penicillin (200 U/mL), and streptomycin (200 μg/mL) for 72 h, on six-well plates.

3. Dilute lipofectin to a total of 100 mL in opti-MEM media at a ratio of 2:1 for lipofectin: RNA (Use 10 mL lipofectin for 5 mg RNA).
4. Allow to stand still at RT for 30–45 min, then dilute AOs to a final volume of 100 μL in opti-MEM media.
5. Combine diluted lipofectin and AOs and mix gently.
6. Incubate at RT for 10–15 min.
7. Remove serum-containing medium from cells and wash them with opti-MEM reduced serum media.
8. Add 0.8 mL opti-MEM media to the tube containing the lipofectin DNA complexes.
9. Mix gently and overlay the complex onto the cells.
10. Return cells to the incubator and after 3 h replace opti-MEM media with differentiation medium and wait 3–10 days until they differentiate into myotubes.

3.3. Intramuscular Injections of Antisense Oligos in Dogs

1. Induce general anesthesia by 20 mg/kg of thiopental sodium injections and maintain by isoflurane inhalation (2.0–3.0%).
2. Cut skin above tibialis anterior (TA) muscle with scalpel.
3. Stitch the fascia of TA muscles at two different points at 2 cm intervals as markers; i.e., inner side distal/outer side proximal.
4. Bend needles (10°) to inject PMOs horizontally using 27 G needle, inject PMO solutions slowly into muscles and wait 1 min before removing the needle to prevent leakage.
5. Inject butorphanol tartrate (0.2 mg/kg) before and after procedure.
6. Administer sepham antibiotics (Cefamezine or Syncl) for three days after surgical procedures.

3.4. Systemic Injections of Antisense Morpholinos

1. Dissolve 120–200 mg/kg of morpholinos Ex6A, Ex6B, and Ex8A at 32 mg/mL in saline.
2. Inject them into saphenous vein of a dog using 22 G indwelling needles for each injection using infusion pumps to inject at 50 mL/20 min.
3. Inject morpholinos for 5–11 times at weekly or biweekly intervals.

3.5. RNA Extraction from Myotubes

1. Remove medium.
2. Put 1 mL Trizol for each well of six-well plates.
3. Wait 10 min.

4. Add 200 μL of chloroform (for RNA)
5. Shake well.
6. Wait 2 min.
7. You can see three layers including the RNA layer (top), DNA layer (middle), and protein layer (bottom).
8. Centrifuge at 12,000×*g* for 15 min at 4°C.
9. Take 400 mL carefully from top layer. Remove supernatant from the top layer, and put in another tube.
10. Add 500 mL isopropanol.
11. Keep in −80°C for O/N.
12. Centrifuge at 12,000×*g*, 10 min, 4°C.
13. Decant fluid. You can see a pellet of RNA in bottom.
14. Wash with 75% EtOH.
15. Centrifuge at 8,000×*g*, 5 min, 4°C.
16. Dry up, keep upside down for 15 min or O/N.
17. Add 15–30 μL water, then quantify RNA concentration.

3.6. RT-PCR

1. Make Reaction mix containing 1.5 μL 10 mM forward primer, 1.5 μL 10 mM reverse primer, 1 μL dNTP, 5 μL one-step PCR kit buffer, 0.7 μL RNAsin, 1 μL enzyme mixture from one-step PCR kit, and 200 ng RNA and add water to the total of 25 μL.
2. Perform RT-PCR in the thermocycler with 1 cycle of 50°C 30 min, 1 cycle of 95°C 15 min, 35 cycles of 94°C 1 min, 60°C 1 min and 72°C 1 min. Finally add 1 cycle of 72°C 10 min and then store PCR product in 4°C.

3.7. cDNA Sequencing

1. Use Qiagen gel extraction kit to excise the band of interest for subsequent cDNA sequencing according to manufacturer's instructions. Exon 6-9 skipped band (101 bp) is identified by electrophoresis using 2% agarose gel (Fig. 1).
2. Use BigDye® Terminator v3.1 cycle sequencing kit for cDNA sequencing with the same primers following manufacturer's instructions (Fig. 1).

3.8. Muscle Sampling from Necropsy of Dogs

1. Inject with thiopental sodium for induction of general anesthesia, then maintain anesthetic status by isoflurane.
2. Euthanize dogs by bleeding from the carotid artery.
3. Collect following muscles by necropsy of dogs 2 weeks after final injection of oligos. These muscles include TA, extensor digitorum longus (EDL), Gastrocnemius, soleus, biceps femoris, rectus femoris, biceps brachii, triceps brachii, deltoid, extensor carpi ulnaris (ECU), extensor carpi radialis (ECR),

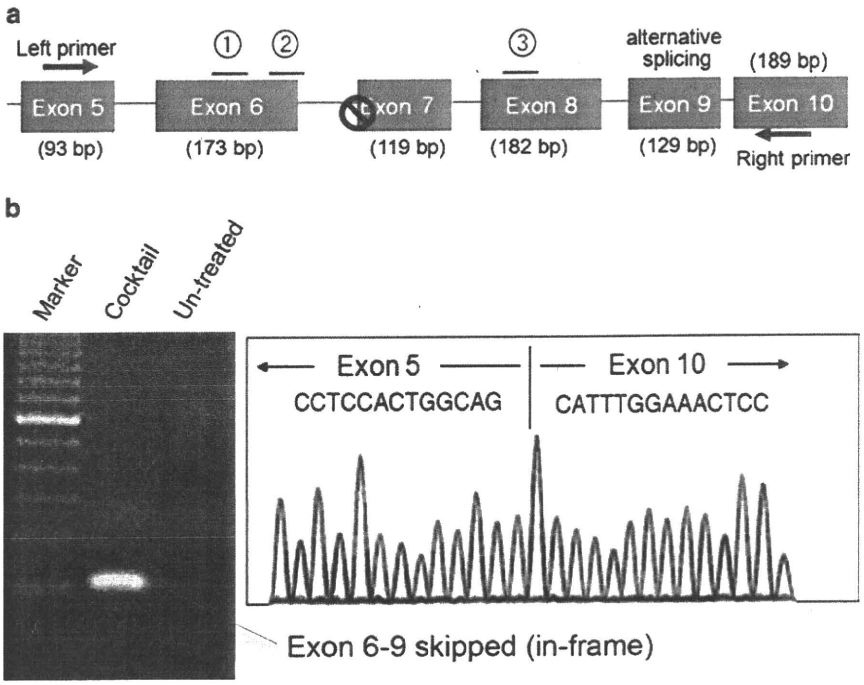

Fig. 1. Multiple exon skipping in dystrophic dogs. (**a**) Schematic outline of the protocol. A splice site mutation in intron 6 leads to deletion of exon 7 at mRNA level in dystrophic dogs. To restore the reading frame, two additional exons (exon 6 and exon 8) need to be skipped (removed) by three oligo cocktail of antisense. Exon 9 is known as alternative splice site. (**b**) RT-PCR and cDNA sequencing after exon skipping in dystrophic dogs. *Left panel*; RT-PCR reveals exon 6–9 skipped in-frame products (101 bp) in dystrophic dogs after the treatment of cocktail oligos. Alternative splice site Exon 9 is also mostly removed from the resulting mRNA. *Right panel*; Exon-skipping patterns are further confirmed by cDNA sequencing.

flexor carpi ulnaris (FCU), flexor carpi radialis (FCR), gracilis, intercostal, abdominal muscles, diaphragm, lateral dorsi, esophagus, sternocleidomastoid, and the heart.

4. Dissect muscles into small portion to stand on cork disks (1.2 cm diameter) labeled with the ID of the animal and muscle name on the back side.
5. Mix a portion of tragacanth gum (10–20 mL) well with equal amount of water until it becomes soft and sticky. Put them into 10 mL or 25 mL syringes. Unused gum in the syringe can be stored in freezer.
6. Put tragacanth gum to fix the muscle specimen on cork disks.
7. Put liquid nitrogen in a metal container and isopentane in a smaller metal container.
8. Lower the isopentane with the container into the liquid nitrogen. Wait for a couple of minutes until it becomes slushy and ready for freezing.
9. Put a portion of gum on the cork.

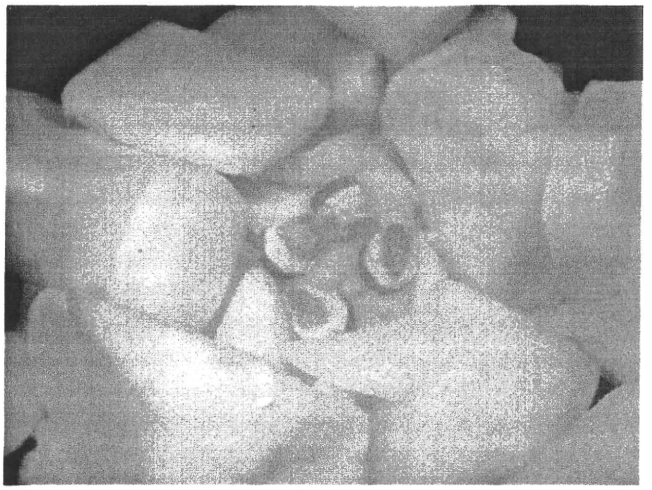

Fig. 2. Frozen muscle samples from dystrophic dogs.

10. Dissect out muscles and put it on the cork at RT. Place it on the cork longitudinally and put some gum blob around the bottom of them so that the longitudinal axis of the muscle is perpendicular to the cork and stable.
11. Place the muscle on cork into the cooled isopentane and shake vigorously for 1 min.
12. Place it on dry ice (Fig. 2).
13. Put samples in a glass vials, and store at –80°C.

3.9. Immunostaining for Dog Muscles

1. Set up cryostat for sectioning. The working temperature should be –25°C. Set the section thickness at 8 μm for immunohistochemistry and 12 μm for HE staining. Put in a blade.
2. Place muscle blocks on dry ice for transportation.
3. Label slide glasses in pencil with animal IDs, cut date, and muscle name.
4. Mount cork with muscle sample block and fix in place with water. Attach the chuck with tissue specimen onto the holder.
5. Start slicing the muscle until approximately one fourth of the way in the muscle.
6. Touch and transfer individual sections onto RT slide glass and leave at RT to dry.
7. Place every sixth section on the same slide (sections 1, 6, 11 on slide #1; sections 2, 7, 12 on slide #2) and cut them at interval of every 200 mm until you have five sections collected per slide. Keep sections clustered as closely as possible to reduce the amount of antibody solutions required.

8. When finished, allow slides to dry at RT for at least an additional 90 min. Slides can be stored at −80°C.
9. For immunohistochemistry, put slides in moisture chamber (and dry them for 30 min if they were stored in a freezer).
10. Blocking; 2 h in PBS with 15% goat serum at RT.
11. Incubate with a primary antibody; antidystrophin rod (DYS-1) or C-terminal monoclonal antibody (DYS-2) for dog dystrophin staining (1:150 dilutions) for overnight at 4°C.
12. Wash with PBS 5 min × 3 times.
13. Incubate with a secondary antibody, Alexa 594 goat antibody against mouse IgG_1 or IgG_2 (highly cross-absorbed) (1:2,500) for 30 min at RT.
14. Wash with PBS 5 min × 5 times.
15. Wipe off excess liquid and mount with DAPI-containing mounting agent for nuclear staining and then put cover glasses.
16. Count the number of positive fibers for DYS1 under fluorescent microscope and compare in sections where their biggest number of the positive fibers were as previously described (see Note 4) (11).
17. Immunohistochemistry is also applicable for myotubes. Use slide glasses with chambers to culture them and fix them by 4% PFA for 10 min (Fig. 3).

3.10. Western Blotting from Dog Muscles

1. Collect the 30–40 of cryo-sections of 15 μm in 1.5 mL tube on dry ice.
2. Add 150 mL of sample buffer and homogenize on ice.
3. Boil them for 3–5 min and centrifuge for 15 min at 16,500 × *g*.
4. Collect supernatant and keep the aliquot at −70°C.

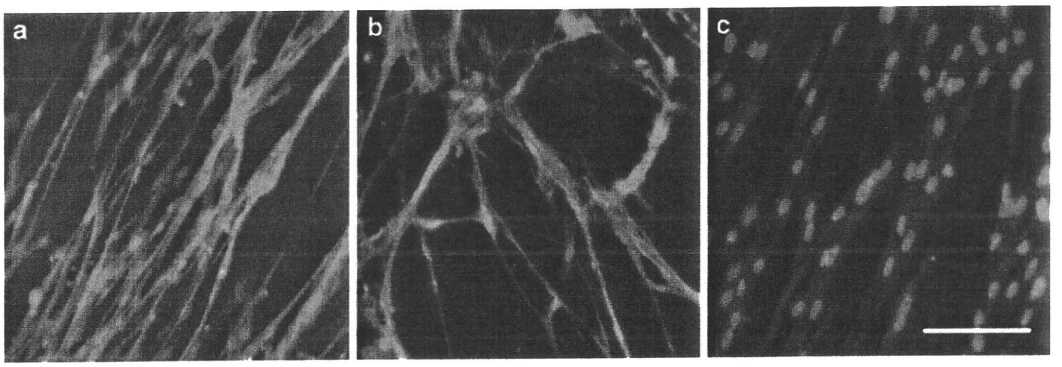

Fig. 3. Recovery of dystrophin expression after cocktail antisense transfection in dystrophic dog myoblast. Dystrophin expression and DAPI nuclear double staining of wild-type myotubes (**a**), cocktail of Ex6A, Ex6B, and Ex8A 2'OMePS transfected myotubes (**b**), and nontreated CXMD myotubes (**c**). Dystrophin C-terminal antibody DYS-2 is used. Bar: 50 μm.

5. Dilute an aliquot of protein 100-fold with distilled water to reduce final SDS concentration less than 0.1%. Measure protein concentration of the diluted protein sample with Bradford protein assay. Specifically, record the absorbance at 570 nm using a photospectrometer and calculate the concentration from standard curve.
6. For SDS-PAGE, set the glass plates for readymade mini-gel (5%).
7. Mix the samples with 2× Laemmli SDS-loading buffer.
8. Boil samples for 3 min, then load 20 mg of samples in each lane.
9. Run the gel at 150 V for approximately 3 h.
10. After running the gel, incubate the gel for 20 min in transfer buffer + 0.1 % SDS (optional for transferring high molecular weight proteins).
11. Wet four pieces of sponge and Whatman paper with ddH_2O, then soak them in the transfer buffer, and soak PVDF membrane using methanol for 1 min to prewet it, and then pour-off methanol and add H_2O, make sure that the membrane does not float. Leave it in water for 3 min.
12. Set the gel and membrane as shown in the manual.
13. Run 40–50 V o/n in cold room.
14. For blotting, prepare 2,000 mL of 0.05% PBS/Tween 20 (PBST). Wash the membrane briefly with 20 mL PBS.
15. Prepare 100 mL of PBST/5% milk powder, and incubate in 50 mL PBST/5% milk powder for 2 h.
16. Incubate the blot with primary antibody in the appropriate dilution with PBS/5% milk powder (1:100 dilution for Dys1 dystrophin antibody) for 1 h or O/N.
17. Wash the blot for 15 min each with 3× 100 mL PBST, then incubate the blot with the HRP conjugated secondary antibody.
18. Wash the blot for 20 min each with 3× 200 mL PBST.
19. Use ECL plus kit for detection. Mix two solutions at 40:1, and incubate with membrane for 1 min. Then use film and developer for the detection.
20. To preserve for the spare blot, rinse the blot with PBST, and store it in PBST at 4°C for a few weeks. Desmin antibody is used to normalize intersample loading amount. Signals are analyzed and quantified using Adobe Photoshop and ImageJ software (Fig. 4).

3.11. Clinical Grading of Dogs

1. Let a dog walk and evaluate gait disturbance: grade 1 = none, grade 2 = sitting with hind legs extended, grade 3 = bunny

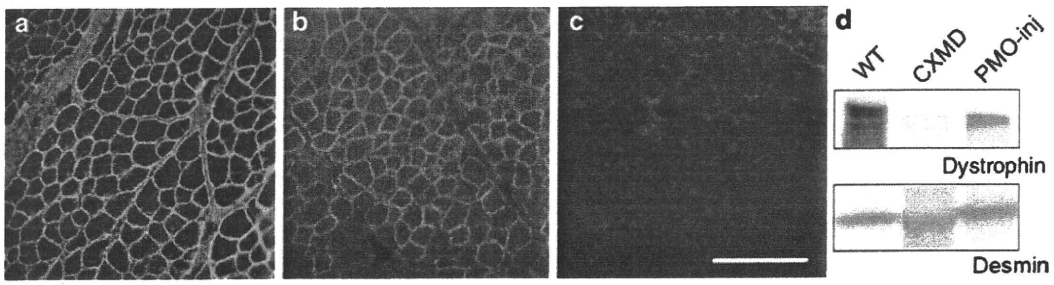

Fig. 4. Recovery of dystrophin expression after 7 × 200 mg/kg intravenous cocktail morpholino injections. Dystrophin expression of wild-type dog muscle (**a**), cocktail of Ex6A, Ex6B, and Ex8A PMOs injected dog muscle (**b**), nontreated CXMD dogs (**c**), Western blotting analysis with dystrophin antibody (**d**). Bar: 100 μm.

hops with hind legs, grade 4 = shuffling walk, and grade 5 = unable to walk (12).

2. Evaluate mobility disturbance: grade 1 = none, grade 2 = lying down more than normal, grade 3 = cannot jump on hind legs; grade 4 = increasing difficulty moving around, and grade 5 = unable to get up and move around.
3. Palpate limb or temporal muscle atrophy: grade 1 = none, grade 2 = suspect hardness, grade 3 = can feel hardness or apparently thin, grade 4 = between grades 3 and 5, and grade 5 = extremely thin or hard.
4. Evaluate drooling: grade 1 = none, grade 2 = occasionally dribbles saliva when sitting, grade 3 = some drool when eating and drinking, grade 4 = strings of drool when eating or drinking, and grade 5 = continuous drool.
5. Evaluate macroglossia: grade 1 = none, grade 2 = slightly enlarged, grade 3 = extended outside dentition, grade 4 = enlarged and slightly thickened, and grade 5 = enlarged and thickened.
6. Evaluate dysphagia: grade 1 = none; grade 2 = takes time and effort in taking food, grade 3 = difficulty in taking food from plate, grade 4 = difficulty in chewing, swallowing, or drinking, and grade 5 = unable to eat.
7. Add up the total score.
8. For running test, encourage each dog to run one time for 15 m, and record elapsed time.

4. Notes

1. Alternatively, one can also use 2'O-MePs (Eurogentec) against exons 6 and 8 of the dog dystrophin gene. These include Ex6A (GUUGAUUGUCGGACCCAGCUCAGG),

Ex6B (ACCUAUGACUGUGGAUGAGAGCGUU), and Ex8A (CUUCCUGGAUGGCUUCAAUGCUCAC).

2. The efficacy of antisense oligos is highly unpredictable, and hence several antisense oligos should be designed for each target exon. A preferred antisense sequence contains 40–60% of GC, does not have more than three consecutive guanine, and does not lead to self dimers or hetero dimers when injected as a cocktail. We have designed more than ten antisense sequences against exon 6 and exon 8 of dogs, and optimized the most efficient combination of cocktail antisense oligos both in vitro and in vivo (Saito et al., Unpublished).
3. For 2'OMePS, U (uracil) is used instead of T (thymidine).
4. Occasionally dystrophin-positive revertant fibers can be detected in dystrophic dog muscles (5, 12). Revertant fibers cannot be distinguished from antisense-mediated dystrophin expression by immunohistochemistry unless an epitope-specific antibody is used. Therefore, the expression level should be carefully compared with untreated controls.

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CHAPTER NINE

Multifunctional Roles of Activins in the Brain

Hiroshi Ageta *and* Kunihiro Tsuchida

Contents

Abstract

Activins, which are members of the TGF-β superfamily, were initially isolated from gonads and served as modulators of follicle-stimulating hormone secretion. Activins regulate various biological functions, including induction of the dorsal mesoderm, craniofacial development, and differentiation of numerous cell types. Activin receptors are highly expressed in neuronal cells, and activin mRNA expression is upregulated by neuronal activity. Activins also exhibit neuroprotective action during excitotoxic brain injury. However, very little is known about the functional roles of activins in the brain. We recently generated various types of transgenic mice, demonstrating that activins regulate spine formation, behavioral activity, anxiety, adult neurogenesis, late-phase long-term potentiation, and maintenance of long-term memory. The present chapter describes recent progress in the study of the role of activin in the brain.

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I. Introduction

In 1986, during inhibin purification, activin, a regulating factor for secretion of follicle-stimulating hormone from pituitary cells was discovered (Ling *et al.*, 1986; Vale *et al.*, 1986). For more than 20 years after its discovery, numerous studies have shown that activins serve as multifunctional growth and differentiation factors in many cell types (Mather *et al.*, 1997; Ying *et al.*, 1997).

Activins are dimeric glycoproteins, which are formed by two of four different β subunits of inhibin in mammals (βA, βB, βC, and βE; Tsuchida, 2004). βA and βB transcripts exist in almost all tissues. In contrast, βC and βE subunits are predominantly expressed in the liver. Homodimers of inhibin βA or βB subunits, activin A and activin B, respectively, exist in various tissues (Nakamura *et al.*, 1992), and heterodimeric activin AB has also been isolated from porcine follicular fluid (Ling *et al.*, 1986; Nakamura *et al.*, 1992).

Activins directly bind to serine/threonine kinase activin type II receptors (ActRII and ActRIIB), which are located on the cell membrane (Pangas and Woodruff, 2000; Fig. 9.1). Once the ligand is bound, type II receptors

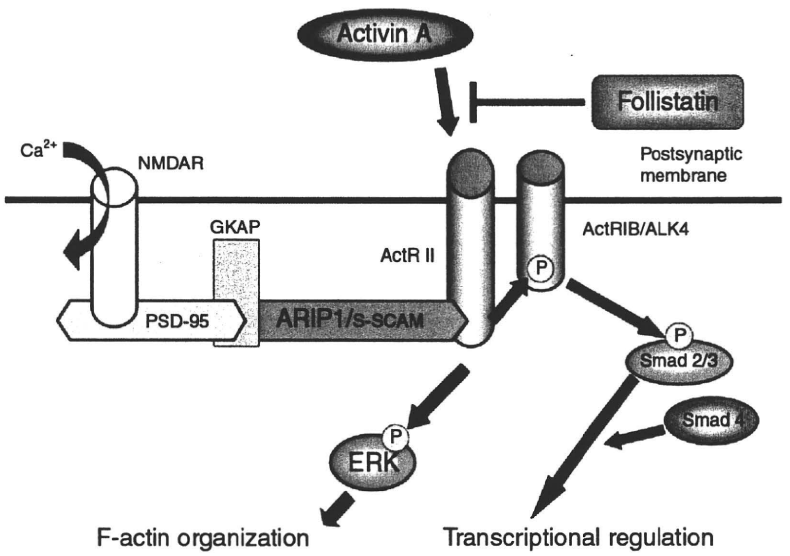

Figure 9.1 Activin-signaling cascade in the postsynaptic region. Activin A binds directly to activin type II receptor (ActRII or ActRIIB). Following binding, ActRIIs phosphorylate activin type I receptors (ActRIB or ALK4). ActRIB phosphorylates the transcriptional factor Smad2/3, and phosphorylated Smad2/3 binds to Smad4. Smad complexes translocate to the nucleus and regulate transcriptional activities. In addition, activins activate ERK signaling and lead to NMDA receptor phosphorylation in neurons. Follistatin specifically binds to activins. The activin-bound follistatin does not have access to ActRIIs. Therefore, follistatins are endogenous inhibitors of activin signals. ActRIIs form large protein complexes, including NMDAR, ARIP1, and PSD95.

recruit and phosphorylate activin type I receptors (ActRIs), termed activin receptor-like kinase 4 (ALK4 also known as ActRIB) for activin A or ALK7 for activin B. Activated ActRIs then phosphorylate transcriptional factors Smad2/3, and phosphorylated Smad2/3 binds to Smad4. Subsequently, Smad complexes are translocated to the nucleus to regulate transcriptional activities. Follistatins, which are also secreted factors, bind to activins with high affinity. The activin-bound follistatin does not have access to activin receptors (Fig. 9.1). Therefore, follistatins are specific inhibitors for the activin-signaling cascade.

Inhibin βA KO mice die within 24 h after birth, and *Follistatin* KO mice exhibit growth retardation and die within hours after birth, which is due to respiratory failure (Matzuk *et al.*, 1995a,b), demonstrating that activin signals are important for normal embryonic and postnatal development. Activin signals are also involved in the pathogenesis of a variety of diseases, including metabolic diseases, musculoskeletal disorders, cancers, and mental disorders. According to recent studies, activin signaling could be a promising target for these disorders (Tsuchida *et al.*, 2009).

Recently, we generated several types of transgenic mice, which demonstrated that activins regulate spine formation, behavioral activity, anxiety, adult neurogenesis, late-phase long-term potentiation (LTP), and maintenance of long-term memory (LTM). This chapter provides an overview of recent progress in the study of the role of activin in the brain.

II. Expression Pattern of Activin and Activin Receptor in the Brain

In 1995, Andreasson and Worley isolated the neural activity-dependent gene *inhibin* βA mRNA and showed that its regulation was dependent upon *N*-methyl-D-aspartate (NMDA) receptor activation (see Section III about NMDA receptor; Andreasson and Worley, 1995). At the same time using the differential display method, *inhibin* βA mRNA was identified as a neural activity-dependent gene in the rat hippocampus (Inokuchi *et al.*, 1996). In contrast, however, *inhibin* α mRNA levels were not affected by neuronal activity (Inokuchi *et al.*, 1996). In addition, activin receptor *ActRII* mRNA is expressed in the adult brain and is specifically abundant in the hippocampus and amygdala (Cameron *et al.*, 1994). Immunohistochemical analysis also revealed ActRII expression in neurons of the cerebral cortex, hippocampus, medial amygdala, and thalamus (Funaba *et al.*, 1997).

III. Activin Receptor and its Regulatory Proteins

The brain is composed of neurons that communicate with one another by transmitting chemicals (neurotransmitters) (Fig. 9.2A). To accomplish this, neurons develop two distinct processes—axons and dendrites (Fig. 9.2A). Information flows from one neuron to another across a synapse, which is a small gap between the neurons. Chemicals (neurotransmitters) are released from the axonal terminal (presynapse), which then bind to cell-surface receptors located on the dendrites (postsynapse; Fig. 9.2A). Spines are small, membranous protrusions on the dendrite. The majority of spines have a bulbous head (the spine head) and a thin neck that connects the head of the spine to the dendrite shaft (Fig. 9.2A). In the spine head, crucial proteins accumulate to respond to presynaptic regional signals, which are linked by scaffold proteins.

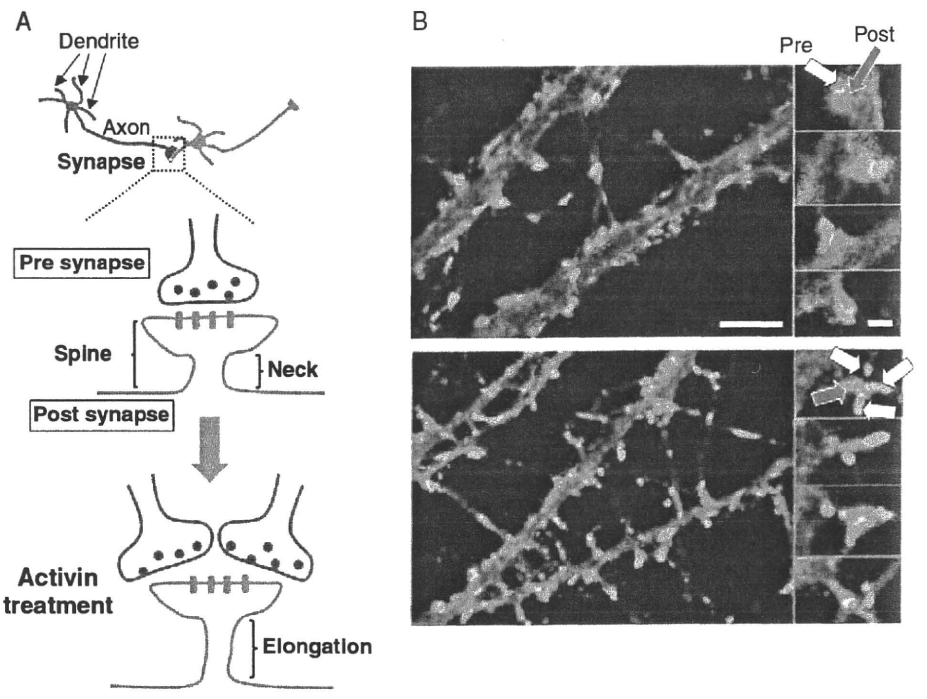

Figure 9.2 Activins regulate spine morphology. (A) Illustration of neuron, synapse, and spine structures. (B) Cultured hippocampal neurons were treated with vehicle (upper) and activins (lower), followed by staining with phalloidin to identify F-actin (red, blue arrows) and antisynaptophysin antibody (green, white arrows) to identify presynaptic regions. Scale bar, 1 μm.

Activin type II receptor, ActRII, binds to a scaffold protein called activin receptor interacting protein 1 (ARIP1)/synaptic scaffolding molecule (S-SCAM)/membrane-associated guanylate kinase, WW and PDZ domain containing 2 (MAGI2), which is localized at the postsynaptic site (Fig. 9.1). In addition, ARIP1 binds to guanylate kinase domain-associated protein (GKAP), Fyn kinase (Kurisaki *et al.*, 2008), and Smad3 (Shoji *et al.*, 2000). Postsynaptic density 95 (PSD95) is a well-characterized scaffold protein in the postsynaptic region, which binds to various protein types, including channel/receptor proteins (e.g., K channel, NMDA receptor, and Erb4), adhesion proteins (e.g., neuroligin), signaling proteins (e.g., synaptic RasGAP (SynGap) and neuronal nitric oxide synthase (nNOS)), and other neuronal function regulators (e.g., Stargazin and GKAP; Hata and Takai, 1999; Sheng and Sala, 2001; Talmage, 2008). GKAP binds to numerous proteins *via* Shank (Tu *et al.*, 1999) and Vesl-1L/Homer-1C (Kato *et al.*, 1998). Therefore, ActRII is a component of the huge PSD-95 protein complex located in the postsynaptic site.

Glutamate is the major excitatory neurotransmitter in the mammalian central nerve system (CNS). It acts *via* two classes of receptors—ligand-gated ion channels (ionotropic receptors) and G-protein-coupled metabotropic receptors. The ionotropic glutamate receptors are subdivided into three groups (AMPA, NMDA, and kainate receptors) based on pharmacological properties. NMDA receptors are highly expressed in the hippocampus, and dysregulated activation and/or inhibition of NMDA receptors influences many CNS disorders, including stroke, Parkinson's disease, Alzheimer's disease, epilepsy, drug dependence, depression, anxiety, and schizophrenia (Muir, 2006; Parsons *et al.*, 1999; Sawa and Snyder, 2002; Skolnick, 1999). Activation of NMDA receptors results in influx of Ca^{2+}. Ca^{2+} influx through NMDA receptors is thought to play critical roles for memory acquisition and LTP (see Section IV.D for details).

Activin treatment induces phosphorylation of NMDA receptors in primary hippocampal cultures, which is dependent on Fyn tyrosine kinase and ARIP1. In addition, activins increase Ca^{2+} influx through these NMDA receptor complexes (Kurisaki *et al.*, 2008). These results indicate that activins influence neuronal activity and are involved in a multitude of CNS disorders. According to our recent work, activins also play a role in anxiety, memory, and LTP (see below).

IV. Functions of Activins in the CNS

A. Activin regulates spine formation

Individual spines, which are comprised of cytoskeletal actin, undergo actin-dependent shape changes that are regulated by neurotransmitter stimulation. This phenomenon could contribute to plasticity of brain circuits (Fischer *et al.*, 1998, 2000; Fukazawa *et al.*, 2003; Honkura *et al.*, 2008; Matus, 2005).

In typical hippocampal cultures, the majority of spine contact takes place at only one presynaptic site. Activin treatment enhances the number of presynaptic contacts per individual spine, which elevates the average length of the spine neck through F-actin organization (Fig. 9.2B; Shoji-Kasai *et al.*, 2007).

In addition to the canonical Smad pathway, activin receptors activate other Smad-independent pathways (p38 MAPK, ERK1/2, and JNK) in a cell type-specific manner (Bao *et al.*, 2005; de Guise *et al.*, 2006; ten Dijke *et al.*, 2000; Werner and Alzheimer, 2006). The influence of activins on spinal morphology is independent of protein and RNA synthesis (Shoji-Kasai *et al.*, 2007). However, it is interesting to note that these phenomena are completely blocked by treatment with MEK inhibitor. Following treatment with activins, ERK1/2 phosphorylation is markedly increased in hippocampal cultures, but not in astroglial-enriched cultures. However, activins do not result in a significant increase in JNK and p38 MAPK phosphorylation. These results suggested that ERK pathways primarily affect activin-dependent spine changes in hippocampal neurons.

B. Activin influences depression and anxiety-related behavior

Mood disorders, such as bipolar disorder and depression, represent one of the most common mental illnesses, affecting as many as 17% individuals in the United States (Kessler *et al.*, 1994). Because depression is a leading cause of suicide, it is considered a serious disorder in today's society.

Previous results have shown that activin A infusion into the rat hippocampus produced an antidepressant-like effect in the forced swimming test, which is typically used to assess the effects of antidepressant drugs (Dow *et al.*, 2005).

We have generated activin and follistatin transgenic mice under the control of the αCaMKII promoter, whose activity is thought to influence postnatal development in the forebrain. Several behavioral analyses were performed on these mice—namely, open field test, light and dark choice test, elevated plus maze test, and novel-area accessing test (Ageta *et al.*, 2008). The open field test is used to measure locomotor activity, and light and dark choice test, elevated plus maze test, and novel-area accessing test are used to measure anxiety levels. Results demonstrated that follistatin overexpression mice (FSM) exhibit decreased general locomotor activity and enhanced anxiety. In contrast, activin overexpression mice (ACM) exhibit more aggressive behavior than wild-type littermates, as well as reduced anxiety-related behavior. These results showed that activin levels in the forebrain affect locomotor activity and anxiety-related behavior (Fig. 9.3 and Table 9.1).

Other studies have generated dominant-negative ActRIB transgenic mice under control of the αCaMKII promoter, which demonstrated that activin signals are negative regulators of anxiety (Zheng *et al.*, 2009). For

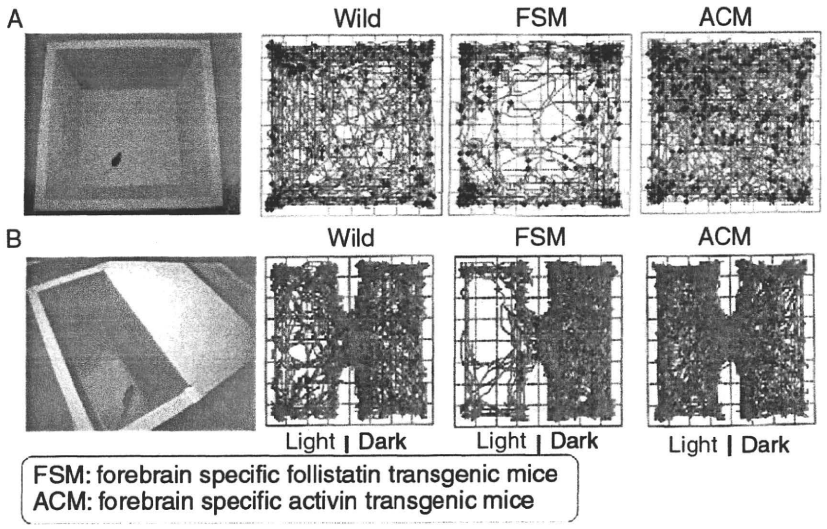

Figure 9.3 Activins influence anxiety-related behavior. (A) Open field test. (B) Light and dark test. Right panels show typical traces for each genotype.

example, these mice visited a greater number of inner fields in the open field test, which represents risk-taking behavior, and the mice also spent more time on lit, elevated places in the light-dark exploration test. These behaviors correlate with the level of anxiety in an animal, and results from this study were consistent with results from our laboratory. However, the conflicting results between the two studies could be due to transgene differences; Zheng *et al.* utilized the dominant-negative receptor ActRIB as a transgene. By contrast, we have utilized secreted factors (activins and follistatins) to determine the role of activin in the brain. GDF11, another member of the TGF-β superfamily, is expressed in the adult brain and it also binds to activin receptor II and IB. (Nakashima *et al.*, 1999). Therefore, differences in results could be due to regulation of GDF11, as well as activins. It is possible that the dominant-negative receptor ActRIB dimerizes with receptors of the TGF-β family members other than activins.

Alternatively, the dominant-negative ActRIB lacks a cytoplasmic kinase domain, thereby blocking the activin-Smad signaling cascade (see above, Fig. 9.1). Recent studies have shown that Smurf1, an E3 ubiquitin ligase, associates with the cytoplasmic domain of the TGF-β type I receptor and induces internalization and degradation of TGF-β receptors (Di Guglielmo *et al.*, 2003; Ebisawa *et al.*, 2001; ten Dijke and Hill, 2004). ActRIB also binds to and is ubiquitinated by the Smad7–Smurf1 complex *via* the ActRIB cytoplasmic domain (Yamaguchi *et al.*, 2006). Furthermore, activin-dependent spine changes are independent of Smad activation in hippocampal cultures (Shoji-Kasai *et al.*, 2007) (see above, Fig. 9.2). *In vivo*, if activin receptors are regulated by this kind of ubiquitination and proteasomal degradation,

Table 9.1 Summary of behavioral analysis of FSM and ACM

	Open field test				Light and dark test	Elevated plus-maze test	Novel-area accessing test
	Walking speed	Time spent in locomotion	Time in rearing	Risk-taking behavior	Time in light compartment	Time in open arm	Time in novel area
FSM	–	↓	↓	↓	↓	–	↓
ACM	–	–	↑	↑	↑	↑	NT

↓ downregulated compared with wild-type mice; ↑ upregulated compared with wild-type mice; – not significant change in behavior; NT, not tested; ACM, forebrain-specific activin transgenic mice; FSM, forebrain-specific follistatin transgenic mice.

expression of dominant-negative ActRIB could lead to inhibited degradation and extension of the activation period, thereby inducing an ACM phenocopy.

Previous studies have suggested correlations between anxiety and depression. Many popularly prescribed antidepressant drugs modulate monoamine neurotransmission, which could take 6–8 weeks to exert effects, and individual drugs are efficacious in only 60–70% of patients. A novel antidepressant, which acts rapidly and safely in a high proportion of patients, would be highly advantageous (Wong and Licinio, 2004). Therefore, activins and the involved signaling pathway could represent novel therapeutic targets for depression, as well as ischemic brain injury. The transgenic mice generated in our laboratory could be useful for screening compounds for newly developed and novel antidepressant drugs (Ageta *et al.*, 2008).

C. Activin is an important factor in adult neurogenesis

Postnatal neurogenesis, which is the production of new neurons in the adult brain, influences a number of physiological roles, including the replacement of damaged neurons, stress responses, and memory formation (Gage, 2002; Kitamura *et al.*, 2009; Kokaia and Lindvall, 2003; Shors *et al.*, 2001; Wang *et al.*, 2004). Several recent reports have demonstrated that neurogenesis is also involved in depression (Malberg *et al.*, 2000; Santarelli *et al.*, 2003).

Bromodeoxyuridine (BrdU), which is a thymidine analog, is incorporated into genomic DNA during S-phase and can be used as a marker of dividing cells. Through double-labeling and immunofluorescent methods utilizing antibodies specific for BrdU and the mature neuronal marker NeuN, newly generated neurons can be easily detected within brain tissue sections (Kuhn *et al.*, 1996).

In 2000, chronic antidepressant treatment was shown to significantly increase the number of BrdU-labeled cells in the hippocampal dentate gyrus and hilus (Malberg *et al.*, 2000). An additional study demonstrated that the disruption of antidepressant-induced neurogenesis actually blocks the behavioral effects of antidepressants (Santarelli *et al.*, 2003). Recently, our laboratory showed that activins are involved in adult neurogenesis (Ageta *et al.*, 2008); FSM and ACM 5-week-old transgenic mice were injected with 75 mg/kg BrdU 3 times per day for 3 days. These mice were sacrificed either 24 h or 4 weeks after the final injection; at 24 h after the final injection, BrdU incorporation was measured in the hippocampal subgranular zone (SGZ), where dividing progenitor cells are located (Gage, 2002). There was no change in the number of BrdU-positive cells in the transgenic mice compared with wild-type mice at 24 h. However, after 4 weeks, the number of BrdU/NeuN-positive cells was significantly decreased in the FSM mice, but there was no neurogenesis effect in the ACM mice.

These results indicated that endogenous activin signals are essential for adult neurogenesis (Ageta *et al.*, 2008). In addition, the level of neurogenesis in FSM/ACM-double transgenic mice was significantly greater than in FSM mice, indicating that reduced neurogenesis in the FSM mice was partially rescued by increased activin expression (Ageta *et al.*, 2008). Similar results have been reported by other groups (Abdipranoto-Cowley *et al.*, 2009). These studies suggested that activin is an important factor for adult neurogenesis.

Follistatin inhibits activins, but it also antagonizes other members of the TGF-β superfamily, namely, GDF11/BMP11 (Gamer *et al.*, 1999) and GDF8/myostatin (Tsuchida, 2004). Because increased neurogenesis was not observed in the activin overexpressing mice (ACM), the possibility of decreased neurogenesis in follistatin overexpressing mice (FSM) due to GDF11 or GDF8 could not be ruled out. However, the likelihood of this is quite low, because of the following: (1) GDF8 is exclusively expressed in skeletal muscle (McPherron *et al.*, 1997; Sharma *et al.*, 1999); (2) GDF11 is mainly expressed in olfactory epithelium (OE) in adult mice; *GDF11* mRNA expression is low in the hippocampus (ALLEN Brain project: http://www.brain-map.org); and (3) GDF11 acts as a negative regulator for neurogenesis in the OE. In fact, GDF11 inhibits production of progenitors and neurons (Wu *et al.*, 2003), which was shown in mice lacking follistatin. In FSM mice, the number of progenitor cells is normal, but survival of newly generated neurons is significantly decreased (Ageta *et al.*, 2008). Therefore, it is likely that activins, not GDF8 and GDF11, regulate adult hippocampal neurogenesis.

Results from our study demonstrated that decreased postnatal neurogenesis, which is due to activin inhibition, results in anxiety-related behavior during adulthood (Fig. 9.8).

Activin treatment in hippocampal cultures suppresses emergence of GAD67(+) GABAergic neurons and increases the percentage of Prox1 (+), dentate granule neurons. In contrast, follistatin treatment increases the percentage of GAD67(+) neurons and decreases the percentage of Prox1(+) neurons (Sekiguchi *et al.*, 2009). These results indicated that activin signaling during postnatal neural development alters neural circuitry composition by regulating the ratio of excitatory to inhibitory neurons. In addition, results have shown that GABAergic neurotransmission is altered in dominant-negative ActRIB transgenic mice (Zheng *et al.*, 2009).

Activins increase synaptic input for each individual spine (Fig. 9.2; Shoji-Kasai *et al.*, 2007), Ca^{2+} influx *via* the NMDA receptor (Kurisaki *et al.*, 2008), ratio of excitatory to inhibitory neurons in newly generated neurons (Sekiguchi *et al.*, 2009), and maintenance of early-phase LTP (E-LTP; see below) (Ageta *et al.*, 2010).

When the summation of excitatory and inhibitory postsynaptic potential from numerous synaptic inputs reaches the triggering threshold, the action

potential propagates through the axon and triggers neurotransmitter release from the presynaptic region. Therefore, these activin-dependent actions positively affect action potential activation; in other words, activins induce activation of the entire neuronal circuit.

Because electroconvulsive therapy (ECT) is used most often to treat severe major depression that does not respond to other treatments (Mukherjee *et al.*, 1994), the resulting activin-dependent changes could positively affect depression. If this were the case, the combination of compounds that enhance activin signals and low-level electroconvulsive stimulation may be suitable for human therapy, because low-level stimulation reduces unpredictable side effects. In addition, the treatment of compounds that enhance activin signals could be utilized to replace ECT.

D. Activin is a key player for maintaining late-phase LTP

LTP, which is thought to underlie learning and memory mechanisms, exhibits two distinct phases, named early-phase LTP (E-LTP) and late-phase LTP (L-LTP). E-LTP persists for several hours and does not depend on protein synthesis, whereas L-LTP persists for weeks and depends on *de novo* RNA transcription and protein synthesis (Abraham *et al.*, 1993; Frey *et al.*, 1988; Nguyen *et al.*, 1994). The formation of LTM also requires *de novo* RNA transcription and protein synthesis (Bourtchuladze *et al.*, 1994; Castellucci *et al.*, 1989; Squire and Barondes, 1973). Therefore, activity-dependent gene expression is expected to play a crucial role in LTM.

To understand the molecular mechanisms of LTM, many studies have isolated a number of neuronal activity-dependent genes, including *inhibin* βA (Andreasson and Worley, 1995; Inokuchi *et al.*, 1996), *vesl-1s/homer-1a* (Brakeman *et al.*, 1997; Kato *et al.*, 1997), *zif268* (Cole *et al.*, 1989), *arc* (Lyford *et al.*, 1995), and *scrapper* (Yao *et al.*, 2007). *Arc* and *zif268*-deficient mice exhibit impaired long-term memory, but not short-term memory (Jones *et al.*, 2001; Plath *et al.*, 2006). Vesl-1S protein is synaptically tagged *via* the ubiquitin-proteasome system (Ageta *et al.*, 2001a,b) and it regulates spinal morphology and synaptic responses (Hennou *et al.*, 2003; Sala *et al.*, 2003). Furthermore, *zif268* and *vesl-1S* knockout mice also exhibit deficient reconsolidation memory processes (see Section IV.E; Bozon *et al.*, 2003; Inoue *et al.*, 2009). These results indicated that neural activity-dependent genes have important roles in the memory process.

Recently, we examined hippocampal dentate gyrus LTP in urethane-anesthetized rats. Results showed that follistatin or antiactivin A antibody inhibits L-LTP formation without affecting E-LTP (Fig. 9.4A). The decay time course is similar to that of animals injected with the protein synthesis inhibitor anisomycin. Activins facilitate E-LTP duration (Fig. 9.4B) (Ageta *et al.*, 2010), and maintenance of CA1 L-LTP, but not E-LTP, in