ARCHIVAL REPORT

Distinct Roles of Adenylyl Cyclases 1 and 8 in Opiate Dependence: Behavioral, Electrophysiological, and Molecular Studies

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Background: Opiate dependence is a result of adaptive changes in signal transduction networks in several brain regions. Noradrenergic neurons of the locus coeruleus (LC) have provided a useful model system in which to understand the molecular basis of these adaptive changes. One of most robust signaling adaptations to repeated morphine exposure in this brain region is upregulation of adenylyl cyclase (AC) activity. Earlier work revealed the selective induction of two calmodulin-dependent AC isoforms, AC1 and AC8, after chronic morphine, but their role in opiate dependence has remained unknown.

Methods: Whole cell recordings from LC slices, behavioral paradigms for dependence, and gene array technology have been used to dissect the role of AC1 and AC8 in chronic morphine responses.

Results: Both AC1 and AC8 knockout mice exhibit reduced opiate dependence on the basis of attenuated withdrawal; however, partially distinct withdrawal symptoms were affected in the two lines. Loss of AC1 or AC8 also attenuated the electrophysiological effects of morphine on LC neurons: knockout of either cyclase attenuated the chronic morphine-induced enhancement of baseline firing rates as well as of regulation of neuronal firing by forskolin (an activator of ACs). The DNA microarray analysis revealed that both AC1 and AC8 affect gene regulation in the LC by chronic morphine and, in addition to common genes, each cyclase influences the expression of a distinct subset of genes.

Conclusions: Together, these findings provide fundamentally new insight into the molecular and cellular basis of opiate dependence.

Key Words: DNA microarray, drug abuse, forskolin, knockout mice, locus coeruleus, opiate withdrawal

The locus coeruleus (LC), the major noradrenergic nucleus in brain, has served as a useful model system in which to understand the molecular and cellular mechanisms underlying the long-term effects of opiates on the brain (1). Locus coeruleus neurons are inhibited by acute opiate exposure, but during a course of chronic drug administration, LC firing rates return to normal levels and increase far above normal levels upon precipitation of withdrawal (1). Several studies have shown that this withdrawal-induced activation of LC neurons contributes to opiate dependence and withdrawal (2–6), although some authors have questioned this view (7). Nevertheless, the observation that the withdrawal activation of LC neurons occurs in vitro as well as in vivo supports the involvement of factors that are intrinsic to these neurons (5,8). Considerable evidence now supports the hypothesis that one of the intrinsic factors is upregulation of the cAMP pathway and, more specifically, induction of adenylyl cyclase (AC) (9,10). Accordingly, LC neurons in brain slices show greater excitatory responses to forskolin (an activator of AC) after prior chronic in vivo morphine treatment (11,12). Furthermore, drugs that inhibit the cAMP pathway, infused directly into the LC, attenuate the development and expression of opiate withdrawal, whereas drugs that activate the cAMP pathway worsen withdrawal and can, in drug naïve animals, even induce certain withdrawal-like behaviors (13,14).

In earlier work, we and others demonstrated that two isoforms of AC, AC1 and AC8, are induced uniquely in the LC after chronic morphine administration, whereas all other isoforms of the enzyme are unaffected (11,15,16). The AC1 and AC8 isoforms belong to a subfamily of ACs that are activated by calcium (Ca^{2+})/calmodulin (17). In addition, several studies suggest that these isoforms are relatively insensitive to inhibition by Gαi (18,19), which raises the possibility that their induction might help LC neurons overcome persistent morphine inhibition of AC activity and thereby contribute to the cellular forms of tolerance and dependence observed electrophysiologically.

In the present study, we characterized the behavioral, cellular, and molecular adaptations to chronic morphine in AC1 and AC8 knockout (KO) mice. Our findings implicate both AC isoforms in the long-term actions of morphine on LC neurons, with loss of each isoform causing distinct effects on opiate dependence and withdrawal, LC neuronal excitability, and changes in gene expression.

Methods and Materials

Animals

Mice derived from heterozygous matings of AC1 or AC8 lines, N7 generation onto C57Bl/6, were used in all behavioral tests. AC1−/− mice and AC8−/− mice backcrossed seven generations to C57Bl/6 were mated to produce individual mice heterozygous for both AC1 and AC8. These mice were then mated to generate individual mice mutant for both AC1 and AC8 as well as wildtype control animals.

For all behavioral, electrophysiological, and molecular studies, we used 2–4-month-old male mice. Animals were housed in

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a temperature and humidity controlled environment with a 12-hour light-dark cycle and had free access to food and water. All studies were conducted according to Animal Care and Use Committees of Yale University and UT Southwestern Medical Center.

Morphine Analgesia and Tolerance

The hot plate test was performed on a platform heated to 52°C with a cutoff time of 40 sec (IITC Life Sciences, Woodland Hills, California). The latency to paw lick or jump was recorded as described elsewhere (20) (see Supplement 1).

Morphine Withdrawal

Mice were injected IP with escalating morphine doses (20, 40, 60, 80, 100, 100, and 100 mg/kg) every 8 h for 2.5 days. Two hours after the last morphine injection, mice received naloxone hydrogen chloride (1 mg/kg SC, Sigma, St. Louis, Missouri). Withdrawal signs (jumps, wet dog shakes, diarrhea, ptosis, tremor, weight loss) were then monitored for 25 min as described (20).

Electrophysiological Recordings

Brain slices from morphine-dependent animals and their control counterparts were prepared as described previously (8,21). Chronic morphine treatment involved the SC implantation of a sham or morphine pellet (containing 25 mg morphine base, National Institute on Drug Abuse) under light anesthesia, and mice were used 72 hours later. A 300-μm-thick coronal slice containing the LC was transferred to the stage of a submerged recording chamber and secured by a fine mesh attached to a platinum wire frame and perfused with oxygenated artificial cerebrospinal fluid (ACSF). After placement of the slice, the bath temperature was raised from room temperature to 33°C. Single-unit extracellular potentials were recorded by the use of patch pipettes filled with ACSF and monitored through a high-input impedance amplifier (Axoclamp 2B; Axon Instruments, Union City, California). Detailed methods for electrophysiological recordings are provided in Supplement 1.

For statistical analysis, the firing rates of 10–14 neurons from a single LC slice were averaged and considered as a single data point. Statistical significance of differences between mutants and control subjects was then tested by two-tailed *t* tests.

Gene Expression Microarrays

Microarray analysis was performed as described previously (22), with chips from Affymetrix (Santa Clara, California), with few modifications. Four groups of animals, AC1 KOs, AC8 KOs, and each of their wildtype littermate control counterparts, received SC sham or morphine pellets (as in preceding text) and were analyzed 72 hours later, making a total of 6 groups (wildtype groups were pooled) for microarray comparison. To reduce variability and increase statistical power, we performed at least three biological replicates (triplicate Affymetrix chips) for each group (depending on the number of mice available) (23). Each biological replicate consisted of 1 μg of pooled total RNA from the LCs of four mice.

Detailed methods for gene expression arrays are provided in Supplement 1. To determine the optimal criteria for considering significant gene regulation, we compared the wildtype sham versus morphine dataset with our previously published microarray and real-time polymerase chain reaction (PCR) geneset from the same brain region and same drug treatment (22). We found that the approach that best recapitulates the previous data was to select our "significantly regulated genelist" by Probe Logarithmic Intensity Error Estimation (PLIER) normalizing the data and selecting only genes with a $\log_2$ ratio of experimental/control to be $> .3$ or $< -.3$ and a comparison *p* value (using *t* test) of $< .01$. These criteria of generating a differentially expressed gene list with fold change primary criteria followed by a non-stringent *p* value cutoff is also recommended by the largest microarray study to date, which consists of > 1300 microarrays from > 50 institutions, including our own (24,25). Our "significantly regulated genelist" and associated values for each condition were imported into Genespring (Agilent Technologies, Santa Clara, California) for data visualization and heatmap generation.

Results

Behavioral Responses to Morphine in AC1 and AC8 KO Mice

To determine the role of AC1 and AC8 in opiate dependence, we first monitored opiate withdrawal behavior in morphine-dependent AC1 KO mice and their respective wildtype littermate control subjects. Animals chronically treated with morphine received an injection of the opioid receptor antagonist naloxone (1 mg/kg SC), and withdrawal behavior was monitored for 25 min. The AC1 KO mice become less dependent on morphine, because several standard opiate withdrawal signs are dramatically decreased compared with wildtype control subjects (Figure 1A). In particular, deletion of the AC1 gene dramatically decreases wet dog shakes, paw tremor, diarrhea, ptosis, and weight loss. Jumps trended toward a decrease as well, but this did not reach statistical significance. There was no effect on general body tremor.

We next assessed the role of AC1 in morphine analgesia and analgesic tolerance by use of a 52°C hot plate assay. As shown in Figure 1B, absence of the AC1 gene had no effect on morphine's initial analgesic effects or on the development of morphine tolerance after repeated drug exposures.

Behavioral responses to morphine were also evaluated in AC8 KO mice and their wildtype littermates. Interestingly, deletion of the AC8 gene caused significantly decreased jumping and general body tremor (Figure 2A), the two signs that are not significantly affected by deletion of the AC1 gene. The AC8 KO mice also exhibited an approximately 50% reduction in diarrhea compared with their wildtype control subjects, similar to the effect observed in AC1 KO mice. As with AC1 KO mice, knockout of the AC8 gene does not affect morphine analgesia or analgesic tolerance in the 52°C hot plate assay (Figure 2B).

To investigate whether deletion of both AC1 and AC8 genes results in a more pronounced decrease in morphine dependence and withdrawal, we generated AC1/8 double KO mice and studied their responses to morphine. Overall, the withdrawal phenotype of the double KOs was complex (Figure 3A). The mice reflected features of the AC1 phenotype (dramatic reductions in paw tremor and diarrhea) as well as features of the AC8 phenotype (reduced jumps), but other signs (wet dog shakes, general body tremor, ptosis, and weight loss)—which were significantly attenuated in one or the other single KO—were no longer significantly affected in the double KO mice. These latter findings are surprising and suggest that loss of both AC isoforms might cause compensatory, developmental changes that alter their responses to morphine. The double KOs showed no phenotype in the morphine analgesia-tolerance paradigm (Figure 3B), as would be expected from the lack of phenotype in the two single KO lines.

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