Impaired expression of postsynaptic density proteins in the hippocampal CA1 region of rats following perinatal hypoxia

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Abstract

Perinatal hypoxia is an important cause of brain injury amongst the newborn, such injury often resulting in an increased risk of impaired performance as regards learning and memory in later life for the affected individual. The postsynaptic density 95 (PSD-95) protein is a cytoskeletal specialization involved in the anchoring of N-methyl-D-aspartate (NMDA) receptors in postsynaptic neurons and has been reported to serve several important functions (e.g., synaptogenesis, synaptic plasticity and learning and memory performance) for the mammalian brain. Herein we investigated the long-term effects of perinatal hypoxia upon the complex of PSD-95 with NMDAR subunits by means of downstream signalling cAMP response element binding protein (CREB) phosphorylation at the Serine-133 locus (CREBSer-133 phosphorylation) within the hippocampal CA1 area (an essential integration area for mammalian learning and memory) within test-rat brains, as well as the effects upon afflicted-individual long-term learning and memory performance. We also assessed the therapeutic efficacy of dopamine D1/D5 receptor (D1/D5R) activation for such study animals. Perinatal hypoxia on postnatal day ten (P10) led to impaired performance as regards long-term spatial learning and memory (as determined on P45) associated with decreases in the level of CREBSer-133 phosphorylation and decreases in the expression of the complex of PSD-95 with NMDAR subunits (NR1, NR2A, and NR2B). In addition, activation of the D1/D5R via A68930 (a selective, CNS-permeable agonist of D1/D5Rs) administration (2 mg/kg/day, P17–23 inclusively) markedly attenuated the hypoxia-induced deleterious effects, suggesting an effective therapeutic efficacy for A68930. Our results demonstrate the long-term effects of perinatal hypoxia upon the developing brain and provide additional insights into the relative vulnerability of postsynaptic density (PSD) proteins to such insult, as well as the impairment of downstream transcription signalling CREBSer-133 phosphorylation following perinatal hypoxia. More importantly, D1/D5R activation following perinatal hypoxia may be an alternative therapeutic strategy to that which is currently available and may offer significant clinical potential for hypoxia sufferers.

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Introduction

Perinatal hypoxia is one of the most important causes of brain injury amongst the newborn, and features consequences that are potentially devastating and life-long, such consequences varying from mild behavioral dysfunctions to severe seizures, mental retardation, and/or cerebral palsy (Jensen et al., 1991; Buwalda et al., 1995; du Plessis and Volpe, 2002).
Although the consequences of perinatal hypoxia have been extensively investigated in the past, the hypoxia-elicited cascades of patho-physiological processes leading to long-term neurological dysfunctions would not appear to have been well documented previously.

The postsynaptic density 95 (PSD-95) protein is an essential synaptic adaptor protein that is involved in the anchoring of NMDA receptor (NMDAR) subunits (Kornau et al., 1995; Niethammer et al., 1996). The complex of PSD-95 with NMDAR subunits has been implicated in various important roles in the regulation of ion-channel function, neuronal differentiation, synaptogenesis, synaptic plasticity and the processes of learning and memory (Tsien et al., 1996; Migaud et al., 1998; Yamada et al., 1999; Kennedy, 2000; Yao et al., 2004). Previous studies have indicated that transient cerebral hypoxia/ischemia triggers a series of patho-physiological changes resulting, ultimately, in the death of neurons in sensitive so-affected brain regions, including the hippocampus (Kirino, 1982; Hu et al., 1998; Takagi et al., 2000). Because the PSD-95 protein plays a central role in the regulation of synaptic function, and is also important as regards linking of the synapse to downstream signalling pathways, the PSD-95 protein could also be involved in the process of hypoxia/ischemia-induced changes that lead to neuronal damage. To the best of our knowledge, however, the cellular and/or molecular mechanisms underlying the perinatal hypoxia-induced changes to synaptic function and neuronal damage within the developing brain would appear to not be fully understood at time of writing. Since the complex of PSD-95 with NMDAR subunits plays a very important role in the developing brain, it highlights the possibility that the behavioral dysfunctions resulting from perinatal hypoxia/ischemia may be associated with alterations to the complex of PSD-95 with NMDAR subunits.

The cAMP-response element binding protein, a leucine zipper-class nuclear transcription factor activated through phosphorylation at the Serine-133 locus (pCREBSer-133), is capable of integrating diverse signalling pathways and it also plays a key role in governing coordinated gene transcription during neuronal differentiation and maturation, synaptogenesis, neuronal survival, and learning and memory processes (Gonzalez and Montminy, 1989; Brindle and Montminy, 1992; Deisseroth et al., 1996; Crino et al., 1998; Silva et al., 1998; Bender et al., 2001; Kandel, 2001; Vo and Goodman, 2001). A number of previous studies have indicated that induction of pCREBSer-133 can promote long-term changes to cellular plasticity, such changes involving myriad postsynaptic density proteins (Berke and Hyman, 2000; Kelley, 2004). In addition, pCREBSer-133 levels can be induced by the activation of the dopamine D1/D5 receptor (D1/D5R) pathway, a pathway which exhibits important neuromodulatory influence within the mammalian adult brain, featuring actions such as the modulation of memory function coupled to the Ras-Mitogen-Activated Protein Kinase/Extracellular-Signal Regulated Kinase (MAPK/ERK) cascade (Levin et al., 1990; Luine et al., 1990; Packard and White, 1991; Sawaguchi and Goldman-Rakic, 1991; Brindle and Montminy, 1992; Schultz et al., 1993; Hersi et al., 1995; Roberson et al., 1999; Davis et al., 2000; Seamans et al., 2001). As best we are aware, there currently exist no effective postnatal intervention modalities to prevent long-term neurological deficits for newborn infants previously exposed to perinatal hypoxia. Thus, the present study was designed to investigate not only the long-term effect of perinatal hypoxia upon CREB Ser-133 phosphorylation, the expression of the complex of PSD-95 with NMDAR subunits, and the performance of study animals as regards spatial learning and memory, but also the therapeutic efficacy of D1/D5R activation following perinatal hypoxia.

Methods

Induction of perinatal hypoxia

Fig. 1 presents a graphic summary of this study. Sprague–Dawley (SD) rats incorporating a total of 16 dams and their litters (nine to 11 pups per dam) were housed in the animal-care facility at our institution and provided with a 12-h light/dark cycle. The animal numbers used in the present study were as follows: vehicle-control (n = 30), A68930 (a selective, CNS-permeable agonist of D1/D5Rs) alone (ie test animals receiving only A68930; n = 30), hypoxia recipients (n = 30), A68930 plus hypoxia (n = 30). For the experiments focusing on the blockade of D1/D5Rs, the animal numbers were as follows: vehicle-control (n = 6), A68930 alone (n = 6), hypoxia (n = 6), SCH23390 (a selective, CNS-permeable antagonist of D1/D5Rs) alone (ie test animals receiving only SCH23390; n = 6), A68930 plus hypoxia (n = 6), and SCH23390 + A68930 + hypoxia (n = 6). All experimental procedures were approved prior to experimental conduct which proceeded in accordance with the guidelines set by the Animal Care and Use Committee (National Science Council, Taiwan). Briefly, the procedures for inducing perinatal hypoxia were as has been described previously (Jensen et al., 1991; Yang et al., 2004; Chen et al., 2006). In brief, on postnatal day 10 (P10), juvenile rats were removed from the litter and placed in an airtight chamber (30×30×30 cm) on a heating pad to maintain temperature at 34 °C. Intra-chamber O2 concentration was measured with an oxygen meter placed inside the chamber. Following this, the O2 concentration was reduced to 5–7% and maintained at this level by constant, regulated infusion of N2 gas into the chamber. A second pulse oxygen meter was placed on the test animal’s skin surface for the simultaneous detection of oxygen saturation in the rat. Hypoxia induced a flurry of myoclonic jerks for test animals, followed by tonic–clonic head and limb movements. The total number of both myoclonic jerks and tonic–clonic movements was counted for each pup by an observer who was blinded to the identity of the study group. After 12 min of such conditions, the oxygen concentration was lowered by approximately 1%/min until the onset of apnea. The total duration of hypoxia ranged from between 12 and 15 min. Only rats exhibiting at least one tonic–clonic seizure and lower blood oxygen saturation (<60%) during hypoxia were used for this study. The hypoxia plus A68930 group received A68930 by subcutaneous (SC) injection at a dose of 2 mg/kg, on the basis of the results of...
previous reports relating to A68930’s pharmacological impact (Gilmore et al., 1995; Hersi et al., 1995; Deveneny and Waddington, 1997; Clifford et al., 1999; Salmi and Ahlenius, 2000). The compound, A68930 (cis-[±]-1-[Aminomethyl]-3,4-dihydro-3-phenyl-1H-2-benzopyran-5,6-diol hydrochloride) is a selective, CNS-permeable agonist of D1/D5Rs and SCH23390 (R[+]7-chloro-8-hydroxy-3-methyl-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine), is a selective, CNS-permeable antagonist of D1/D5Rs. Where appropriate, SCH23390 was administered to test animals (0.05 mg/kg, by subcutaneous injection) 30 min prior to each administration of A68930 (2 mg/kg as administered by subcutaneous injection), both agents being purchased from Tocris Bioscience (UK) and dissolved in phosphate-buffered saline and administered to test animals for a consecutive seven-day period (from P17 to P23 inclusively, a single injection per day) (Gilmore et al., 1995; Hersi et al., 1995; Deveneny and Waddington, 1997; Clifford et al., 1999; Salmi and Ahlenius, 2000).

Water-maze task

Two independent observers who were blinded to the entire study performed the water-maze experiments. We used an open-field water maze (2 m in diameter; opaque water; 28±1 °C; automated swim-path monitoring) (Migaud et al., 1998; Huang et al., 2002; Komiyama et al., 2002; Yang et al., 2003; Yang et al., 2006). Visible platform-trained: rats were trained to a randomly located platform (30-cm-diameter) marked with a striped flag protruding above, such training being conducted between P37 and P39 inclusively (four trials per day for 3 days; curtains drawn around the pool in order to occlude extra-maze cues; maximum trial duration was 90 s; intertrial interval (ITI), 10 min). For hidden-platform training, a hidden platform with the extra-maze cues visible was used between P40 and P44 inclusively (four trials per day for a period of 5 days; platform/pool area was 1/44; a period of 30 s was allowed for test animals on the platform at the end of each trial; ITI, 10 min). On P45, the measure of time spent in the annular zone within the training quadrant was observed as a final record.

Hippocampal slice preparation

Animals (P45) were scarified by decapitation immediately following the behavioral test. The hippocampal slices (400 μm) were cut transversely to the long axis of the hippocampus using a vibroslicer (Campden Instruments, Sileby, Loughborough, UK) and were immediately transferred to artificial cerebrospinal fluid (ACSF) in an incubating chamber featuring a humidified 95% O2/5% CO2 gas at 34.0±0.5 °C for an equilibrium period of at least one hour. The control ACSF consisted of (in mM): NaCl (124), KCl (3.5), CaCl2 (2), MgCl2 (1), NaH2PO4 (1.25), NaHCO3 (26), D-glucose (10), and prepared at a pH 7.4. The osmolarity of the solution was kept at 305±5 mOsm. Unless otherwise stated, drugs were dissolved in sterile water for all stock solutions which were then stored at −20 °C until required for use. All drugs in ACSF were prepared immediately prior to each experiment from available frozen stocks and were administrated via bath application. For CREB\textsuperscript{Ser-133} phosphorylation experiments, hippocampal slices were incubated with L-glutamate (50 μM) for 15 min, a process which served as a pseudo-conventional synaptic stimulus to detect the efficacy of CREB\textsuperscript{Ser-133} phosphorylation (Deisseroth et al., 1996; Huang et al., 2002; Yang et al., 2003; Yang et al., 2006). In order to obtain hippocampal CA1 sub-region tissue, a hippocampal slice was incubated with ice-cold oxygenated ACSF and was then cut into a tissue block (0.1×0.5 cm) within 15 s of tissue slicing. Subsequently, the hippocampal CA1 sub-regions were immediately frozen at −80 °C until analysis was undertaken, and all such tissue was only ever thawed once.
Co-immunoprecipitation and western-blot analysis

For co-immunoprecipitation analyses, PSD-95 and NMDAR subunits were co-immunoprecipitated in hippocampal CA1 sub-regions from the same rat. The tissues were homogenized to obtain nuclear protein extracts and cytoplasmic supernatant as has been previously described (Yang et al., 2004; Chen et al., 2006; Yang et al., 2006). Briefly, the tissue was homogenized in 50 mM Tris–HCl, pH 7.5, 10 mM EGTA, 5 mM EDTA, 1% sodium deoxycholate, 50 mM NaF, 20 mM ZnCl2, 1 mM sodium orthovanadate, 0.5 mg/ml PMSF, and protease inhibitors (Roche Molecular Biochemicals, Germany). The membranous components of PSD-95 and the NMDAR subunits (the P2 membrane fractions) of the homogenates were centrifuged at 800×g for a period of 10 min at 4 °C. The resulting pellets were re-homogenized and centrifuged as described above. The supernatants were combined and centrifuged at 11,000×g at 4 °C for 20 min in order to obtain the P2 pellet. This pellet was then re-suspended in 0.32 M sucrose containing phosphatases and protease inhibitors as has been described previously (Yang et al., 2004; Chen et al., 2006; Yang et al., 2006). The protein concentrations were measured by the use of Bio-Rad DC protein-assay kits (Cat. No.500-0112, Bio-Rad Laboratories, Hercules, CA, USA). Subsequently, co-immunoprecipitation was performed with the Catch and ReleaseTM Immunoprecipitation System (Upstate, USA). A quantity of protein (500 μg) was incubated with a specific antibody and antibody capture-affinity ligand for 15 minutes at room temperature and then transferred onto spin columns. Following a 6-min period of centrifugation at 1500×g, a volume of 2× samples buffer was added to the homogenate, and the sample was incubated in a 95 °C water bath for a period of 10 min. Samples were then loaded onto 10% SDS-polyacrylamide gels and resolved by standard electrophoresis (Novex, Carlsbad, CA, USA). The gels were then transferred onto PVDF filters that had been incubated with a specific primary antibody: phosphorylated CREBSerine-133 (1:1000) or PSD-95 (1:2000), and the NR1, NR2A, and NR2B (1:1000) subunits of NMDARs. Following this, filters were visualized by chemiluminescence. Subsequently, the filters were stripped and reprobed with anti-total CREB (1:1000) or anti-β-tubulin (1:2000) both of which served as the internal standard. For quantification of immunoblot signals, the band intensity was measured using a Kodak Digital Science 1D program (Rochester, NY, USA).

Statistical analysis

All data in the present study are presented as mean ± standard error of mean (SEM). Statistical differences were determined by analysis of variance OVA with repeated measurements followed by application of the Bonferroni’s t-test for post hoc multiple comparisons. A statistical significance level of α=0.05 and p<0.05 was applied to all tests.

Results

Induction of hypoxia

Heart rate and oxygen saturation were simultaneously measured for the animals at the onset of perinatal hypoxia-induced seizure activity with apnea (n=60). Both the mean heart rate (HR: 117.4±3.7 beats/min) and oxygen saturation level (92.7±2.1%) measured at the onset of hypoxia-induced seizure activity with apnea were significantly lower than the corresponding value prior to the induction of hypoxia (HR: 188.8±4.8 beats/min, p<0.05; oxygen saturation level: 99.2±0.5%, p<0.05).

Impaired performance as regards long-term learning and memory following perinatal hypoxia

In order to determine whether activation of D1/D5Rs via A68930 at an effective dosage of 2 mg/kg (during the period of from P17 to P23 inclusively, incorporating a single injection per day) exerted any beneficial effects upon the performance as regards spatial learning and memory amongst test animals following perinatal hypoxia having been induced, the water-maze test was applied to the test animals following earlier experimentation (P45). Four experimental groups (n=30 animals, for each experimental group) were trained for cued and spatial versions of the water-maze test. In the NMDAR-independent cue task, the four groups learned to approach a randomly located platform marked by a visible cue with featuring an equivalent and progressive reduction in path length over successive trials (Fig. 2A). We further tested spatial learning using the hidden-platform version of the water maze. In this NMDAR-dependent spatial task, the four experimental groups (vehicle-control, A68930 alone, hypoxia, and A68930 plus hypoxia) revealed a decrease in overall path length over the course of 20 trials (vehicle-control: F(19,551)=3.97; p<0.01; A68930 alone: F(19,551)=2.83; p<0.01; hypoxia: F(19,551)=1.78; p<0.05; hypoxia plus A68930: F(19,551)=3.62; p<0.05). In addition, the swim paths of animals from the hypoxia group revealed significantly longer overall path lengths than was the case for animals from the hypoxia group (p<0.05, Fig. 2B). As compared to the hypoxia group, the differences in mean escape latencies for the hypoxia plus A68930 group proved to be significant over the final four testing trials (p<0.05). There appeared to be no statistical inter-group difference in swimming speed on any test day (p>0.1). Subsequent to 20 training trials, performance at a transfer test (with the platform removed) was used as a final index of learning and memory (P45, Figs. 2C, D). The use of the proportion of time spent in an annular zone centred around the target platform relative to time spent at all four possible platform locations (dashed circles, Fig. 2C), the hypoxia group demonstrated significantly less-focused swimming, and test animals spent substantially less time in the annular zone than was the case for animals from the other three
experimental groups (Fig. 2D, p < 0.05). For some experiments, A68930 (1 mg/kg, subcutaneous single injection per day, P17–23 inclusively) failed to improve both learning and memory performance and the expression of cellular protein analysis for study rats. In addition, A68930 (at 3 mg/kg) was noted to improve the hypoxia-induced PSD-95 and pCREBSer-133 impairment; however, test animals revealed odd behavioral changes (e.g., frequent muscle twitching, intake refusal, locomotion impairment) from P17 to P23 while high-dosage A68930 (at 3 mg/kg) was administered to the animals (data not shown). We therefore considered that A68930 (at 3 mg/kg) may be a toxic dosage to be used for this study, in that such dosage may introduce unwanted neurobehavioral alterations for participating test animals. Together with the activation of D1/D5Rs via A68930 (2 mg/kg, P17-23 inclusively) subsequent to the induction of perinatal hypoxia effectively improved test-animal performance as regards long-term spatial learning and memory without introducing associated unwanted neurobehavioral changes.

**PSD-95 and pCREBSer-133 following perinatal hypoxia**

We analyzed the levels of CREBSer-133 phosphorylation and PSD-95 expression in the hippocampal CA1 sub-region of test animals (on P45), the results of which can be seen in Fig. 2. The right- and left-side hippocampal CA1 sub-regions from the same rat were prepared for CREBSer-133 phosphorylation and PSD-95 expression, respectively. As indicated in Fig. 3, significant decreases in CREBSer-133 phosphorylation and PSD-95 expression (n = 10, p < 0.05) were observed for the hypoxic animals, as compared to the vehicle-control animals (n = 10). The use of A68930 alone appeared to elicit no discernible effect upon both CREBSer-133 phosphorylation and PSD-95 expression (n = 10, p > 0.1). For the hypoxia plus A68930 group (n = 10), A68930 markedly reversed the hypoxia-elicited decreases in level of CREBSer-133 phosphorylation and PSD-95 expression for animals, they featuring improved performance as regards long-term learning and memory on P45 (p < 0.05).

In order to determine whether the A68930-induced effects seen in Fig. 3 were actually D1/D5R-mediated responses, SCH23390, a selective CNS-permeable antagonist of D1/D5Rs (0.05 mg/kg, SC injection 30 minutes prior to each A68930 administration, P17-23 inclusively) was applied to the animals. As indicated in Fig. 4 (n = six animals for each experimental group), the use of SCH23390, by itself, only elicited a mild reduction in the level of CREBSer-133 phosphorylation and PSD-95 expression. The application of A68930 (2 mg/kg) in the presence of SCH23390 failed to generate significant restorations as regards the level of CREBSer-133 phosphorylation and...
PSD-95 expression as can be seen in Fig. 3, such a result suggesting that the effect of A68930 proceeded primarily through D1/D5Rs.

The complex of PSD-95 and NMDAR subunits following perinatal hypoxia

Since PSD-95 is an essential synaptic adaptor protein that is involved in the anchoring of NMDAR subunits (Kornau et al., 1995; Niethammer et al., 1996), we examined a possible alteration of the NMDAR subunits in the present study. As indicated in Fig. 5A (n=ten animals for each experimental group), activation of D1/D5Rs via A68930 alone resulted in no significant changes arising as regards the expression of the NMDAR subunits for the test animals by P45, as compared to the vehicle-control group (p>0.1). In addition, decreased total protein expression for NR1, NR2A, and NR2B NMDAR subunits were observed for the animals exhibiting impaired performance as regards learning and memory, as compared to those test animals from the vehicle-control group (p<0.05).

We further investigated as to whether the protein interaction in the complex of PSD-95 with NMDAR subunits (NR1, NR2A, or NR2B) altered as regards activity level for test animals participating in the present study. The complex of PSD-95 with NMDAR subunits within the hippocampal CA1 subregion was co-immunoprecipitated from the same rat. Figs. 5B–D illustrate the P2 membrane fractions which were extracted and immunoprecipitated with antibodies against NR1, NR2A, NR2B or PSD-95 (n=ten for each group). For those animals which revealed impaired performance as regards learning and memory, it can be seen from Fig. 3, that when PSD-95 was the immunoprecipitation antibody, the quantity of NR1, NR2A, and NR2B that co-precipitated with PSD-95 was always less for the perinatal hypoxia group (p<0.05), this being especially the case for the complex formed between PSD-95 and NR2A which featured the largest decrease in the expression level for all test animals (Fig. 5C). The use of A68930 alone (at 2 mg/kg) appeared to exert no discernible effect upon the complex of PSD-95 with NMDAR subunits, however, activation of D1/D5Rs via A68930 markedly reversed the perinatal hypoxia-induced decreases in the formation of the complex of PSD-95 with NMDAR subunits, as compared to the case for the vehicle-control group (n=ten, p<0.05).

Discussion

For this study, we principally investigated the long-term effects of perinatal hypoxia upon the level of postsynaptic density protein and also upon spatial learning and memory performance. The mammalian newborn brain has been considered to be less sensitive to deficits in oxygen supply than the adult brain (Rice et al., 1981; Grafe, 1994; Towfighi et al., 1997). In addition, it has also been reported that hypoxia without apparent ischemia does not appear to elicit significant brain damage (Vannucci, 1990). Nevertheless, perinatal hypoxia did lead to a long-term reduction in the level of CREB<sup>Ser-133</sup> phosphorylation, PSD-95 (Figs. 3 and 4) and the complex of PSD-95 with NMDAR subunits (Fig. 5) in the absence of apparent neuronal loss of hippocampal CA1 subregion for animals exhibiting impaired performance as regards long-term spatial learning and memory (Fig. 2). More importantly, activation of D1/D5Rs via A68930 provided beneficial effects upon such perinatal hypoxia-induced neuro-pathological changes.

Alterations to the density and subunit composition of the NMDAR play important roles in regulating fundamental processes within the mammalian CNS during early life (Kirson and Yaari, 1996), including neurotransmitter release (Augustine et al., 1987), neuronal plasticity (Tsien et al., 1996), growth-cone development and synaptogenesis (Mattson, 1996), behavioral sensitization (Karl er et al., 1994), and learning and memory processes (Morris et al., 1986; Tsien et al., 1996).
It has been often reported that some aspects of NMDAR signal transduction depend upon receptor interactions that involve certain intracellular signalling proteins mediated by the scaffold protein within the postsynaptic density complex (Kennedy, 2000). A prominent organizing protein in this complex is the PSD-95 protein, a protein which couples the C terminus of the modulatory NMDAR subunits to various cytoplasmic proteins and enzymes (Kornau et al., 1995; Niethammer et al., 1996). The changes of protein-protein interaction between the receptors and submembranous scaffolding/signalling proteins

Fig. 4. Effect of D1/D5R activation in the presence of the selective D1/D5R antagonist SCH23390 upon level of CREB\(^{\text{Ser-133}}\) phosphorylation and PSD-95 expression amongst test animals subsequent to the induction of perinatal hypoxia. Representative immunoblots show the changes to CREB\(^{\text{Ser-133}}\) phosphorylation and PSD-95 expression for experimental rats as assessed on P45. Total CREB (rCREB) and β-tubulin served as internal standard control and the quantity present did not appear to differ significantly across lanes. The labels for vehicle-control, SCH23390, hypoxia alone, hypoxia plus A68930, and hypoxia+A68930+SCH23390 groups are, respectively, C, S, H, H+A, and H+A+S. Participating test-animal numbers include six animals for each experimental group. * \(p<0.05\) as compared with the vehicle-control group.

Fig. 5. Effect of D1/D5R activation upon NMDAR subunits in the animals subsequent to the induction of perinatal hypoxia. (A) Summary of total NR1, NR2A, and NR2B expression with representative immunoblots for rats as assessed on P45. The labels for vehicle-control (\(n=10\)), hypoxia alone (\(n=10\)), A68930 alone (2 mg/kg, \(n=10\)), and hypoxia plus A68930 (2 mg/kg, \(n=10\)) groups include, respectively, C, A, H, and H+A. β-tubulin served as a control and did not appear to differ significantly as regards quantity present across individual lanes. (B–D) PSD-95 and NMDAR subunits from the same rat (on P45) for a total of ten animals per experimental group were co-immunoprecipitated as follows: (A) PSD-95 with NR1; (B) PSD-95 with NR2A; (C) PSD-95 with NR2B, as indicated. Summary of the normalized ratios for PSD-95/NR1, PSD-95/NR2A, and PSD-95/NR2B with representative co-immunoprecipitations. Bar graphs represent the average ratios ±SEM. * \(p<0.05\) as compared with the vehicle-control group.
within the PSD protein would likely interfere with transmission of the PSD apparatus to variable extracellular signals to precise postsynaptic activity and plasticity (Ostwald et al., 1993; Hu et al., 1998; Martone et al., 1999; Wyneken et al., 2001; Wyneken et al., 2003). Truncation of the C-terminal domain of NMDAR subunits by calpain, a calcium-dependent protease (Bi et al., 1998), which is activated by a hypoxia challenge, results in the loss of the ability of the subunits to bind to PSD-95 for adult animals. We here provide further evidence that decreased protein expression for the PSD-95 protein (Figs. 3 and 4) and total NMDAR level (Fig. 5A) occurred, as well as reduced interaction between the PSD-95 and NMDAR subunits (Figs. 5B–D) within the hippocampal CA1 sub-region from the animals exhibiting impaired performance as regards long-term spatial learning and memory (Fig. 2). Indeed, PSD-95 is dynamically involved in regulating and modulating a range of synaptic functions and activities (Migaud et al., 1998; Sattler et al., 1999; Kennedy, 2000; Takagi et al., 2000; Yao et al., 2004). Altered PSD-95 expression is involved in hypoxia-induced changes leading to neuronal injury for the adult brain (Hu et al., 1998; Takagi et al., 2000). As indicated in the present study, perinatal hypoxia resulted in a reduced interaction between PSD-95 and NMDAR subunits with the largest decrease in interaction occurring for the complex formed between PSD-95 and NR2A (Fig. 5). These findings raise the possibility that changes as regards protein–protein interactions between PSD-95 and NMDA subunits may contribute to such impaired performance as regards learning and memory.

As the maturation of a newly formed neuron becomes integrated into the surrounding nervous-system network, a process requiring the formation and refinement of neuronal connections, CREB^Ser-133^ phosphorylation, and a NMDAR-mediated downstream signalling process (Deisseroth et al., 1996; Bading, 1999; Roberson et al., 1999), would appear to contribute to functional/morphological synaptogenesis and synaptic plasticity within the mammalian hippocampus. Specifically, the hippocampal CA1 sub-region is essential for spatial memory amongst rodents (Morris et al., 1986; Tsien et al., 1996). Moreover, the hippocampal CA1 sub-region is known for its sensitivity to hypoxia-induced injury during early development (Sanchez et al., 2001). For instance, during the second and third postnatal week, hippocampal granule cells in the dentate gyrus undergo important morphological and physiological changes indicating their functional maturation, including the formation of synaptic networks and morphological maturation (Bayer, 1980; Cowan et al., 1980; Bayer et al., 1982; Zafirov et al., 1994; Sans et al., 2002). Previous studies have indicated that the activation of NMDARs, which typically results in a major influx of calcium into the neuron, could activate multiple kinase pathways, including protein kinase C, calmodulin kinase, and ERK/MAP/RSK kinases that interact with each other, and converge upon key transcriptional elements such as CREB (Deisseroth et al., 1996; Bading, 1999; Roberson et al., 1999). Further, CREB is also a member of a family of DNA-binding transcription factors and is involved in the transcriptional regulation of certain immediately-early genes such as c-fos and JunB (Herdegen and Zimmermann, 1994).

Thus, phosphorylated CREB^Ser-133^ would likely regulate the transcription of various genes, including those relating to ion channels, receptors of growth or neurotrophic factors or synaptic proteins, and thus phosphorylated CREB^Ser-133^ likely contributes to synaptogenesis, synaptic plasticity, and neuronal survival in the central nervous system (CNS) (Gonzalez and Montminy, 1989; Brindel and Montminy, 1992; Deisseroth et al., 1996; Crino et al., 1998; Silva et al., 1998; Roberson et al., 1999; Davis et al., 2000; Bender et al., 2001; Kandel, 2001; Vo and Goodman, 2001). CREB mRNA transcription is also involved in synaptic differentiation and the maturation of the hippocampus during a critically important development period (the second and third postnatal weeks for test rats) (Hatalski and Baram, 1997; Murphy and Segal, 1997; Crino et al., 1998). A recent human study has demonstrated that a decrease in level of CREB^Ser-133^ phosphorylation in fibroblasts correlated with intelligence level in patients suffering from Coffin-Lowry syndrome, an x-linked mental-retardation syndrome (Harum et al., 2001). Here we provided additional evidence that decreased CREB^Ser-133^ phosphorylation was associated with a significant reduction in PSD-95/NMDAR expression for study-participating animals exhibiting impaired performance as regards learning and memory (on P45), such an outcome suggesting the down-regulated involvement of NMDAR/MAPK/ERK/pCREB^Ser-133^/PSD-95 cascade of patho-physiological processes leading to long-term neurological dysfunctions (Berke and Hyman, 2000).

It has been often reported, and would appear to be accepted clinically, that the window for effective clinical treatment of hypoxia-induced brain injury could be particularly narrow for the newborn (Jensen et al., 1991; Koh and Jensen, 2001; Sanchez et al., 2001; du Plessis and Volpe, 2002). In this study, activation of the D1/D5R pathway by the selective CNS-permeable agonist A68930 during the period P17-24 inclusively, significantly improved not only the cognitive performance as regards the water-maze task but also the levels of PSD-95/NMDAR expression with incorporating downstream signalling CREB^Ser-133^ phosphorylation. Indeed, the dopamine D1/D5R pathway exhibits a significant level of importance as regards neuromodulatory actions (e.g., modulation of memory formation) within the mammalian brain (Levin et al., 1990; Luine et al., 1990; Packard and White, 1991; Sawaguchi and Goldman-Rakic, 1991; Schultz et al., 1993; Hersi et al., 1995; Seamans et al., 2001). Several previous studies have indicated that activation of D1/D5Rs triggers “a cascade of events” involving MAPK up-regulation of CREB^Ser-133^ phosphorylation within the hippocampal CA1 sub-region (English and Sweatt, 1997; Bach et al., 1999; Roberson et al., 1999). Previously, we have undertaken investigations involving three different experimental protocols in order to understand an effective therapeutic period within which A68930 (2 mg/kg, a single subcutaneous daily injection) was injected into hypoxic rats over one of three different time periods (namely P10-P16, P12-18, or P14-P20 inclusively). From such experimentation, we noted no significant beneficial effects as regards behavioral performance (water-maze) and cell-protein level (PSD-95/pCREB^Ser-133^ expression) for such perinatal hypoxia-induced...
alterations occurring for test rats as has been witnessed for the present study (data not shown). As has been indicated in the present study, the use of A68930 markedly reversed the level of perinatal hypoxia-induced alterations to the CNS when the A68930 was injected into the hypoxic animals during the period P17-23 inclusively, as compared to the corresponding outcomes for the controls. It is likely that a “critical therapeutic window” may exist for the developing brain following perinatal hypoxia; however, further experiments are clearly needed to address this issue. Although the possible mechanisms underlying A68930’s beneficial effects as observed in this study are, as best we are aware, not clear, a previous study has reported altered dopaminergic activity within the prefrontal-striatal circuitry of rat pups subjected to postnatal hypoxia (P7-11 inclusively), such alterations being associated with impaired working memory (Decker et al., 2003). In addition, it has also been reported that activation of D1/D5Rs generates a persistent enhancement of NMDAR-mediated synaptic currents within hippocampal CA1 pyramidal neurons (Yang, 2000). Taken together, such results raise the possibility that activation of the D1/D5R pathway may play a role, at least in part, in the triggering of certain NMDAR-mediated functions (e.g., pCREBSer-133) which, in turn, promotes the beneficial up-regulation of certain mechanisms (e.g., myriad postsynaptic density proteins) responsible for synaptic differentiation and stabilization, and the maturation of neuronal networks within the hippocampus of the developing brain subsequent to the occurrence of perinatal hypoxia (Sawaguchi and Goldman-Rakic, 1991; Hersi et al., 1995; Bender et al., 2001; Seamans et al., 2001; Hardingingham et al., 2002).

In summary, our results provide experimental evidence for the notion that certain molecular and cellular mechanisms underlying the long-term existence of decreased CREBSer-133 phosphorylation, PSD-95 and NMDAR protein expression associated with the decreased interaction of the complex between the PSD-95 protein and the NMDAR subunits for animals exhibiting impaired performance as regards long-term spatial learning and memory. More importantly, activation of D1/D5Rs following perinatal hypoxia significantly alleviates such long-term neurological deficits, demonstrating the beneficial actions of this signalling pathway. Thus, D1/D5R activation may be a useful therapeutic strategy for perinatal hypoxia-induced encephalopathy. Although specific extrapolation of the results of rodent experiments to the human condition appears difficult, a good understanding of the important elements within the postsynaptic apparatus may provide more information to help to open new initiative that couples such experimental conditions to related downstream signalling pathways for humans and provides further refined therapeutic strategies for perinatal hypoxia-induced encephalopathy at cellular and/or molecular levels.

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