Stabilization of morphine tolerance with long-term dosing: Association with selective upregulation of mu-opioid receptor splice variant mRNAs

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Chronic morphine administration is associated with the development of tolerance, both clinically and in animal models. Many assume that tolerance is a continually progressive response to chronic opioid dosing. However, clinicians have long appreciated the ability to manage cancer pain in patients for months on stable opioid doses, implying that extended dosing may eventually result in a steady state in which the degree of tolerance remains constant despite the continued administration of a fixed morphine dose. Preclinical animal studies have used short-term paradigms, typically a week or less, whereas the clinical experience is based upon months of treatment. Chronic administration of different fixed morphine doses produced a progressive increase in the ED_{50} that peaked at 3 wk in mice, consistent with prior results at shorter times. Continued morphine dosing beyond 3 wk revealed stabilization of the level of tolerance for up to 6 wk with no further increase in the ED_{50}. The degree of tolerance at all time points was dependent upon the dose of morphine. The mRNA levels for the various mu opioid receptor splice variants were assessed to determine whether stabilization of morphine tolerance was associated with changes in their levels. After 6 wk of treatment, mRNA levels of the variants increased as much as 300-fold for selected variants in specific brain regions. These findings reconcile preclinical and clinical observations regarding the development of morphine tolerance.

analgesia | opiate receptor | opioid | splice variant | MOR-1

Preclinical studies have established that tolerance progressively increases over time (1). However, clinicians have long appreciated the ability to manage cancer pain in patients for months on stable opioid doses, implying that extended dosing may eventually result in a steady state in which the degree of tolerance remains stable despite the continued administration of a fixed morphine dose (2, 3). However, the animal studies used short-term opioid exposure, which differs from the clinical experience, where patients are often treated on steady opioid doses for many months. In an effort to reconcile these divergent observations, we extended morphine treatment of mice for up to 6 wk and determined morphine ED_{50} values and changes in mRNA levels of the splice variants of the mu-opioid receptor gene *Oprm1* in a range of brain regions.

Morphine tolerance is a complex response involving many processes (3). Blockade of the NMDA/nitric oxide cascade provided the first evidence that tolerance could be minimized and/or reversed pharmacologically (4–13). Morphine tolerance also can be prevented with delta-opioid receptor antagonists (14, 15) by antisense down-regulation of the delta-opioid receptor (16) and in a delta-opioid receptor KO mouse (17, 18). A number of cellular factors have been implicated, including desensitization and trafficking (19–21). Even dispositional factors influence tolerance (22–26). Chronic morphine up-regulates the expression of P-glycoprotein and multidrug resistant-associated protein, which are ATP transporters involved in maintaining the blood–brain barrier that impedes morphine entry into the brain. Down-regulation of either transporter enhanced morphine potency and prevents the development of morphine tolerance.

Although many factors have an impact on tolerance, prior studies have failed to observe significant changes in mu-opioid receptor expression, at either the protein or mRNA level. Early studies examining the binding of the mu-agonist ^{3}H-dihydromorphine following morphine pellet implantation revealed small increases of 40–50% as early as 2 h that did not change over 108 h (27). Single doses of morphine revealed similar increases as early as 20 min, and the antagonist naloxone increased binding over 60%. Thus, these modest changes in binding seen with acute or short-term treatment were not a consequence of tolerance. Long-term heroin self-administration also increased opioid receptor binding in selected brain regions by as much as 30% (28) and following withdrawal (29). Similarly, many laboratories examined the effects of morphine treatment on mu-opioid receptor mRNA levels and failed to find a significant effect (20, 21, 30–36). These studies examined whole-brain levels of the predominant mu opioid receptor variant MOR-1 following relatively short durations of treatment. However, the mu-opioid receptor gene Oprm1 generates a host of variants due to alternative splicing that fall into three classifications: (*i*) full-length 7 transmembrane (TM) variants, (*ii*) truncated single TM (1TM) variants, and (*iii*) truncated 6TM variants (3), which were not examined individually. The current studies explore the full range of splice variants of the mu-opioid receptor gene following prolonged morphine exposure over 6 wk to see if stabilization of tolerance is associated with changes in their levels.

Significance

Many assume that morphine tolerance is a continually progressive response, based mainly upon preclinical studies typically lasting a week or less. Yet, clinicians have long appreciated the ability to manage cancer pain in patients with stable opioid doses for months, implying that extended dosing may eventually stabilize the level of tolerance. To reconcile these differences, we examined tolerance over 6 wk and show that extended morphine dosing leads to progressive tolerance for 3 wk that then stabilizes for up to 6 wk and is associated with increases in the abundance of mu opioid receptor splice variant mRNA levels of as much as 300-fold.

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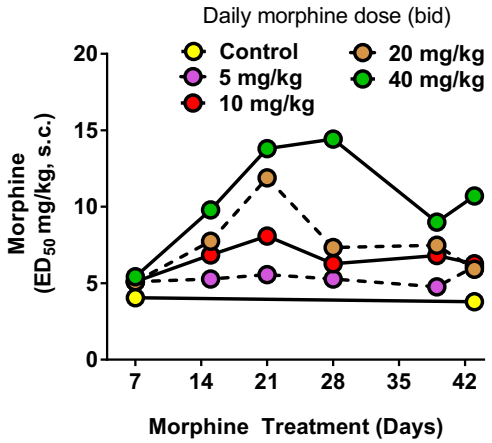

Fig. 1. Effect of extended morphine dosing on the development of tolerance. Groups of CD-1 mice (n = 60) were s.c. injected with saline (control group, n = 20) or the indicated morphine dose (5, 10, 20, or 40 mg/kg) twice daily (0830 hours and 2030 hours). Each morphine group started with 60 mice. Ten mice were removed from each group on days 7, 15, 21, 28, 39, and 43 for determination of their respective morphine ED_{50} values as described in *Methods* and were then removed from the study. bid, twice daily.

Results

Tolerance following morphine pellet implantation increases from the first day to the third day (1), even though the blood morphine levels decline after the first day (37). Daily morphine injection paradigms yield measurable tolerance within 5 d (10, 38). However, these paradigms used relatively short durations of treatment. The current study examined tolerance following several doses of morphine over a period of up to 6 wk. Tolerance developed to all morphine doses over time, although it was modest at the lower dose of 5 mg/kg (Fig. 1 and Fig. S1). The degree of tolerance depended upon the dose, with higher morphine doses producing greater tolerance. Tolerance increased in a linear fashion over the first 3 wk of dosing. As the dosing continued beyond 3 wk, tolerance stabilized, with the ED_{50} values remaining constant and/or declining. Thus, fixed doses of morphine failed to increase tolerance beyond 3 wk of treatment. The degree of tolerance depended upon the daily dose, with higher doses maintaining higher tolerance levels. Tolerance reached peak levels at 3 wk. At 3 wk, there was a close correlation between the daily dosing level and the degree of tolerance (Fig. S2). These results were consistent both with the prior preclinical literature showing progressive tolerance over short durations of treatment (1) and with clinical experience, where patients can often be maintained on fixed doses of opioids for months (39).

The leveling off of tolerance after 3 wk suggested that long-term dosing may induce compensatory mechanisms that oppose further tolerance development. Prior studies failed to identify significant changes in MOR-1 mRNA expression levels with chronic morphine exposure, but the duration of dosing was relatively short, examining whole brain and only levels of MOR-1 (20, 21, 30–32, 34–36, 40). We therefore examined the mRNA levels of a series of mu-opioid receptor splice variants after 6 wk of morphine treatment using RT-SYBR green quantitative PCR (qPCR) assays in a number of brain regions (41) (Table 1). MOR-1 undergoes extensive alternative splicing that can be divided into three categories in mice, rats, and humans (3). The 5′ splicing divides the variants into those variants generated by exon 1 promoter or exon 11 promoter. The exon 1 promoter generates a series of full-length 7TM and 1TM variants due to exon skipping or insertion. Although the exon 11 promoter produces three 7TM variants, it also yields a series of truncated 6TM variants and a 1TM variant. Within all three categories, 3′ splicing leads to a range of variants differing at the C terminus.

First, we quantified the levels of the different variants in the saline-treated controls (Table 1). The exons comprising the mouse MOR-1 variant (mMOR-1) (exons 1/2/3/4) are also contained within mMOR-1H, mMOR-1i, and mMOR-1J. This lack of a unique primer set for mMOR-1 makes it difficult to assess the levels of mMOR-1 alone. We therefore used primers in exons 1 and 2 (mE1–2) to amplify the complete repertoire of full-length 7TM variants. Subtracting the levels of the other 7TM variants provided an approximate estimate of the percentage of mE1–2 corresponding to mMOR-1 (Table S1). In whole brain, mMOR-1 accounted for about 45% of the mE1–2 levels, with widely varying percentages from region to region. The mMOR-1 levels corresponded to between 65% and 75% of mE1–2 levels in the striatum, periaqueductal gray, brainstem, and spinal cord, but accounted for less than 15% of mE1–2 levels in the prefrontal cortex, thalamus, and hypothalamus.

Morphine treatment elicited profound changes in mRNA levels, but only for some variants in some regions (Figs. 2 and 3 and Tables S2–S4). With the exception of the modest up-regulation of mMOR-1S ($P < 0.05$), we failed to see significant changes in whole brain. However, a very different pattern emerged within discrete regions. Whereas many regions failed to reveal significant changes in mRNA levels, others had profound increases as high as 300-fold. Only the levels of mMOR-1P and mMOR-1Z showed significant decreases and only in the brainstem. Although changes in *Oprm1* transcription are probably important, the varying patterns among the variants imply modulation of alternative splicing as well because a simple up-regulation of transcription would be expected to retain the same relative abundance of the different variant mRNAs in the control saline and morphine groups. Furthermore, there was no correlation between the various mRNA levels in the control groups and the degree of change with morphine treatment. Some of the greatest increases for the 7TM variants were seen with variants constitutively expressed at low levels, such as mMOR-1D, which showed the greatest increase.

In the saline control animals, the calculated relative abundance of mMOR-1 varied among the regions (Table S1). Although we still observed regional differences in the calculated relative abundance of mMOR-1 following morphine treatment (40 mg/kg twice daily), not all regions were affected similarly. The percentage of mMOR-1 changed little in some regions, such as the prefrontal cortex, hippocampus, periaqueductal gray, brainstem, and spinal cord. Other regions showed much larger differences. For example, relative abundance of mMOR-1 levels in the striatum following morphine dropped to a third of control levels, whereas the relative abundance of mMOR-1 in the thalamus and hypothalamus increased markedly. Although interesting, these changes should be assessed cautiously because the levels were not measured directly.

The three supraspinal regions most commonly showing increased mRNA levels were the striatum, hypothalamus, and hippocampus, despite the absence of highly significant changes in whole brain. Interestingly, regions typically associated with analgesia, such as the thalamus and periaqueductal gray, showed few significant changes. The elevations in the hypothalamus and hippocampus were dose-dependent, with the higher morphine dose showing greater changes. However, the reverse was observed in the striatum, where the ~20- to 150-fold increases produced by the lower morphine dose were lost at the higher morphine dose, indicating a bell-shaped dose–response curve for their up-regulation.

The truncated variants also showed a bell-shaped curve in the striatum, with the exception of mMOR-1G and mMOR-1Q, which showed no stimulation at either dose. mMOR-1G and mMOR-1M stood out from the others with their 39-fold and 47-fold increases, respectively, in the brainstem. The increases for the 1TM variants mMOR-1Q and mMOR-1R were more

Table 1. Expression of Oprm1 splice variants in different brain regions of CD-1 mice

Variant	Normalized expression levels [$E^{-(\Delta CT)} \times 10^5$]									
	Prefrontal cortex	Striatum	Thalamus	Hypothalamus	Hippocampus	PAG	Brainstem	Cerebellum	Spinal cord	Whole brain
7TM variants (exons 1/2/3/variable)										
mE1-2	532 ± 139	4,328 ± 1,010	2,721 ± 278	1,237 ± 412	4,429 ± 986	4,010 ± 848	6,019 ± 1,139	159 ± 38.4	2,612 ± 480	604 ± 108
mMOR-1A	96.2 ± 22	295 ± 67	614.1 ± 85	376 ± 93	657 ± 204	470 ± 67	579 ± 134	34.5 ± 8.6	186 ± 27	52.5 ± 9.3
mMOR-1B1	139 ± 1.5	269 ± 8.3	1,104 ± 247	306 ± 24	80.1 ± 2.0	309 ± 59	308 ± 25	15.2 ± 0.2	205 ± 27	71.8 ± 1.8
mMOR-1B2	8.4 ± 1.6	11.8 ± 0.6	23.5 ± 7.4	11.7 ± 3.4	5.6 ± 1.8	12.4 ± 1.0	15.0 ± 1.6	2.1 ± 0.7	6.6 ± 2.6	2.8 ± 1.0
mMOR-1B3	29.6 ± 8.6	36.0 ± 1.8	68.9 ± 20	43.4 ± 11	139 ± 57	62.2 ± 11	95.5 ± 32	4.4 ± 1.7	59.7 ± 15	9.2 ± 2.4
mMOR-1B4	62.1 ± 2.7	107 ± 23	226.5 ± 68	84.5 ± 29	624 ± 242	143 ± 42	169 ± 47	16.3 ± 7.6	197 ± 23	49.4 ± 9.0
mMOR-1B5	28.7 ± 1.6	91.9 ± 17	117.9 ± 34	65.8 ± 19	61.1 ± 18	99.2 ± 9.5	110 ± 29	3.8 ± 1.8	49.9 ± 11	15.8 ± 4.2
mMOR-1C	41.9 ± 9.7	72.8 ± 17	156.3 ± 56	89.3 ± 43	142 ± 37	134 ± 15.2	171 ± 48	8.4 ± 2.6	140 ± 24	34.1 ± 0.8
mMOR-1D	0.7 ± 0.2	1.3 ± 0.5	2.7 ± 0.8	1.2 ± 0.03	7.2 ± 3.7	3.7 ± 0.9	9.4 ± 1.5	0.2 ± 0.03	1.4 ± 0.6	0.6 ± 0.3
mMOR-1E	31.4 ± 4.8	23.5 ± 4.8	17.5 ± 2.9	9.7 ± 1.8	72.0 ± 11	12.7 ± 4.7	14.5 ± 5.2	7.8 ± 0.6	6.9 ± 2.1	4.0 ± 1.3
mMOR-1F	11.9 ± 2.4	66.2 ± 3.7	101.7 ± 8.2	66.2 ± 12	8.9 ± 2.9	85.0 ± 14.9	103 ± 13	1.4 ± 0.3	13.9 ± 2.9	12.9 ± 3.7
mMOR-1H*	1.8 ± 0.6	2.2 ± 0.5	4.9 ± 1.2	2.0 ± 0.5	1.7 ± 1.0	2.1 ± 0.7	3.0 ± 1.1	0.1 ± 0.0	24.5 ± 8.9	1.1 ± 0.3
mMOR-1i*	14.7 ± 4.5	16.1 ± 0.8	47.1 ± 15	21.0 ± 4.9	11.8 ± 2.9	21.6 ± 1.7	21.1 ± 1.9	1.0 ± 0.2	20.0 ± 1.0	5.4 ± 1.1
mMOR-1J*	1.7 ± 0.6	2.5 ± 0.4	8.3 ± 2.8	3.5 ± 1.0	3.4 ± 1.0	2.6 ± 0.4	3.1 ± 0.8	0.1 ± 0.0	1.6 ± 0.3	0.7 ± 0.2
mMOR-1O	217 ± 64	101 ± 16	192 ± 77	29.4 ± 3.5	1,359 ± 201	64.1 ± 9.0	61.6 ± 12	23.4 ± 7.9	68.3 ± 14	84.6 ± 30
mMOR-1P	3.6 ± 0.4	7.9 ± 1.6	12.0 ± 2.1	5.4 ± 0.2	1.0 ± 0.7	5.3 ± 0.7	7.4 ± 0.5	0.2 ± 0.1	4.4 ± 1.0	1.6 ± 0.3
mMOR-1U	4.0 ± 1.3	5.9 ± 0.8	17.7 ± 5.5	8.0 ± 2.1	11.7 ± 3.5	6.1 ± 0.8	7.2 ± 1.8	0.3 ± 0.0	3.9 ± 0.8	1.9 ± 0.5
mMOR-1W	5.5 ± 1.7	8.0 ± 1.1	23.0 ± 6.9	10.6 ± 2.7	11.7 ± 3.1	8.2 ± 1.0	9.6 ± 2.4	0.4 ± 0.1	5.3 ± 1.1	2.6 ± 0.7
6TM variants (exons 11/2/3/variable)										
mMOR-1G*	21.1 ± 9.0	9.3 ± 1.2	11.0 ± 4.6	5.3 ± 1.7	4.6 ± 1.5	9.6 ± 2.3	15.6 ± 3.8	3.9 ± 1.3	65.2 ± 10.0	5.4 ± 1.5
mMOR-1M*	5.6 ± 1.8	3.5 ± 0.1	7.7 ± 1.2	3.5 ± 0.3	7.5 ± 1.6	2.6 ± 0.4	3.5 ± 1.2	0.9 ± 0.5	25.0 ± 6.1	1.0 ± 0.1
mMOR-1N*	0.3 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.2 ± 0.03	0.9 ± 0.2	0.4 ± 0.1	0.5 ± 0.1	0.1 ± 0.02	1.5 ± 0.3	0.1 ± 0.03
mMOR-1K*	17.4 ± 4.5	11.2 ± 5.5	38.3 ± 1.4	9.0 ± 1.6	96.1 ± 24	27.3 ± 3.9	36.2 ± 3.4	1.1 ± 0.3	16.4 ± 5.0	3.6 ± 0.6
mMOR-1L*	8.0 ± 1.9	8.2 ± 0.6	23.3 ± 4.9	10.1 ± 0.7	10.0 ± 3.4	22.9 ± 2.9	21.1 ± 3.7	1.0 ± 0.1	10.7 ± 3.4	2.2 ± 0.6
1TM variants (exons 1/variable)										
mMOR-1Q	14.2 ± 2.5	51.6 ± 9.1	99.4 ± 18	44.8 ± 9.7	29.8 ± 9.4	71.3 ± 10.5	72.0 ± 13	1.4 ± 0.6	32.1 ± 5.0	8.3 ± 1.7
mMOR-1R	6.4 ± 1.9	9.3 ± 1.2	26.2 ± 7.7	12.3 ± 3.0	24.8 ± 5.9	9.6 ± 1.2	11.2 ± 2.7	0.5 ± 0.1	6.2 ± 1.2	3.1 ± 0.8
mMOR-1S	12.0 ± 3.0	11.9 ± 0.4	30.2 ± 2.7	15.7 ± 3.7	104.2 ± 12.8	39.0 ± 11.7	16.2 ± 6.5	1.6 ± 0.2	12.3 ± 2.3	5.2 ± 0.4
mMOR-1T*	7.5 ± 1.7	18.0 ± 0.6	31.1 ± 8.8	17.6 ± 2.8	23.5 ± 4.0	33.6 ± 8.4	25.0 ± 5.7	1.0 ± 0.4	3.8 ± 1.4	3.0 ± 0.5
mMOR-1Z	2.6 ± 0.8	3.8 ± 0.6	12.1 ± 3.9	5.2 ± 1.4	3.6 ± 1.4	4.0 ± 0.5	4.7 ± 1.2	0.1 ± 0.03	2.5 ± 0.6	1.3 ± 0.2

*Generated through the exon 11 promoter.

modest at ~10-fold but still highly significant ($P < 0.0001$). Only four full-length variants were up-regulated in the brainstem, with mMOR-1H showing the greatest increase (31-fold). Of the full-length 7TM variants, only two that are associated with the exon 1 promoter were increased. In contrast, all five of the 6TM variants and two of the three full-length 7TM variants associated with exon 11 were significantly increased in the brainstem. Thus, seven of the nine variants up-regulated in the brainstem are under the control of the exon 11 promoter.

All of the exon 11-associated variants and many of the exon 1-associated variants were significantly up-regulated in the hypothalamus. Large increases were seen with mMOR-1G (91-fold) and mMOR-1C (58-fold). mMOR-1D (312-fold) had the highest increase of all of the variants in any of the regions despite its low relative abundance compared with the other 7TM variants.

The mouse expresses five 1TM variants. With the exception of mMOR-1Q, they all show a bell-shaped stimulation in the striatum and significantly increased expression in the hypothalamus, with mMOR-1S levels increasing by 45-fold and mMOR-1Z levels increasing by 30-fold. mMOR-1T and mMOR-1Z levels increased ~30-fold in the hippocampus, whereas mMOR-1S and mMOR-1R levels increased over 10-fold in the brainstem.

Discussion

The current studies provide interesting insights into morphine tolerance. The long-term use of opioids clinically is controversial. Many suggest that continued development of tolerance over time would require continually progressive increases in drug doses to maintain pain relief, thereby making its long-term use counterproductive. Countering this suggestion has been extensive clinical experience, particularly in the cancer pain area, showing that patients can be maintained for long periods of time at constant drug doses (39). Our results reconcile these disparate observations and are consistent with both the clinical observations in patients with cancer and the preclinical studies. Morphine tolerance progressed in a linear fashion for up to 3 wk and then stabilized. The failure of tolerance to increase beyond 3 wk supports the feasibility of long-term opioid use in selected patients for whom it is appropriate and needed.

The results also suggest that compensatory mechanisms to minimize tolerance may include changes in the levels of mu-opioid receptors. The changes in whole-brain levels did not achieve statistical significance. However, specific regions revealed dramatic increases as high as 300-fold. Overall, the changes were not very widespread, perhaps explaining why they were not evident in whole brain. Several patterns among the variants and the regions emerged. Almost all of the variants showed a bell-shaped response in the striatum, with greater increases induced by the lower morphine dose. At the higher morphine dose, many mRNAs revealed major increases in the hypothalamus and hippocampus, particularly the exon 11-associated variants.

In the thalamus, changes were more limited, with significant increases in only mE1-2 and mMOR-1C mRNA levels among

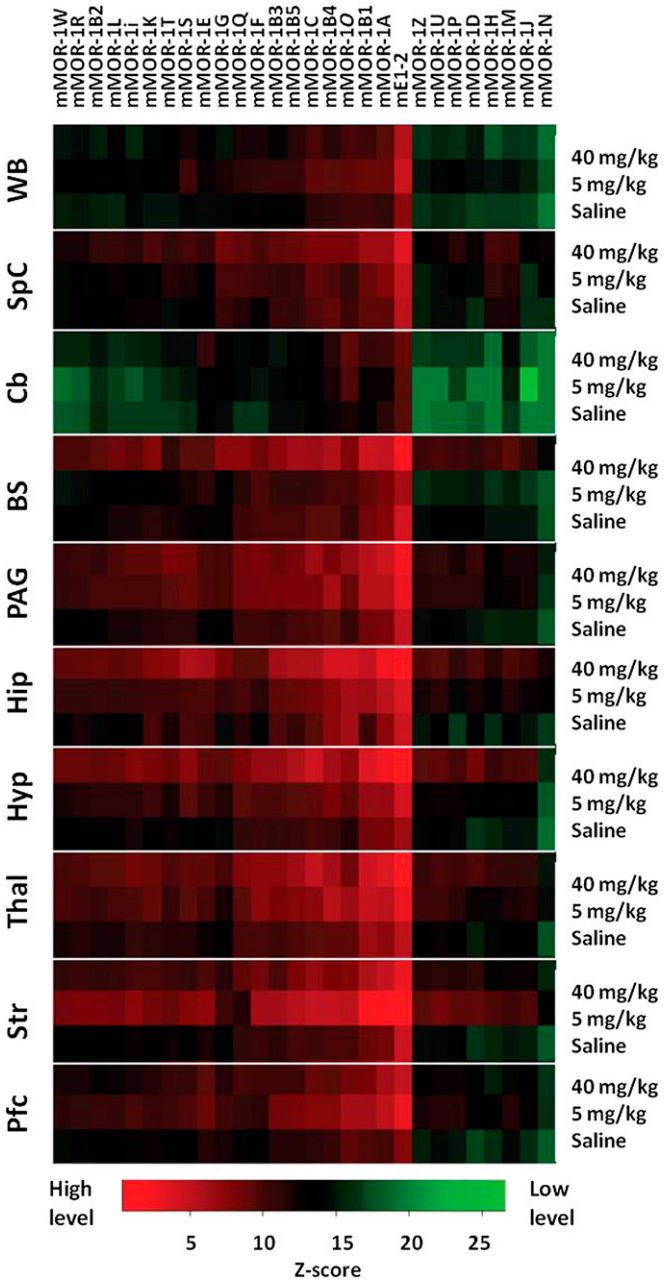

Fig. 2. Heat map of the changes in mRNA levels of MOR-1 splice variants by region. The relative abundance of the different variants in saline and both morphine treatment groups is presented in various brain regions. The heat map cluster is based upon the values [Log_2 ($E^{-\Delta C(t)}$] plotting from the highest level (left, red) to the lowest level (right, green). BS, brainstem; Cb, cerebellum; Hip, hippocampus; Hyp, hypothalamus; PAF, periaqueductal gray; PFC, prefrontal cortex; SpC, spinal cord; Str, striatum; Thal, thalamus; WB, whole brain.

the full-length variants. Estimates of the relative abundance of MOR-1 (Table S1) suggest that a major portion of the increase is related to up-regulation of mMOR-1 (Table S1). There were few changes in the periaqueductal gray, another important area for opioid pain modulation.

In the brainstem, all of the 6TM variants were significantly increased, as well as two of the three full-length exon 11-associated 7TM variants, whereas only two of 17 full-length exon 1-associated 7TM variants were elevated. This result suggests a greater effect upon the exon 11 promoter than upon the exon 1 promoter. However, in some regions, such as the low morphine dose in the striatum and the high morphine dose in the hypothalamus, the majority of the variants were increased, consistent with the transcriptional up-regulation of both the exon 11 and exon 1 promoters. The mu-opioid receptor gene is subjective to epigenetic control (42, 43). Our results raise intriguing questions regarding epigenetic regulation by long-term morphine treatment. At the same time, changes in the relative expression pattern among the variants, both within and between each of the exon 1-associated and exon 11-associated groups, indicate the importance of changes in the regulation of splicing itself.

These studies also give insights into the splice variants themselves. Whereas the mRNA levels of many are far lower than mMOR-1, their dramatic increases in selected brain regions argue for their importance in opioid action. Despite the low abundance of individual variants, together, they represent a significant fraction of the full-length variant mRNAs. In whole brain, we estimate that mMOR-1 corresponds to approximately half of the sum of all of the full-length variants. mMOR-1 appears to correspond to the vast majority of full-length variant mRNA in the striatum, the periaqueductal gray, and the brainstem, but only to a small fraction of full-length mRNA in the prefrontal cortex, thalamus, hypothalamus, and hippocampus. These relative levels changed with morphine treatment in a number of regions, particularly the thalamus and hypothalamus.

It is not clear whether these changes with chronic morphine dosing are responsible for the leveling off of tolerance over time, but it is possible that the increased mRNA levels lead to elevated receptor expression that may be countering the factors responsible for tolerance, leading to its stabilization. Tolerance develops to virtually all opioid actions, suggesting that the changes in mRNA levels in some regions may be associated with analgesic tolerance, whereas others may have an impact on tolerance to respiratory depression and other opioid actions. It is important to note that these chronically treated animals are also highly physically dependent. Thus, many of the changes seen in the current study may correlate with aspects of physical dependence as opposed to analgesia and tolerance. Morphine analgesia is independent of the exon 11-associated variants, whereas other mu-opioids rely heavily upon them (3, 44, 45). It will be interesting to see if these exon 11-dependent drugs produce similar effects on tolerance and mRNA levels.

One remaining question is whether or not these mRNA changes translate into increases in the variants at the protein level. One study examining long-term heroin self-administration showed increases in ^{3}H-naloxone binding in many of the same areas with elevated mRNA levels, including the brainstem and hypothalamus (28). However, ^{3}H-naloxone is not selective because it labels all of the 7TM variants with similar affinities. A similar issue arises with a recent report showing increased ^{3}H-DAMGO ([D-Ala^2,MePhe^4,Gly(ol)^5]enkephalin binding following heroin withdrawal (29). Epitopes that have been used to examine the receptors also are not selective. The cloning of the truncated variants revealed the same 3′ splicing in the 6TM variants as in the 7TM ones, meaning that identical C-terminal epitopes exist in more than one variant. Additional approaches will be needed to resolve this question.

Methods

Animals. Charles River Laboratory:Institute for Cancer Research (Crl:ICR) CD-1 male mice at 6–7 wk of age were obtained from Charles River Laboratories. All mice were housed in groups of five, maintained on a 12-h light/dark cycle, and given ad libitum access to food and water. All animal studies were approved by the Institutional Animal Care and Use Committee of the Memorial Sloan–Kettering Cancer Center. Morphine sulfate was a gift from the Research Technology Branch of the National Institute on Drug Abuse.

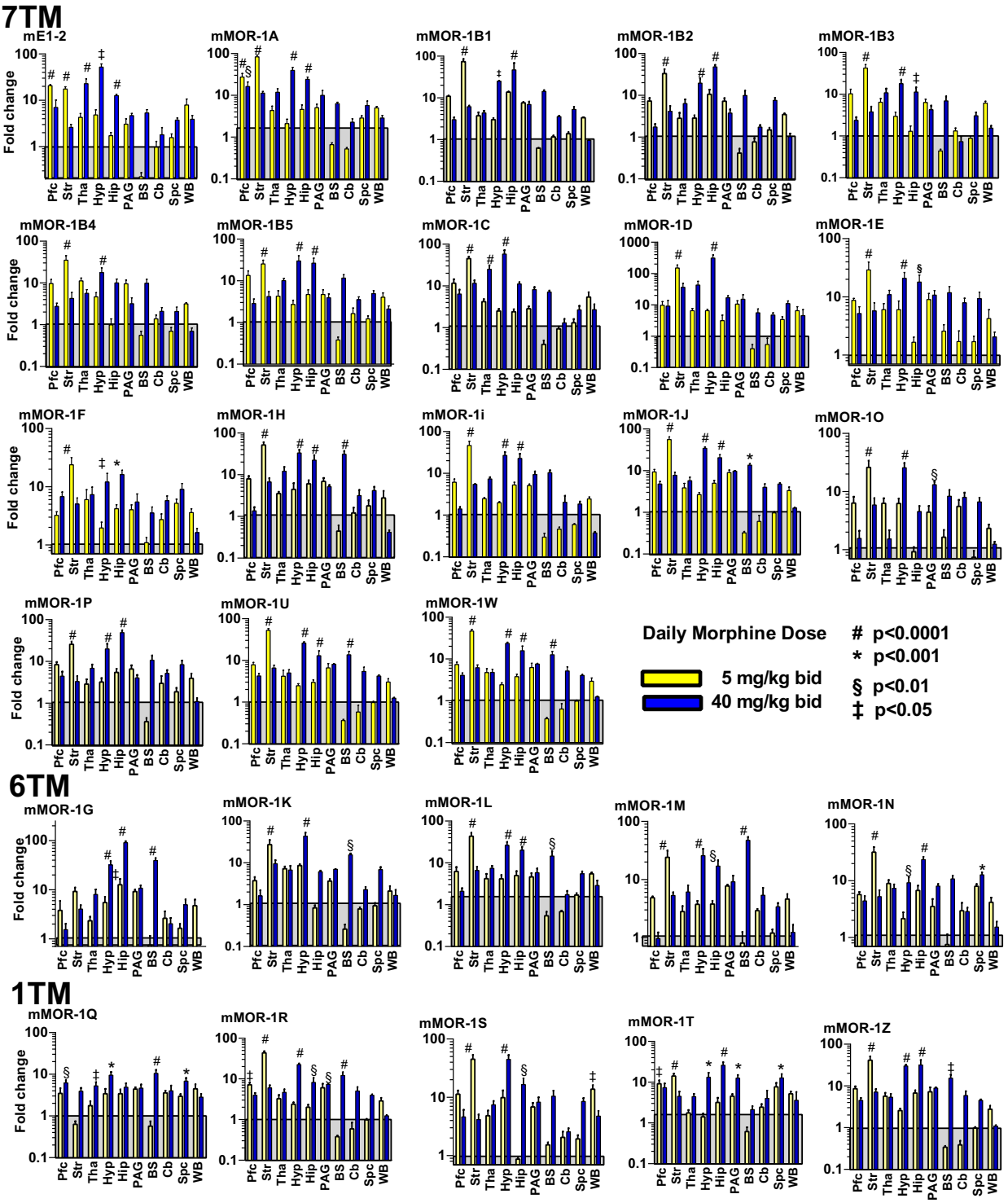

Fig. 3. Changes in mRNA levels of mMOR-1 splice variants after 6 wk of morphine administration in whole brain and brain regions. The levels of the full-length variants following morphine administration were determined as described in *Methods*, and the changes were normalized to saline levels. The changes on the *y* axis are presented on a log scale to accommodate the wide range of differences among the variants and among the regions. Values of 1 correspond to no change from saline controls. Values less than 1 (within the gray zone) correspond to decreases, whereas values greater than 1 represent increases. Significance was determined by ANOVA with Bonferroni's multiple comparison test.

Chronic Morphine Treatment and the Radiant Heat Tail-Flick Assay. Groups of CD-1 mice were s.c. injected with saline (control group, $n = 20$) or morphine (5 mg/kg s.c., $n = 60$; 10 mg/kg s.c., $n = 60$; 20 mg/kg s.c., $n = 60$; or 40 mg/kg s.c., $n = 60$) twice daily (0830 hours and 2030 hours). Each morphine group started with 60 mice, with 10 mice per group removed on days 7, 15, 21, 28, 39, and 43 for determination of their respective morphine ED_{50} values. Morphine ED_{50} values were assessed at the indicated day using a cumulative dose–response paradigm (1, 2.5, 5, 10, 20, and 50 mg/kg) in the radiant heat tail-flick assay and were calculated using probit analysis. Analgesia was assessed quantally (doubling or greater of the baseline tail-flick latency for the particular animal) with a maximal latency of 10 s to minimize any tissue damage, as previously described (26, 46, 47). Similar ED_{50}

results were obtained using graded responses (percent maximal possible effect or %MPE): %MPE ([latency − baseline]/[10 s − baseline]) ED_{50}.

Determination of mRNA Levels. Brain regions, including the prefrontal cortex, striatum, thalamus, hypothalamus, hippocampus, brainstem, and periaqueductal gray, were dissected on a mouse Plexiglas brain mold using the atlas of Paxinos and Franklin (48) as a reference. The spinal cord from L1 to L5 and whole brain were also evaluated. Immediately after isolation, the dissected tissues were homogenized in QiAzol Reagent (Qiagen) and stored at −80 °C. To obtain sufficient material, each region sample was obtained by pooling three to four mice, enabling us to obtain three independent samples for each region from a total of 10 mice. Total RNAs were extracted using a miRNeasy kit (Qiagen) following the manufacturer's protocol. RNA concentrations were determined by using a Qubit 2.0 Fluorometer (Invitrogen). Using the manufacturer's protocols, RNAs were first treated with Turbo-DNA free reagent (Ambion) to remove potential genomic DNA contamination and then reverse-transcribed with SuperScript II reverse transcriptase (Invitrogen) using random hexamers. SYBR Green qPCR was performed using the first-strand cDNA from reverse transcriptase as a template, appropriate primers, and HotStart-IT SYBR green qPCR Master Mix (Affymetrix) with an Opticon 2 DNA Engine System (Bio-Rad), as previously described (41). Table S5 lists all qPCR primers and conditions. Three reference genes, succinate dehydrogenase subunit A (SDHA), TATA box binding protein (TBP), and glyceraldehyde 3-phosphate dehydrogenase (G3PDH), were selected to obtain a normalization factor (NF) for the data (49–51): $NF = (C(t)_{SDHA} \times C(t)_{TBP} \times C(t)_{G3PDH})^{1/3}$ where C(t) is the cycle threshold. Normalized expression (NE) for each variant was calculated using the equation $NE_{variant} = E^{-(C(t)variant\ -\ C(t)NF)}$, where E is efficiency determined by standard curves using serial dilutions of corresponding cDNAs as templates in qPCR assays. Comparisons were carried out using two-way ANOVA with Bonferroni's multiple comparison test.

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1. Way EL, Loh HH, Shen F-H (1969) Simultaneous quantitative assessment of morphine tolerance and physical dependence. *J Pharmacol Exp Ther* 167(1):1–8.
2. Pasternak GW (2007) When it comes to opiates, just say NO. *J Clin Invest* 117(11): 3185–3187.
3. Pasternak GW, Pan Y-X (2013) Mu opioids and their receptors: Evolution of a concept. *Pharmacol Rev* 65(4):1257–1317.
4. Gutstein HB, Trujillo KA, Akil H (1995) Does chronic nociceptive stimulation alter the development of morphine tolerance? *Brain Res* 680(1-2):173–179.
5. Trujillo KA, Akil H (1991) Inhibition of morphine tolerance and dependence by the NMDA receptor antagonist MK-801. *Science* 251(4989):85–87.
6. Trujillo KA, Akil H (1994) Inhibition of opiate tolerance by non-competitive N-methyl-D-aspartate receptor antagonists. *Brain Res* 633(1-2):178–188.
7. Tiseo PJ, Inturrisi CE (1993) Attenuation and reversal of morphine tolerance by the competitive N-methyl-D-aspartate receptor antagonist, LY274614. *J Pharmacol Exp Ther* 264(3):1090–1096.
8. Ben-Eliyahu S, et al. (1992) The NMDA receptor antagonist MK-801 prevents long-lasting non-associative morphine tolerance in the rat. *Brain Res* 575(2):304–308.
9. Kolesnikov YA, Pick CG, Pasternak GW (1992) NG-nitro-L-arginine prevents morphine tolerance. *Eur J Pharmacol* 221(2-3):399–400.
10. Kolesnikov YA, Pick CG, Ciszewska G, Pasternak GW (1993) Blockade of tolerance to morphine but not to kappa opioids by a nitric oxide synthase inhibitor. *Proc Natl Acad Sci USA* 90(11):5162–5166.
11. Elliott K, Minami N, Kolesnikov YA, Pasternak GW, Inturrisi CE (1994) The NMDA receptor antagonists, LY274614 and MK-801, and the nitric oxide synthase inhibitor, NG-nitro-L-arginine, attenuate analgesic tolerance to the mu-opioid morphine but not to kappa opioids. *Pain* 56(1):69–75.
12. Pasternak GW, Kolesnikov YA, Babey AM (1995) Perspectives on the N-methyl-D-aspartate/nitric oxide cascade and opioid tolerance. *Neuropsychopharmacology* 13(4):309–313.
13. Kolesnikov YA, et al. (1997) Functionally differentiating two neuronal nitric oxide synthase isoforms through antisense mapping: Evidence for opposing NO actions on morphine analgesia and tolerance. *Proc Natl Acad Sci USA* 94(15):8220–8225.
14. Abdelhamid EE, Sultana M, Portoghese PS, Takemori AE (1991) Selective blockage of delta opioid receptors prevents the development of morphine tolerance and dependence in mice. *J Pharmacol Exp Ther* 258(1):299–303.
15. Fundytus ME, Schiller PW, Shapiro M, Weltrowska G, Coderre TJ (1994) The novel and highly selective d-opioid antagonist TIPP(psi) attenuates morphine tolerance and dependence: Comparison with the effects of naltrindole and TIPP. *Regul Pept* 54:97–98.
16. Kest B, Lee CE, McLemore GL, Inturrisi CE (1996) An antisense oligodeoxynucleotide to the delta opioid receptor (DOR-1) inhibits morphine tolerance and acute dependence in mice. *Brain Res Bull* 39(3):185–188.
17. Zhu Y, et al. (1999) Retention of supraspinal delta-like analgesia and loss of morphine tolerance in delta opioid receptor knockout mice. *Neuron* 24(1):243–252.
18. Nitsche JF, et al. (2002) Genetic dissociation of opiate tolerance and physical dependence in delta-opioid receptor-1 and preproenkephalin knock-out mice. *J Neurosci* 22(24):10906–10913.
19. Whistler JL, Chuang HH, Chu P, Jan LY, von Zastrow M (1999) Functional dissociation of mu opioid receptor signaling and endocytosis: Implications for the biology of opiate tolerance and addiction. *Neuron* 23(4):737–746.
20. Christie MJ (2008) Cellular neuroadaptations to chronic opioids: Tolerance, withdrawal and addiction. *Br J Pharmacol* 154(2):384–396.
21. Williams JT, et al. (2013) Regulation of μ-opioid receptors: Desensitization, phosphorylation, internalization, and tolerance. *Pharmacol Rev* 65(1):223–254.
22. Aquilante CL, Letrent SP, Pollack GM, Brouwer KLR (2000) Increased brain P-glycoprotein in morphine tolerant rats. *Life Sci* 66(4):PL47–PL51.
23. Dagenais C, Zong J, Ducharme J, Pollack GM (2001) Effect of mdr1a P-glycoprotein gene disruption, gender, and substrate concentration on brain uptake of selected compounds. *Pharm Res* 18(7):957–963.
24. Letrent SP, Pollack GM, Brouwer KR, Brouwer KLR (1999) Effects of a potent and specific P-glycoprotein inhibitor on the blood-brain barrier distribution and antinociceptive effect of morphine in the rat. *Drug Metab Dispos* 27(7):827–834.
25. King M, Su W, Chang A, Zuckerman A, Pasternak GW (2001) Transport of opioids from the brain to the periphery by P-glycoprotein: Peripheral actions of central drugs. *Nat Neurosci* 4(3):268–274.
26. Su W, Pasternak GW (2013) The role of multidrug resistance associated protein (MRP) in the blood-brain barrier and opioid analgesia. *Synapse* 67(9):609–619.
27. Pert CB, Pasternak G, Snyder SH (1973) Opiate agonists and antagonists discriminated by receptor binding in brain. *Science* 182(4119):1359–1361.
28. Sim-Selley LJ, Selley DE, Vogt LJ, Childers SR, Martin TJ (2000) Chronic heroin self-administration desensitizes mu opioid receptor-activated G-proteins in specific regions of rat brain. *J Neurosci* 20(12):4555–4562.
29. Seip-Cammack KM, Reed B, Zhang Y, Ho A, Kreek MJ (2013) Tolerance and sensitization to chronic escalating dose heroin following extended withdrawal in Fischer rats: Possible role of mu-opioid receptors. *Psychopharmacology (Berl)* 225(1):127–140.
30. Ammon-Treiber S, Höllt V (2005) Morphine-induced changes of gene expression in the brain. *Addict Biol* 10(1):81–89.
31. Ammon S, Mayer P, Riechert U, Tischmeyer H, Höllt V (2003) Microarray analysis of genes expressed in the frontal cortex of rats chronically treated with morphine and after naloxone precipitated withdrawal. *Brain Res Mol Brain Res* 112(1-2):113–125.
32. Brodsky M, Elliott K, Hynansky A, Jenab S, Inturrisi CE (1995) Quantitation of mu-opioid receptor (MOR-1) mRNA in selected regions of the rat CNS. *Neuroreport* 6(5):725–729.
33. Buzas B, Rosenberger J, Cox BM (1996) Mu and delta opioid receptor gene expression after chronic treatment with opioid agonist. *Neuroreport* 7(9):1505–1508.
34. Castelli MP, et al. (1997) Chronic morphine and naltrexone fail to modify mu-opioid receptor mRNA levels in the rat brain. *Brain Res Mol Brain Res* 45(1):149–153.
35. Grice DE, et al. (2007) Transcriptional profiling of C57 and DBA strains of mice in the absence and presence of morphine. *BMC Genomics* 8:76.
36. Meuser T, et al. (2003) Mu-opioid receptor mRNA regulation during morphine tolerance in the rat peripheral nervous system. *Anesth Analg* 97(5):1458–1463.
37. Yoburn BC, Chen J, Huang T, Inturrisi CE (1985) Pharmacokinetics and pharmacodynamics of subcutaneous morphine pellets in the rat. *J Pharmacol Exp Ther* 235(2):282–286.
38. Kolesnikov YA, Jain S, Wilson R, Pasternak GW (1996) Peripheral morphine analgesia: Synergy with central sites and a target of morphine tolerance. *J Pharmacol Exp Ther* 279(2):502–506.
39. Foley KM (1996) Controlling the pain of cancer. *Sci Am* 275(3):164–165.
40. Korostynski M, Piechota M, Kaminska D, Solecki W, Przewlocki R (2007) Morphine effects on striatal transcriptome in mice. *Genome Biol* 8(6):R128.
41. Xu J, et al. (2014) Differential Expressions of the Alternatively Spliced Variant mRNAs of the μ opioid Receptor, OPRM1, Gene in Brain Regions of Four Inbred Mouse Strains. *PLoS ONE* 9(10):e111267.
42. Hwang CK, et al. (2010) Up-regulation of the mu-opioid receptor gene is mediated through chromatin remodeling and transcriptional factors in differentiated neuronal cells. *Mol Pharmacol* 78(1):58–68.
43. Hwang CK, et al. (2009) Epigenetic programming of mu-opioid receptor gene in mouse brain is regulated by MeCP2 and Brg1 chromatin remodelling factor. *J Cell Mol Med* 13(9B):3591–3615.
44. Majumdar S, et al. (2011) Truncated G protein-coupled mu opioid receptor MOR-1 splice variants are targets for highly potent opioid analgesics lacking side effects. *Proc Natl Acad Sci USA* 108(49):19778–19783.
45. Pan YX, et al. (2009) Involvement of exon 11-associated variants of the mu opioid receptor MOR-1 in heroin, but not morphine, actions. *Proc Natl Acad Sci USA* 106(12):4917–4922.
46. Faskowitz AJ, Kramskiy VN, Pasternak GW (2013) Methadone-induced hypoglycemia. *Cell Mol Neurobiol* 33(4):537–542.
47. Kolesnikov YA, Oksman G, Pasternak GW (2010) Topical methadone and meperidine analgesic synergy in the mouse. *Eur J Pharmacol* 638(1-3):61–64.
48. Paxinos G, Franklin K (2001) *The Mouse Brain in Stereotaxic Coordinates* (Academic Press, London), 2nd Ed.
49. Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 29(9):e45.
50. Vandesompele J, et al. (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 3(7):RESEARCH0034.
51. Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP (2004) Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper–Excel-based tool using pair-wise correlations. *Biotechnol Lett* 26(6):509–515.