of NF-E2-related factor-2 (Nrf2) protein, which regulates transcription of the gene encoding the anti-oxidant protein hemeoxygenase I (HO-1) (Espada et al. 2009; Ma et al. 2013). Therefore, activation of that anti-oxidant pathway through Nrf2 stimulation likely underlies mAChR-dependent neuroprotection. Likewise, the M1-type mAChR-selective agonist AF267B rescues rat primary hippocampal neurons exposed to amyloid-β (Aβ) from cell death by inhibiting increases in GSK-3β (Farías et al. 2004). On the other hand, the mAChR antagonist scopolamine does not block neuroprotection by acetylcholinesterase inhibitors on glutamate (1 mM) toxicity in primary rat cortical neurons (Takada-Takatori et al. 2009). Thus, how mAChRs promote neuroprotection is not entirely clear.

9.3   Neuroprotective Action Mediated by nAChRs

Nine different nAChR subunits (α2-7 and β2-4) are expressed in mammalian brain, and in mouse brain major nAChRs are comprised of homomeric α7 AChR and heteromeric α4β2 complexes (Dani and Bertrand 2007; Dineley et al. 2015; Yakel 2013). Many studies in cultured neurons support the idea that nAChRs have neuroprotective effects. For example, nicotine (10 μM) treatment protects cultured rat primary cortical neurons from cell death by glutamate (1 mM) exposure by activating α4β2 and α7 nAChRs (Kaneko et al. 1997). In addition, the α4β2 inhibitor dihydro-β-erythroidine (DHβE) and α7 inhibitor methyllycaconitine (MLA) both block neuroprotective effects of acetylcholinesterase inhibitors on glutamate (1 mM)-induced excitotoxicity in cultured neurons, an effect not seen following treatment of cells with the mAChR antagonist scopolamine (Takada-Takatori et al.

2009). In vivo, galantamine treatment prevents death of gerbil hippocampal CA1 pyramidal neurons following transient bilateral common carotid artery occlusion (BCCAO), an effect blocked by the non-selective nAChR inhibitor mecamylamine (MEC) (Lorrio et al. 2007). Combined neostigmine and anisodamine treatment are neuroprotective against middle cerebral artery occlusion in wild type- but not in α7 nAChR knock-out mice (Qian et al. 2015). We recently observed that the acetylcholinesterase inhibitor donepezil antagonizes loss of cholinergic neurons in the medial septum (MS) of OBX mice through nAChR stimulation (Yamamoto and Fuknaga 2013). In addition, Hijioka et al. (2012) reported that the α7-specific agonist PNU-282987 but not the α4-specific agonist RJR-2403 blocks neuronal loss following intracerebral hemorrhage in mouse striatum. Since MEC, DHβE and MLA do not block neuroprotective effects of galantamine following ocular hypertension surgery in rat RGCs, neuroprotection mediated by nAChRs may play a more predominant role in CNS than in peripheral neurons. We previously reported that galantamine stimulates glutamatergic and GABAnergic synaptic transmission via nAChR stimulation in rat cortical neurons (Moriguchi et al. 2009). Interestingly, galantamine increases hippocampal insulin-like growth factor 2 expression via the α7 nAChR in mice (Kita et al. 2013). Similarly, stimulation of α7 by the selective agonist PHA-543613 or galantamine treatment enhances α7 channel activity and improves Aβ-induced cognitive deficits in mice (Sadigh-Eteghad et al. 2015). In addition, galantamine treatment promotes survival of newborn neurons in the hippocampal dentate gyrus (DG) viaα7 nAChR but not via M1 mAChR activity (Kita et al. 2014). Taken together, the neuroprotective effect of galantamine is mediated both by mAChRs and nAChRs in the CNS.

## 9.4   Development of the Novel nAChR Modulator SAK3

T-type calcium channels, which are encoded by the *CACNA1G* (Cav3.1), *CACNA1H* (Cav3.2) and *CACNA1I* (Cav3.3), are voltage-gated calcium channels that give rise to low-threshold calcium spikes, which in turn trigger burst firing mediated by sodium channels in many neurons (Huguenard 1996; Perez-Reyes 2003). Recently, we found that a novel AD therapeutic candidate, ST101 (spiro[imidazo[1,2-a] pyridine-3,2-indan]-2(3H)-one), increases Cav3.1 T-type calcium channel currents (Moriguchi et al. 2012). ST101 accelerated ACh release in the hippocampus of OBX mice, an effect inhibited by the T-type calcium channel blocker mibefradil and by nAChR inhibitors (Yamamoto et al. 2013). Moreover, intraventricular injection of mecamylamine inhibited ST101-elicited neurogenesis in the hippocampal DG of OBX mice (Shioda et al. 2010), suggesting that ST101 may activate nAChR and promote ACh release. However, clinical trials showed that administration of ST101 alone was not sufficient to improve memory deficits in AD patients (Gauthier et al. 2015). Therefore, we sought a more potent Cav3.1 and Cav3.3 T-type calcium channel enhancer, resulting in development of SAK3 (Yabuki et al. 2017b). We found that SAK3 promoted more potent ACh release in mouse hippocampal CA1 than did ST101 (Yabuki et al. 2017b).

9.5 SAK3-Induced Neuroprotection in Brain Ischemia

We confirmed SAK3 neuroprotection using a 20-min BCCAO mouse model. To do so, we administered SAK3 (at 0.1, 0.5 or 1.0 mg/kg, p.o.) orally to mice 24 h after BCCAO ischemia. SAK3 administration at 0.5 or 1.0 mg/kg/day significantly blocked loss of hippocampal CA1 neurons and memory deficits seen in BCCAO mice. Treatment with the α7 nAChR-selective inhibitor methyllycaconitine (MLA: 6.0 mg/kg/day, i.p.) antagonized both neuroprotection and memory improvement seen in SAK3 (0.5 mg/kg/day, p.o.)-treated mice (Fig. 9.1). Since excess calcium influx enhances excitotoxic and proapoptotic pathways to induce ischemic neuronal death (Berliocchi et al. 2005; Bano and Nicotera 2007), the impact of T-type channel regulators on neuroprotection is unclear. For example, intraventricular injection of mibefradil and pimozide 6 h before 10-min BCCAO ischemia antagonizes hippocampal injury in rats (Bancila et al. 2011). Other T-type calcium channel blockers, such as U-92032 and flunarizine, administered 1 h prior to BCCAO inhibit delayed neuronal death in the gerbil hippocampal CA1 region (Ito et al. 1994). Such varied effects of T-type calcium channel blockers may be due to differences in timing of drug administration. We administered SAK3 to animals 24 h after BCCAO, whereas others have administered T-type calcium channel blockers before brain ischemia (Bancila et al. 2011; Ito et al. 1994). Moreover, some T-type calcium channel inhibitors, such as mibefradil and flunarizine have affinities to other channel types such as L-type calcium, sodium or potassium channels (Liu et al. 1999; Bloc et al. 2000; McNulty and Hanck 2004). Therefore, SAK3 is neuroprotective against brain ischemia by a mechanism that differs from that of other drugs.

9.6 SAK3 Ameliorates Methimazole-Induced Cholinergic Neuronal Damage

The drug methimazole (MMI) is widely used to antagonize hyperthyroidism and manage Graves' disease, an autoimmune condition promoting hyperthyroidism (Cano-Europa et al. 2011; Wu et al. 2013). Biochemically, MMI acts by preventing iodine incorporation into the thyroid hormone precursor, thyroglobulin, and thus interferes with conversion of thyroxine (T4) to triiodothyronine (T3) (Cooper 1984; Amara et al. 2012; Parisa and Fahimeh 2015). Importantly, treatment with moderate doses of MMI reportedly impairs olfactory function in rats, while high doses cause complete destruction of the olfactory epithelium (OE) (Genter et al. 1995). The OE is a critical site of regeneration of physically- or chemically-injured olfactory sensory neurons (OSNs) (Schwob et al. 1992; Suzukawa et al. 2011). Thyroid hormone deficiency also causes significantly reduced levels of choline acetyltransferase (ChAT), a marker of cholinergic neurons, in various brain regions (Kojima et al. 1981; Oh et al. 1991; Sawin et al. 1998). Since cholinergic neurons in the MS innervate the olfactory bulb and hippocampus (Mesulam et al. 1983a), olfactory
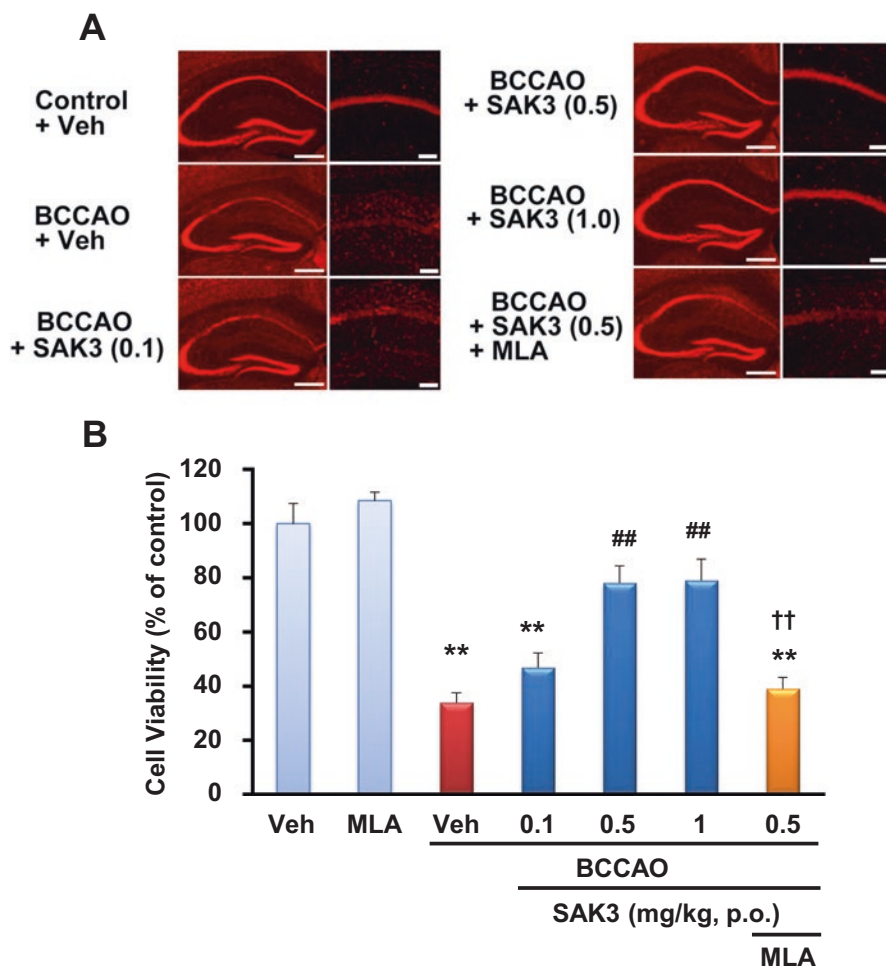
Fig. 9.1 Oral SAK3 administration antagonizes loss of CA1 neurons after BCCAO through α7 nAChR stimulation. (**a**) Representative histological sections of hippocampus in control, vehicle-administered BCCAO, SAK3 (0.1, 0.5 or 1.0 mg/kg, p.o.)-administered BCCAO mice or SAK3 (0.5 mg/kg, p.o.)-administered BCCAO mice treated with MLA. Mice were sacrificed 11 days after BCCAO for histopathological analysis. *Scale bars*: low magnification, 500 μm; high magnification, 100 μm. (**b**) Cell viability is expressed as a percent of the average number of viable hippocampal CA1 cells from control mice (n = 12–23 per group). *Error bars* represent SEM. ** $p < 0.01$ vs. control mice. ## $p < 0.01$ vs. vehicle-administered BCCAO mice. †† $p < 0.01$ vs. SAK3 (0.5 mg/kg, p.o.)-administered BCCAO mice. MLA, methllycaconitine (6.0 mg/kg, i.p.) treatment; SAK3 (0.1), SAK3 (0.1 mg/kg, p.o.) administration; SAK3 (0.5), SAK3 (0.5 mg/kg, p.o.) administration; SAK3 (1.0), SAK3 (1.0 mg/kg, p.o.) administration; and Veh, vehicle administration. (Modified from Yabuki et al. 2017a)
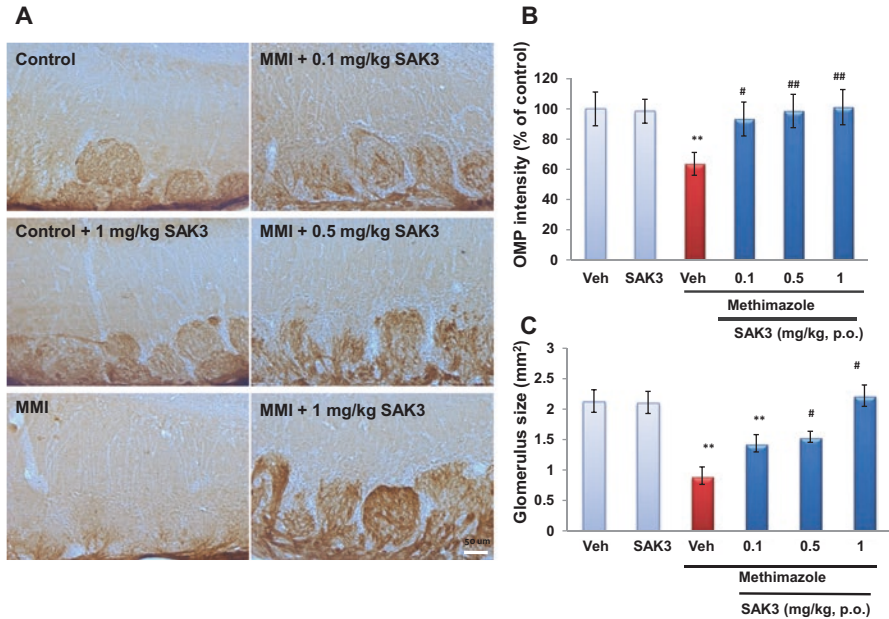
Fig. 9.2 MMI-induced decreases in OMP expression in olfactory bulb glomeruli are antagonized by SAK3 administration. (**a**) Coronal sections of olfactory bulb from indicated control (c), MMI-treated, or MMI-treated and SAK3-treated (0.1, 0.5 and 1 mg/kg) mice were incubated with OMP antibody. (**b**) SAK3 treatment significantly restored OMP staining intensity (**b**) and increased glomerulus size (**c**) in the OB glomerular layer. Scale bar, 50 μm. Error bars represent S.E.M. (**$p < 0.01$ vs control, #$p < 0.05$ and ##$p < 0.01$ vs MMI). n = 7 per group. (Modified from Noreen et al. 2017)

bulbectomy leads to anterograde degeneration of MS cholinergic neurons and con-comitant loss of hippocampal cholinergic nerve terminals (Han et al. 2008). Loss of MS cholinergic neurons is also associated with cognitive deficits seen in Alzheimer's disease (Robinson et al. 2011). Indeed, single administration of MMI (75 mg/kg, i.p.) promotes hypothyroidism in mice, and SAK3 treatment prevents hypothyroid-ism-induced loss of MS cholinergic neurons, thereby improving memory deficits seen in MMI-treated mice (Noreen et al. 2017). In humans, adult onset hypothyroid-ism is associated with impaired spatial memory performance and cognitive function (Tong et al. 2007; Artis et al. 2012), although mechanisms underlying these impair-ments remain unclear.

Our recent analysis of MMI-treated mice showed that SAK3 may be neuropro-tective and antagonize these cognitive deficits (Fig. 9.2). We found that perturbation of OSN maturation by a single dose of MMI is accompanied by a decrease in the number of MS cholinergic neurons (Fig. 9.3), a loss that likely causes memory and cognitive deficits seen in these mice. Importantly, SAK3 administration to MMI-treated mice rescued degeneration of MS cholinergic neurons and improved deficits in spatial reference memory and cognition.
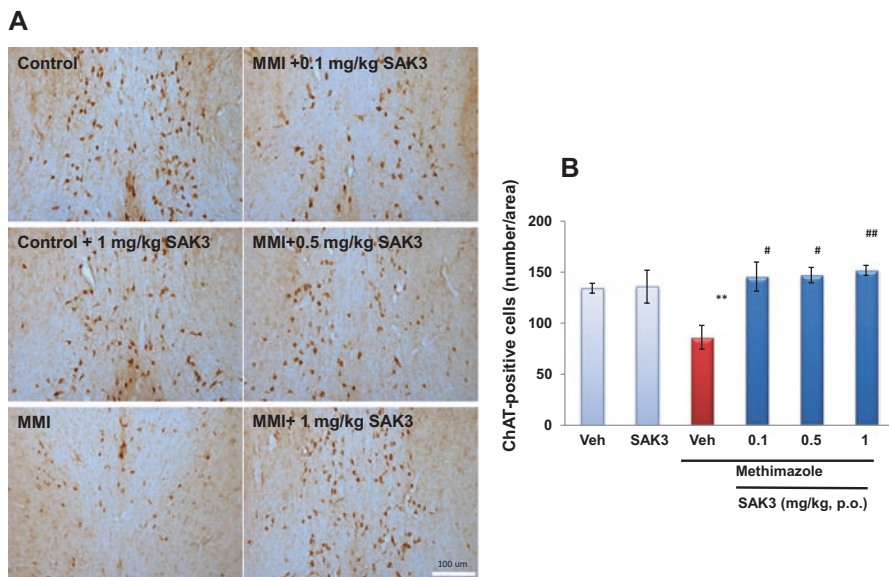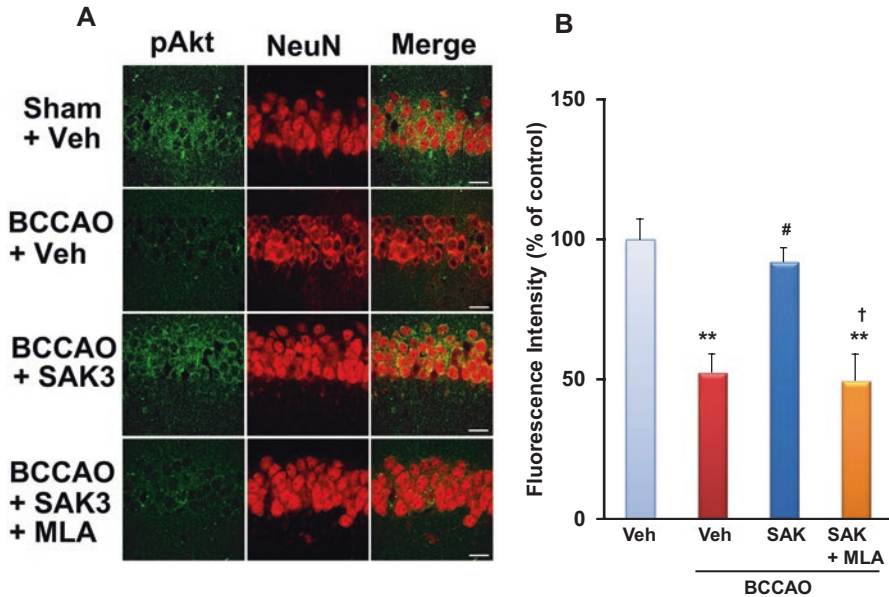
Fig. 9.3 SAK3 administration rescues MMI-induced decreases in the number of ChAT-positive cells in the medial septum. Photomicrographs showing anti-ChAT staining in the medial septum (MS) area. (**b**) ChAT-positive cells were counted in the MS of control or MMI-treated mice with or without SAK3 administration (0.1, 0.5 and 1 mg/kg). *Scale bar*, 100 μm. Error bars represent S.E.M. (**p < 0.01 vs control, #*p* < 0.05 and ##*p* < 0.01 vs MMI). n = 7 per group. (Modified from Noreen et al. 2017)

9.7   SAK3 Is Neuroprotective Via nAChRs

Several reports indicate that nAChR neuroprotective activity requires activation of protein kinase B (Akt) signaling, a critical cell survival pathway (Davis and Pennypacker 2016; Fan et al. 2017). The α7 but not the α4 nAChR subunit interacts with the non-receptor-type tyrosine kinase Fyn and janus-activated kinase 2 (JAK2) (Kihara et al. 2001; Shaw et al. 2002), and α7 nAChR stimulation triggers activation of both kinases and subsequently upregulates phosphatidylinositol 3 kinase (PI3K) (Kihara et al. 2001; Shaw et al. 2002). Activated PI3K in turn promotes Akt activity and downstream survival signaling, including Nrf2/HO-1 signaling in neurons (Franke et al. 1997; Kihara et al. 2001; Navarro et al. 2015; Niture and Jaiswal 2012; Shaw et al. 2002). By contrast, α7 nAChR activation in microglia and/or astrocytes is neuroprotective by promoting release of anti-inflammatory cytokines and blocking release of inflammatory cytokines (Di Cesare et al. 2015; Shin and Dixon 2015). The observation that both SAK3-induced ACh release and SAK3-induced neuroprotection are blocked by α7 nAChR inhibitors supports the idea that SAK3 effects are in large part mediated by nAChRs. SAK3-induced neuroprotection is closely associated with enhanced Akt rather than ERK activities (Yabuki et al. 2017a, b) (Fig. 9.4). In this context, α7 nAChR activation by SAK3 administration is critical for neuroprotection.

Fig. 9.4 Acute SAK3 administration rescues Akt phosphorylation in CA1 pyramidal neurons of BCCAO mice through α7 nAChR stimulation. (**a**) Representative images showing fluorescent immunostaining with phospho-Akt (Ser-473: green) and NeuN (red) antibodies. Phosphorylated Akt immunoreactivity decreased in CA1 NeuN-positive neurons 24 h after BCCAO. Treatment with MLA (6.0 mg/kg, i.p.) blocked SAK3-dependent increases in Akt phosphorylation in NeuN-positive neurons. Scale bars: 20 μm. (**b**) Fluorescence intensity of Akt phosphorylation was measured in the hippocampal CA1 region. Immunofluorescence intensity of phosphorylated Akt significantly decreased in CA1 pyramidal cells (n = 4–5 per group). Error bars represent SEM. ** $p < 0.01$ vs. control mice. # $P < 0.05$ vs. vehicle-administered BCCAO mice. † $p < 0.05$ vs. SAK3 (0.5 mg/kg, p.o.)-administered BCCAO mice. MLA, methllycaconitine (6.0 mg/kg, i.p.) treatment; SAK3 (0.5), SAK3 (0.5 mg/kg, p.o.) administration; and Veh, vehicle administration. (Modified from Yabuki et al. 2017a)

9.8 Conclusion

Here, we have discussed neuroprotective activity of AChR signaling based on analysis of the novel modulator SAK3. SAK3 enhances activity of T-type calcium channels, promoting ACh release and activating hippocampal nAChRs, which are critical for memory formation. However, off-target analysis is required to determine whether SAK3 modulates nAChRs directly or indirectly. Since SAK3 activity in the CNS differs from that of cholinesterase inhibitors and from the nAChR modulator memantine, SAK3 is an attractive candidate to antagonize CNS neurodegenerative disorders such as Alzheimer's or Lewy body Diseases.

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