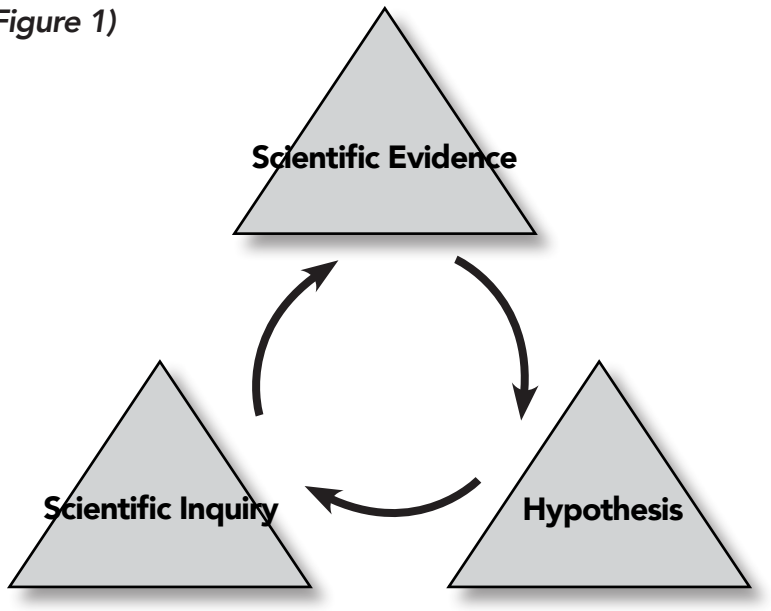
THE SCIENTIFIC EVIDENCE, SMITH HYPOTHESIS, & PREDICTIONS

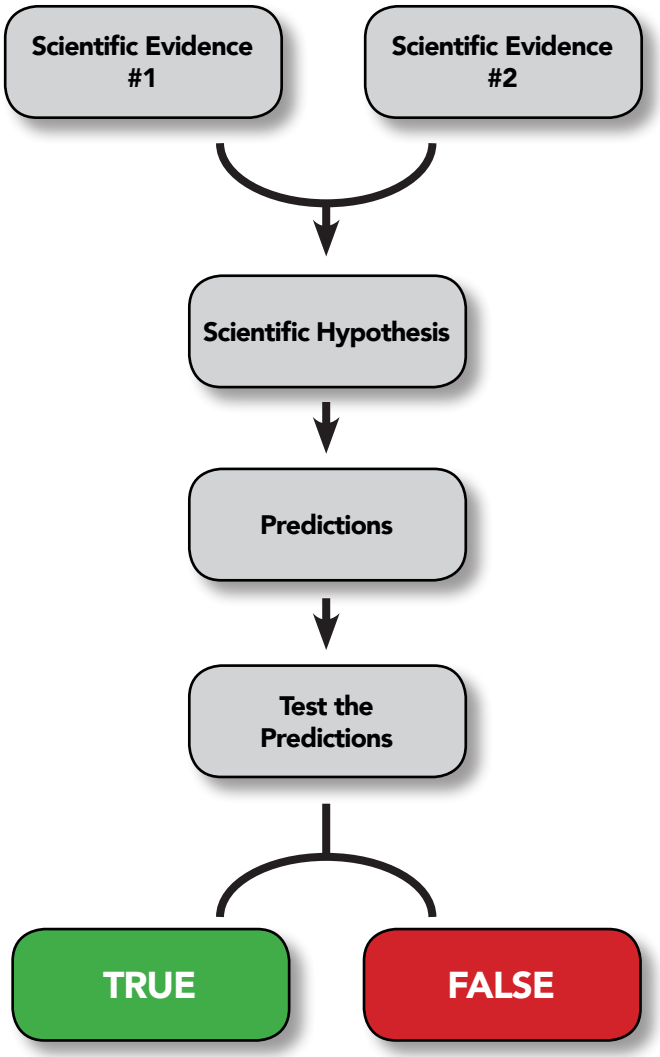
(Figure 1)



ABSTRACT

A critical mass of scientific evidence can lead to a hypothesis. This hypothesis can lead to future scientific inquiry. Further scientific inquiry can lead to further scientific evidence. And so the process continues (Figure 1). We look now to a series of scientific studies which has led to the need for further scientific inquiry. The subject matter is all unified by an inquiry into the nature of the opioids and a possible dysfunction within the Autonomic Nervous System. Some of this scientific evidence goes back decades. And the further evidence that we are looking to gain could be considered basic science.

(Figure 2)



We will be looking into two separate areas of study into the opioids ***(Figure 2)***. We will do our best to unify these two areas of study into a single hypothesis. With this single hypothesis we will make a series of predictions. We will then test to see if we can find evidence supporting or not supporting this prediction. This is how the current scientific method works.

(Figure 3)

Scientific Evidence For a Dysfunction in the Autonomic Nervous System Due to the Opioids

The first area of study we will be looking into is scientific evidence of a dysfunction within the Autonomic Nervous System and due to opioid dependence ***(Figure 3)***.

(Figure 4)

Scientific Evidence For Damage to the DNA Known as Methylation Due to the Opioids

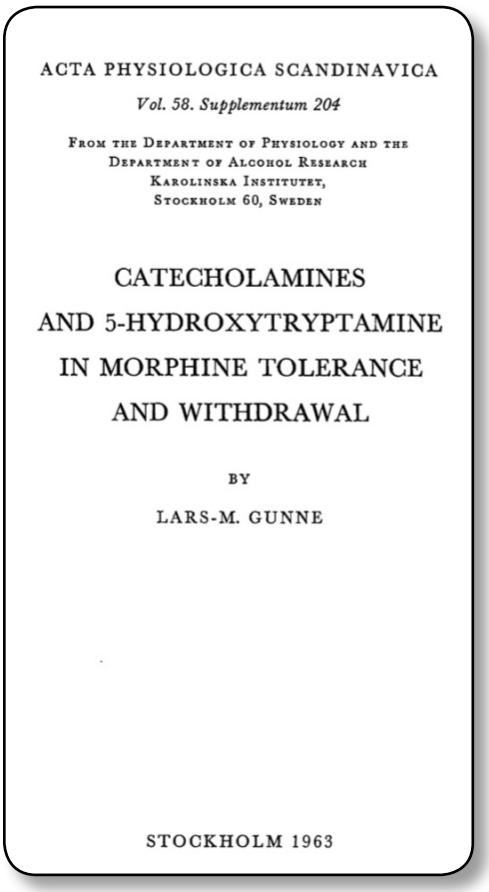
We will begin back into the 1960s and work our way towards the present time. The second area of study we will be looking into is scientific evidence of a type of genetic damage to the DNA known as methylation, and again in the opioid dependent individual. ***(Figure 4)***.

(Figure 5)

Clinical Trial Involves NO Deviation From The Standard of Care

And, once again, after reviewing this known scientific evidence, we will be putting forth a hypothesis. And from this hypothesis, we are proposing a clinical trial for the sole purpose of gaining additional scientific information. It is vital to note that this clinical trial proposal involves **NO DEVIATION FROM THE STANDARD OF CARE** ***(Figure 5)***. The participants in the clinical trial will be treated with a standard medication and at standard dosing regiments. Other than the collection of two blood samples and one DNA sample, there is simply no deviation from the standard of care for the opioid dependent individual. Thus no additional risk or harm is present in this clinical trial.

SCIENTIFIC EVIDENCE FOR DYSFUNCTION WITHIN THE AUTONOMIC NERVOUS SYSTEM AND DUE TO OPIOID DEPENDENCY



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FROM THE DEPARTMENT OF PHYSIOLOGY AND THE DEPARTMENT OF ALCOHOL RESEARCH KAROLINSKA INSTITUTET, STOCKHOLM 60, SWEDEN

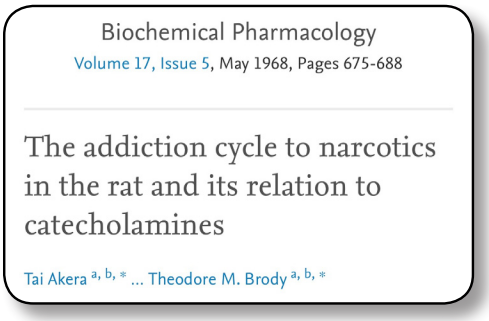
CATECHOLAMINES AND 5-HYDROXYTRYPTAMINE IN MORPHINE TOLERANCE AND WITHDRAWAL

BY

LARS-M. GUNNE

STOCKHOLM 1963

(Figure 6)



Biochemical Pharmacology

Volume 17, Issue 5, May 1968, Pages 675-688

The addiction cycle to narcotics in the rat and its relation to catecholamines

Tai Akera [a, b, *] ... Theodore M. Brody [a, b, *]

(Figure 7)

While there were some studies as early as the 1950s, we have chosen to begin with the study by Gunne (1963). ***(Figure 6)***:

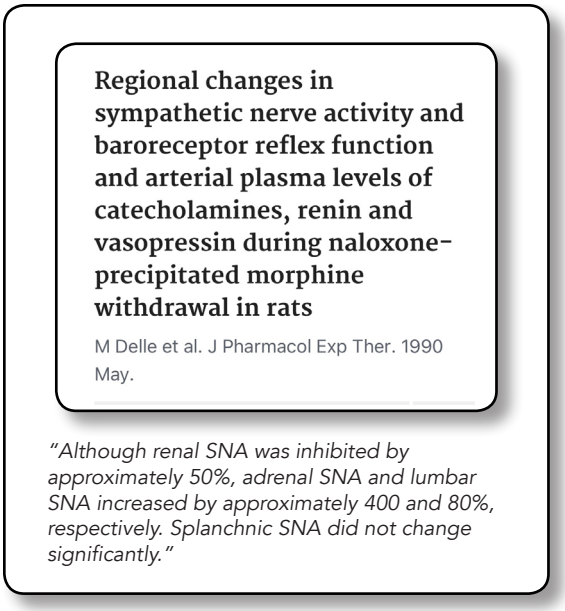
"The content of adrenaline (Epi) in adrenal glands was depleted in chronic morphine-treated rats which experienced withdrawal symptoms 48 hr after abrupt morphine withdrawal" (Gunne, 1963).

We believe that we are interpreting this quote as the author intended. Gunne was making the observation that when opioid dependent mice were allowed to go into a withdrawal state and due to the abstinence of more morphine, that one of the findings was a depletion of epinephrine from the adrenal gland. This raises the question of a possible dysfunction in at least the Sympathetic Branch of the Autonomic Nervous System. And again, this was 1963. What dysfunction led to the depletion of epinephrine in the adrenal glands and during opioid withdrawal? That was the next question.

Our next study for review is by Akera and Brody and from 1968 ***(Figure 7)***. Once again, mice physically dependent upon the opioids were studied. Among the findings by Akera and Brody was an increased level of particularly epinephrine detected in urine of the mice and during a state of opioid withdrawal due to a withholding of the opioids.

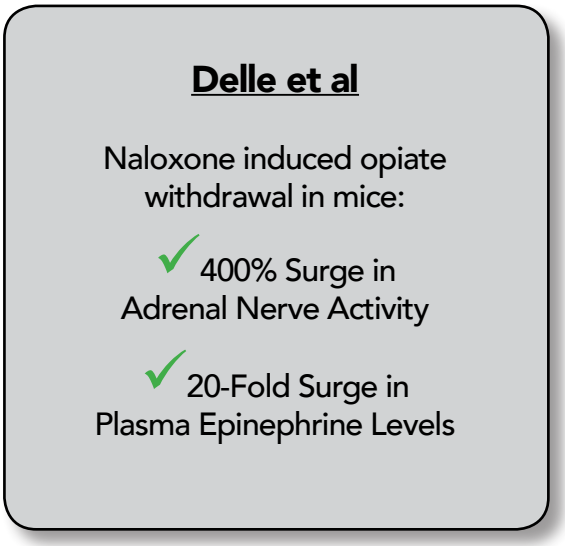
"During withdrawal after chronic (opioid) drug treatment, larger amounts of epinephrine and norepinephrine were excreted, epinephrine being the primary free amine excreted." Akera and Brody (1968)

With these two studies we now have a strong suggestion that in a state of opioid withdrawal, epinephrine is being released from the adrenal glands and in a large concentration. So large in fact, that the epinephrine in the adrenal gland is essentially depleted in a couple of days. This would certainly be suggestive of a dysfunction within the Sympathetic Nervous System. But at this point in time, no direct measurements on the various branches of the Sympathetic Nervous System had been undertaken. For this information, we had to wait until the year 1990. And in the year 1990, we see two excellent studies reported.



(Figure 8)

The first of the two studies from 1990 is the Delle et al study ***(Figure 8)***. Delle (1990) is a complex study with multiple variables. And again, Delle is studying opioid dependent mice and during a state of opioid withdrawal. In the Delle study, opioid withdrawal was accomplished not by a period of opioid abstinence but rather by administration of naloxone, a common opioid antagonist. Either method produces a state of opioid withdrawal. And it is important to note in every study exactly how the opioid withdrawal was obtained, either by a period of opioid abstinence or by the administration of naloxone.



(Figure 9)

But we feel, and apparently Delle et al agree, that the single most significant finding came from a direct measurement of the Sympathetic Nerve innervating the adrenal glands along with a direct measurement of plasma epinephrine levels. This type of direct measurement of the activity of the sympathetic nerve to the adrenal gland could only be accomplished surgically. And this is precisely what Delle and team accomplished ***(Figure 9)***. And what they discovered is nothing short of remarkable. Delle and team discovered a 400% increase in Sympathetic Nerve Activity specifically to the branches of the Sympathetic Nervous System innervating the adrenal glands and during the naloxone induced opioid withdrawal. This is truly a remarkable scientific achievement. Corresponding to this 400% surge in Sympathetic Nerve Activity to the adrenal glands was a simultaneous twenty-fold surge in plasma epinephrine levels. Delle (1990) greatly advanced our knowledge of dysfunction within the Autonomic Nervous during opioid withdrawal. But three strong questions remained after the Delle (1990) study:

1. Would the same type of surge in activity in the sympathetic nerve to the adrenal gland be seen in a withdrawal state obtained simply by opioid abstinence? The Delle (1990) study only utilized mice in opioid withdrawal due to the administration of naloxone.

2. Would a similar increase in plasma epinephrine levels be seen in a withdrawal state obtained simply by opioid abstinence? Again, the Delle(1990) study only utilized mice in opioid withdrawal due to the administration of naloxone.

3. What evidence do we have that the rise in plasma epinephrine is from epinephrine actually derived from the adrenal gland?

Role of plasma catecholamines in eliciting cardiovascular changes seen during naloxone-precipitated withdrawal in conscious, unrestrained morphine-dependent rats

A P Chang et al. J Pharmacol Exp Ther. 1990 Sep.

"After removal of adrenal glands from morphine-dependant rats, naxolone injection produced no change in the BP or plasma Epi."

(Figure 10)

And to address these three questions we turn to the second study from 1990, this time by Chang et al (1990) ***(Figure 10)***. Again, like Delle (1990), the Chang (1990) study is complex and multifaceted. But we remain focused on scientific evidence for dysfunction within the Autonomic Nervous System during a state of opioid withdrawal. And we find plenty of scientific evidence within the Chang (1990) study. Let's take each of the three questions separately and one at a time.

1. Would the same type of surge in the activity in the sympathetic nerve to the adrenal gland be seen in a withdrawal state obtained simply by opioid abstinence? Chang (1990) DOES NOT answer this question as Chang did not do surgical measurements of the sympathetic nervous system. So we simply do not have any further knowledge as to this question.

Chang et al

Naloxone induced opiate withdrawal in mice:

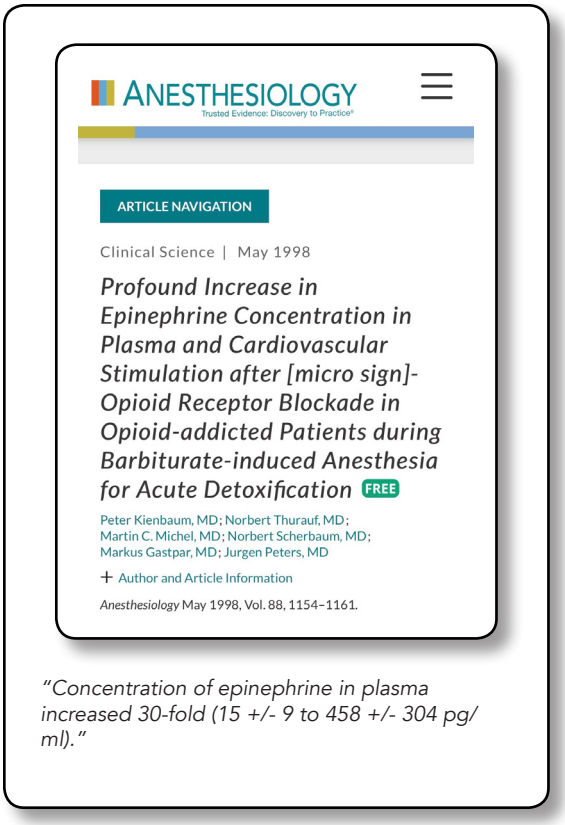
✓ Opioid abstinence withdrawal increased plasma epinephrine levels

✓ Surgical resection of the adrenal glands in mice prevented the surge in plasma epinephrine levels in opioid withdrawal

(Figure 11)

2. Would a similar increase in plasma epinephrine levels be seen in a withdrawal state obtained simply by opioid abstinence? And here Chang (1990) does provide an answer. And this answer is YES ***(Figure 11)***. Chang (1990) found that plasma epinephrine levels increased daily over the three days of measurement (Day 0, 0.183 +/- 0.038 ng/ml), (Day 1 of opioid abstinence withdrawal, 0.665 +/- 0.061 ng/ml), (Day 2 of opioid abstinence withdrawal, 0.730 +/- 0.071 ng/ml), (Day 3 of opioid abstinence withdrawal, 1.00 +/- 0.091 ng/ml). This is strong scientific evidence. And this would be highly consistent with the findings of epinephrine depletion from the adrenal glands due to opioid withdrawal in the Gunne (1963) study. And this would be highly consistent with the findings of epinephrine in the urine due to opioid withdrawal in the Akera and Brody (1968) study.

3. What evidence do we have that the rise in plasma epinephrine is from epinephrine actually derived from the adrenal gland? And once again, Chang et al provide clarity through scientific evidence. Chang et al simply repeated the naloxone induced opioid withdrawal but this time in mice whom had undergone a surgical resection of the adrenal glands. And when the adrenal glands had been surgically removed, there was no surge in plasma epinephrine levels following the administration of naloxone to opioid dependent mice. An answer had been obtained. And this is taken as strong scientific evidence that the measured surge in plasma epinephrine levels during opioid withdrawal is from epinephrine actually derived from the adrenal glands.



(Figure 12)

The next study for our review was reported by Kienbaum et al in 1998 ***(Figure 12)***. And this will be the first study involving human participants. So far, we have focused on studies involving mice as participants. But from the mice we have learned much. We first learned that epinephrine was depleted from the adrenal gland during opioid withdrawal (Gunne (1963) ***(Figure 13)***. We then saw that elevated epinephrine was seen in the urine of mice during opioid withdrawal (Akera and Brody (1968)). Our knowledge was greatly expanded by Delle (1990) who gave us the scientific evidence that opioid withdrawal was associated with a 400% increase in Sympathetic Nerve Activity in the branch of the sympathetic nerve going to the adrenal gland. Furthermore, this markedly increased Sympathetic Nerve Activity was associated with a twenty-fold surge in plasma epinephrine levels and all during a naloxone-induced state of opioid withdrawal. Lastly with the mice studies, we learned from Chang (1990) that the elevated plasma epinephrine levels were present regardless if the state of opioid withdrawal was due to the administration of naloxone or if the state of opioid withdrawal was due to a period of opioid abstinence. Furthermore, surgical removal of the adrenal glands prevented the surge in plasma epinephrine levels. This was taken as strong scientific evidence that it was the surge in the Sympathetic Nerve Activity that was producing the surge in plasma epinephrine levels. And it is with this knowledge base that we now encounter our first study involving human participants.

Kienbaum et al (1998) was looking to determine if it was safe to purposely put a human subject into a naloxone induced state of opioid withdrawal. At the time, 1998, and still today, a small minority of healthcare professionals advocate for a treatment form known as the rapid opioid detoxification. Further discussion of this treatment is beyond the scope of this manuscript. Kienbaum was merely trying to assess the safety of an opioid withdrawal, in this instance one induced by the administration of naloxone. Kienbaum was careful in his final assessment and said merely:

What We HAVE Learned From the Mice Studies

✓ Plasma epinephrine surges in **BOTH** Naloxone-induced withdrawal **AND** opioid abstinence withdrawal (Delle 1990, Chang 1990)

✓ Epinephrine is depleted from the adrenal gland by opioid withdrawal (Gunne 1963)

✓ Epinephrine increases in the urine during opioid withdrawal (Akera, Brody 1968)

✓ Surgical removal of the adrenal glands prevents the surge in epinephrine during opioid withdrawal

(Figure 13)

"Most important, a 30-fold increase in concentration of epinephrine in plasma, a small increase in concentration of norepinephrine in plasma, and profound cardiovascular alterations were observed after mu-opioid receptor blockade despite maintenance of general anesthesia. Because of the attendant cardiovascular stimulation, we suggest that acute detoxification of patients addicted to opioids should be performed by trained anesthesiologists or intensivists."
Kienbaum (1998)

What We HAVE Learned From the Human Studies

✓ Naloxone-induced opioid withdrawal is associated with a "profound" surge in plasma epinephrine

? But what about opioid abstinence withdrawal? Is there also an increase in plasma epinephrine in humans in opioid abstinence withdrawal?

THAT IS THE PURPOSE OF THIS CLINICAL TRIAL

(Figure 14)

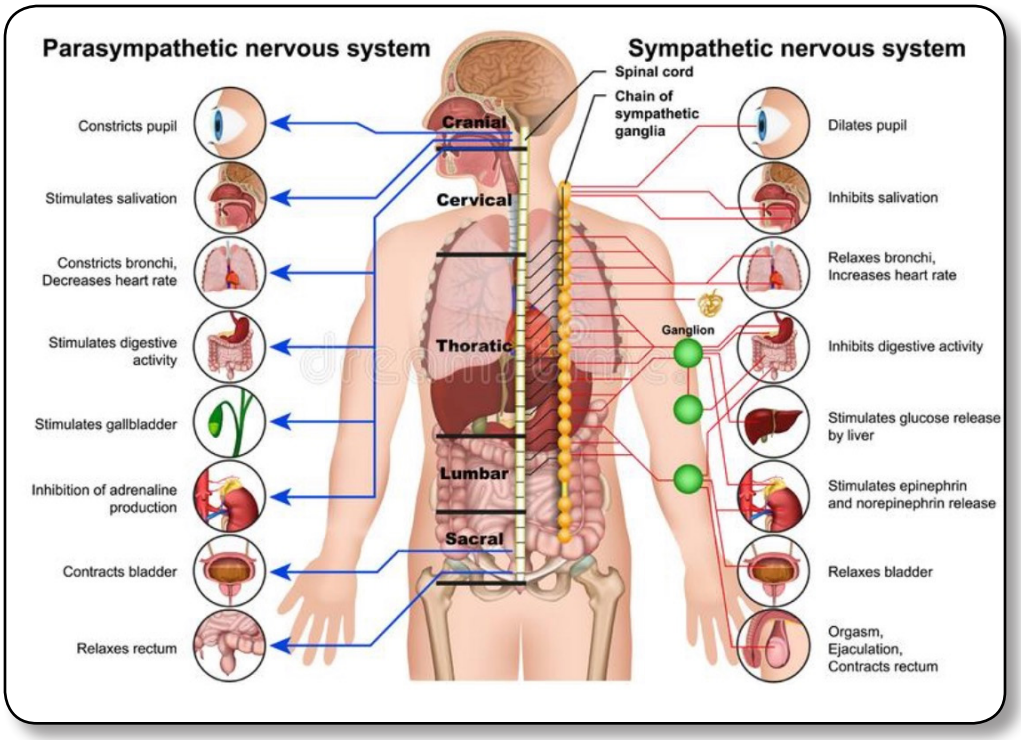
As is evident from this quote, Kienbaum found a thirty-fold surge in plasma epinephrine levels during a naloxone induced state of opioid withdrawal. Furthermore, Kienbaum further reported "profound cardiovascular alterations". The emphasis is on the word "profound". It is worth noting, Kienbaum in 1998 was advising naloxone be administered only by "trained anesthesiologists and intensivists". Contrast these words to today when naloxone is carried by all paramedics, many police officers, and some citizens. Granted, this is for use only in lifesaving situations and has saved many lives. But it raises an interesting question.

Kienbaum answered the question whether or not naloxone-induced opioid withdrawal in the human is associated with a surge in plasma epinephrine levels. And the answer is a resounding yes ***(Figure 14)***. Naloxone-induced opioid withdrawal is associated with a "profound" thirty-fold surge in plasma epinephrine levels. The next question is whether or not the state of opioid withdrawal due to a period of opioid abstinence in the human is also associated with a surge in plasma epinephrine levels. And this is one of the questions we are looking to answer with this clinical trial.

Again, and we emphasize, the protocol of this study exactly follows the established protocol for the induction of Buprenorphine in the opioid dependent individual. It is a requirement that the opioid dependent individual be in a state of opioid abstinence withdrawal prior to the initiation of Buprenorphine. Otherwise, a state of withdrawal known as a Precipitated Withdrawal occurs with Buprenorphine. This is a widely known fact in the literature. All we are doing is simply drawing a blood level for epinephrine before Buprenorphine and one hour later. No deviation from normal Buprenorphine induction exists in this clinical trial.

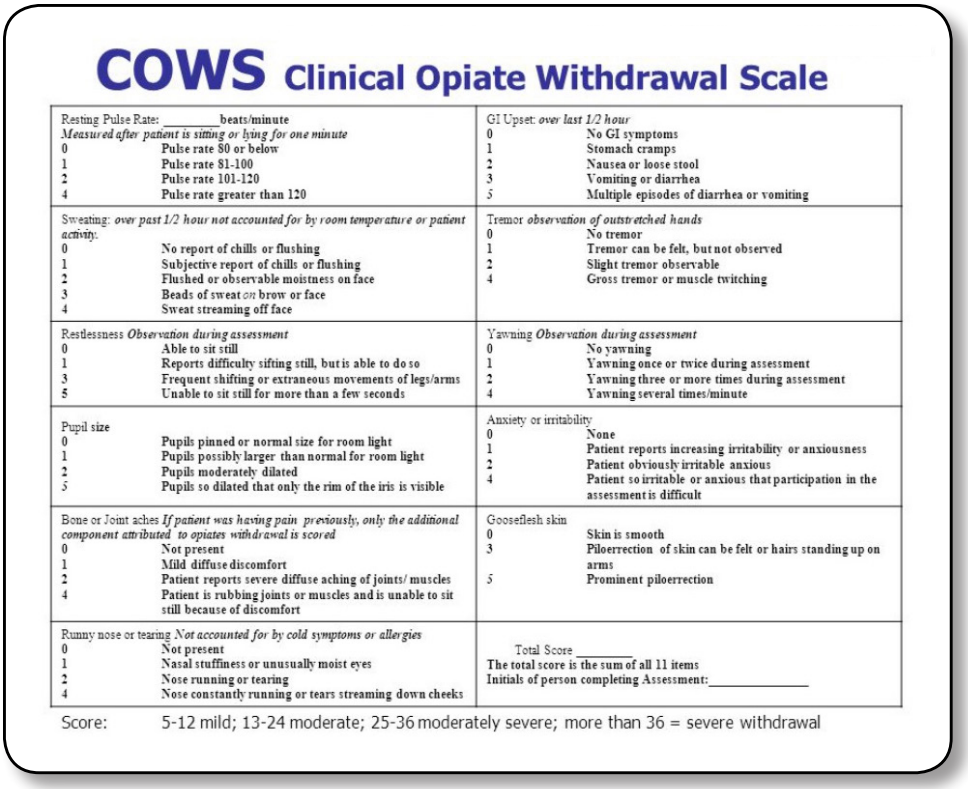
Before we leave the Kienbaum (1998) study, it is worth the time to note an additional finding made by Kienbaum and team:

"The clinical signs of [micro sign]-opioid receptor blockade were observed in all patients: marked gastrointestinal secretion with 500–1,000 ml of fluids draining from the gastric tube and rectal discharges of 200–500 ml during the 180-min observation period." Kienbaum (1998)



(Figure 15)

Why are these findings of "marked gastrointestinal secretion.....and rectal discharges" deemed to be so important? Because these are known to be bodily functions associated with the Parasympathetic Nervous System, the other branch of the Autonomic Nervous System ***(Figure 15)***. We saw direct measurements made surgically upon the mice during opioid withdrawal that revealed a marked dysfunction in the Sympathetic Nerve Activity in the Sympathetic Nerve to the adrenal glands Delle (1990). This dysfunction within the Sympathetic Nervous System resulted in a twenty-fold surge in plasma epinephrine levels. And this was taken to be excellent and direct evidence of dysfunction within the Sympathetic Nervous System, one of the two branches of the Autonomic Nervous System. Why do we say this is direct evidence? Because Delle (1990) surgically measured the activity in various areas of the Sympathetic Nervous System. This was how the 400% surge in activity in the Sympathetic Nerve going to the adrenal glands was measured, by direct measurement. The other branch of the Autonomic Nervous System is the Parasympathetic Nervous System. And with these large amounts of gastric secretions and rectal discharge, we are seeing indirect evidence suggesting a dysfunction also occurring within the Parasympathetic Nervous System. We are not aware of any studies done to date revealing direct measurements of any branches of the Parasympathetic Nervous System. The type of direct measurement that Delle (1990) did upon the Sympathetic Nervous System has simply not been done to date on the Parasympathetic Nervous System.



COWS Clinical Opiate Withdrawal Scale

Resting Pulse Rate: _____beats/minute *Measured after patient is sitting or lying for one minute* 0 Pulse rate 80 or below 1 Pulse rate 81-100 2 Pulse rate 101-120 4 Pulse rate greater than 120	GI Upset: *over last 1/2 hour* 0 No GI symptoms 1 Stomach cramps 2 Nausea or loose stool 3 Vomiting or diarrhea 5 Multiple episodes of diarrhea or vomiting
Sweating: *over past 1/2 hour not accounted for by room temperature or patient activity.* 0 No report of chills or flushing 1 Subjective report of chills or flushing 2 Flushed or observable moistness on face 3 Beads of sweat *on* brow or face 4 Sweat streaming off face	Tremor *observation of outstretched hands* 0 No tremor 1 Tremor can be felt, but not observed 2 Slight tremor observable 4 Gross tremor or muscle twitching
Restlessness *Observation during assessment* 0 Able to sit still 1 Reports difficulty sifting still, but is able to do so 3 Frequent shifting or extraneous movements of legs/arms 5 Unable to sit still for more than a few seconds	Yawning *Observation during assessment* 0 No yawning 1 Yawning once or twice during assessment 2 Yawning three or more times during assessment 4 Yawning several times/minute
Pupil size 0 Pupils pinned or normal size for room light 1 Pupils possibly larger than normal for room light 2 Pupils moderately dilated 5 Pupils so dilated that only the rim of the iris is visible	Anxiety or irritability 0 None 1 Patient reports increasing irritability or anxiousness 2 Patient obviously irritable anxious 4 Patient so irritable or anxious that participation in the assessment is difficult
Bone or Joint aches *If patient was having pain previously, only the additional component attributed to opiates withdrawal is scored* 0 Not present 1 Mild diffuse discomfort 2 Patient reports severe diffuse aching of joints/ muscles 4 Patient is rubbing joints or muscles and is unable to sit still because of discomfort	Gooseflesh skin 0 Skin is smooth 3 Piloerrection of skin can be felt or hairs standing up on arms 5 Prominent piloerrection
Runny nose or tearing *Not accounted for by cold symptoms or allergies* 0 Not present 1 Nasal stuffiness or unusually moist eyes 2 Nose running or tearing 4 Nose constantly running or tears streaming down cheeks	Total Score _____ The total score is the sum of all 11 items Initials of person completing Assessment:_____

Score: 5-12 mild; 13-24 moderate; 25-36 moderately severe; more than 36 = severe withdrawal

(Figure 16)

But even without the direct measurement of dysfunction within the Parasympathetic Nervous System, we do have evidence to further consider. First, we can evaluate the symptoms seen clinically in opioid withdrawal. Whether or not the opioid withdrawal is due to the administration of an opioid receptor antagonist such as naloxone or if the opioid withdrawal is due simply to a period of opioid abstinence, the clinical signs and symptoms are the same. As a guidance, we shall use the Clinical Opiate Withdrawal Scale, commonly referred to as the COWS ***(Figure 16)***. And we shall go down the list and classify each entry into the COWS as either sympathetic, parasympathetic, or other:

Changes in cardiac vagal tone as measured by heart rate variability during naloxone–induced opioid withdrawal

Charles J Levin et al. Drug Alcohol Depend. 2019.

CONCLUSIONS: These preliminary data indicate that a large reduction in cardiac vagal tone occurs during naloxone-induced withdrawal. This finding underscores the need for further research into the role of the parasympathetic nervous system in opioid withdrawal.

(Figure 17)

Pulse rate - sympathetic; sweating - sympathetic; restlessness - sympathetic; pupil size - sympathetic; bone/joint aches - other; runny nose - parasympathetic; tearing - parasympathetic; vomiting - parasympathetic; diarrhea - parasympathetic; tremor - sympathetic; yawning - parasympathetic; anxiety/irritability - sympathetic; goose-flesh skin - sympathetic.

And we can see that other than an abnormality in pain perception, the entire COWS can be seen as nothing more than a dysfunction within both branches of the Autonomic Nervous System, the Sympathetic and Parasympathetic Nervous Systems. Amazingly, and to the best of our knowledge, this is simply the first time the COWS has ever been viewed as merely evidence for dysfunction within the Autonomic Nervous System. This raises the possibility that the opioids, if taken long enough, are producing a dysfunction within a major organ system - the Autonomic Nervous System.

Before we leave our discussion of the Parasympathetic Nervous System, there is one additional study available for our consideration. We turn our attention now to a study by Levin et al (2019) ***(Figure 17)***. Levin worked with opioid dependent human subjects put into a state of opioid withdrawal by the administration of an intramuscular injection of naloxone. Levin was looking to gain some level of understanding of a dysfunction within the Parasympathetic Nervous System during opioid withdrawal. This study utilized a relatively new technique for evaluation of the Autonomic Nervous System - the Heart

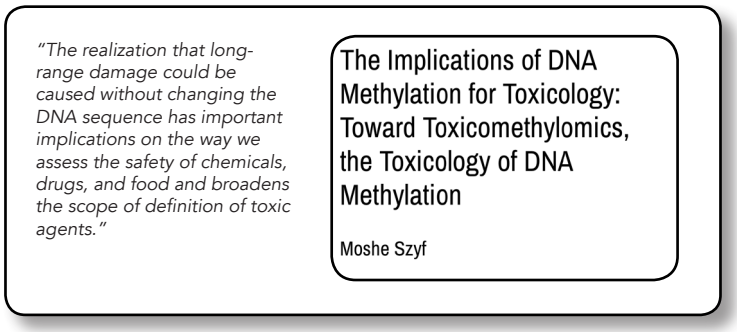
Rate Variability (HRV). Decreased HRV is believed to be a function of both increased sympathetic nerve activity and decreased parasympathetic nerve activity. And we can see here the findings by Levin et al (2019):

<u>CONCLUSIONS:</u> These preliminary data indicate that a large reduction in cardiac vagal tone occurs during naloxone-induced withdrawal. This finding underscores the need for further research into the role of the parasympathetic nervous system in opioid withdrawal.

Clearly Levin and the fellow researchers believed they were seeing an immediate dysfunction within the Parasympathetic Nervous System during opioid withdrawal. But what is it that the opioids were doing that could cause such a major dysfunction within both branches of the Autonomic Nervous System? We turn now to our second area of study - opioid induced methylation within the promoter region of the OPRM1 gene.

OPIOID INDUCED METHYLATION WITHIN THE PROMOTER REGION OF THE OPRM1 GENE

We shall begin this discussion with a quick review of the excellent article by Moshe Szyf and from 2011 ***(Figure 18)***. Szyf 2011 was viewing methylation within the DNA as a form of toxicity to the body:



(Figure 18)

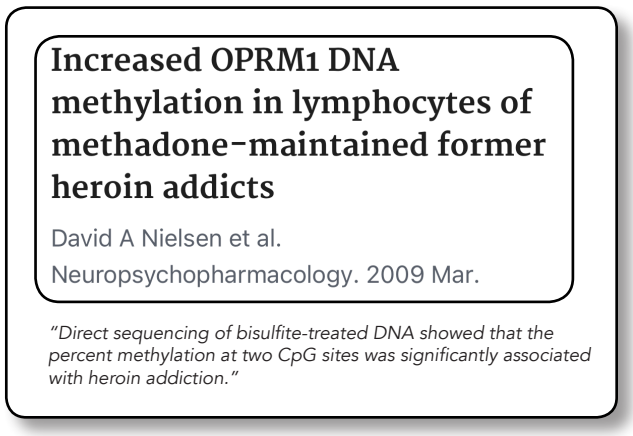
"The realization that long-range damage could be caused without changing the DNA sequence has important implications on the way we assess the safety of chemicals, drugs, and food and broadens the scope of definition of toxic agents."

And we wish to draw the readers attention to the phrase "long-range damage" There is simply no better way to describe the toxicity of methylation than the descriptive phrase "long-range damage". Are the opioids causing the type of long-range damage of which Szyf was warning? The answer is a resounding- yes. This toxicity to the DNA known as methylation has been well studied. We present now, and briefly, the scientific evidence of the opioids and DNA methylation. We have divided the scientific evidence into three broad categories: association, correlation, and causation between the opioids and DNA methylation.

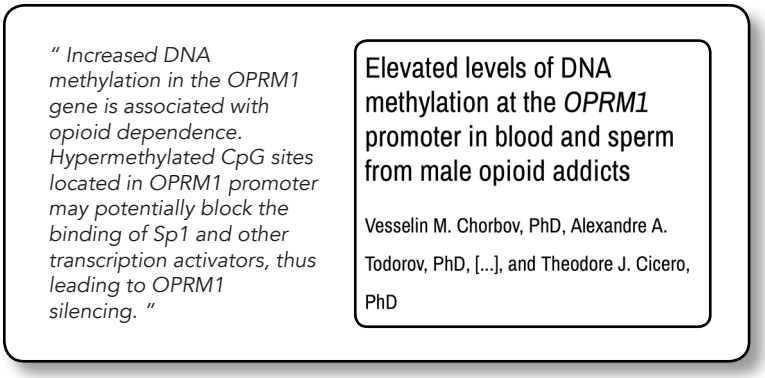
ASSOCIATION BETWEEN THE OPIOIDS & DNA METHYLATION WITHIN THE PROMOTER REGION OF THE OPRM1 GENE

We turn first to two separate studies that we feel are reflective of an association between the opioids and DNA methylation within the promoter region of the OPRM1 gene. The first study is by Nielsen et al and from 2008. Nielsen (2008) studied participants formerly using heroin, now stabilized on Methadone ***(Figure 19)***. Nielsen compared the DNA of these participants to a set of controls who had no dependency upon any of the opioids. And what Nielsen found was astonishing:

"Both the -18 and the +84 CpG sites are located in potential Sp1 transcription factor-binding sites.



(Figure 19)



(Figure 20)

Methylation of these CpG sites may lead to reduced OPRM1 expression in the lymphocytes of these former heroin addicts." Nielsen (2008)

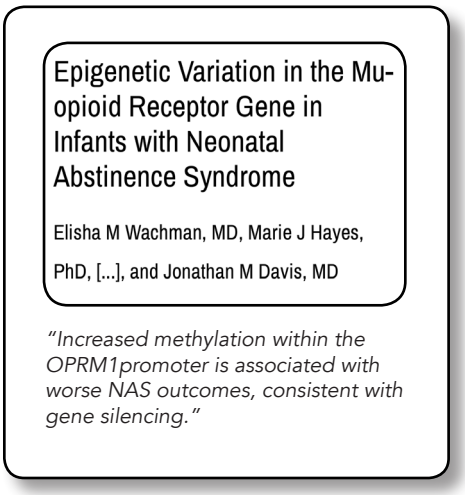
Not only was methylation present within the promoter region of the OPRM1 gene, but methylation was present within the actual SP1 binding sites. This type of gene silencing could be of a high significance. The OPRM1 gene encodes for the (mu) opioid receptor. Methylation within the promoter region, and more specifically, within the SP1 binding sites could be a disaster for the individual. (Note, detailed discussion of methylation, promoter region, gene silencing, and SP1 binding sites is beyond the scope of this manuscript.)

Could this most important of scientific findings be replicated? The answer again is- yes. These findings were replicated and by Chorbov et al and in 2011 ***(Figure 20)***. Chorbov also studied opioid dependent volunteers from the local Methadone Clinic and compared these opioid dependent participants to a set of controls who were not opioid dependent. By so doing, Chorbov replicated the findings of Nielsen:

<u>CONCLUSIONS:</u> "Increased DNA methylation in the OPRM1 gene is associated with opioid dependence. Hypermethylated CpG sites located in OPRM1 promoter may potentially block the binding of Sp1 and other transcription activators, thus leading to OPRM1 silencing."

We now have seen replicating scientific evidence for an association between the opioids and DNA methylation within the promoter region of the OPRM1 gene. We next turn our attention to the concept of a correlation between the opioids and DNA methylation within the promoter region of the OPRM1 gene. In other words, we know that the methylation is present in those individuals dependent upon the opioids. But is there any scientific evidence that this methylation is actually doing anything to the body? And for this concept of correlation between the opioids and DNA methylation within the promoter region of the OPRM1 gene, we turn now to work done by Dr. Elisa Wachman and at the Boston Medical Center.

<u>CORRELATION BETWEEN THE OPIOIDS AND DNA METHYLATION WITHIN THE PROMOTER REGION OF THE OPRM1 GENE</u>



(Figure 21)

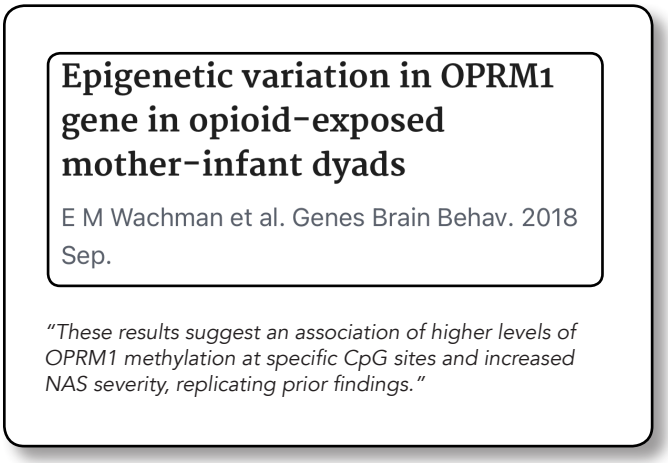
The first study by Dr. Wachman et al is from 2014 ***(Figure 21)***. Dr. Wachman is a pediatrician. Specifically, Dr. Wachman studied the infants exposed to opioids during their gestation and were therefore at risk for opioid withdrawal following delivery. Opioid withdrawal in the newborn is known as Neonatal Abstinence Syndrome (NAS). In her own words:

"We correlated DNA methylation levels in the mu-opioid receptor (OPRM1) promoter in opioid-exposed infants and correlate them with NAS outcomes."

This is to say, Wachman (2014) was looking for a correlation between the level of methylation measured in the infant and the severity of the symptoms experienced by the infant. And again, it is to be

emphasized, this methylation is found within the promoter region of the OPRM1 gene. And again, further emphasize is placed by Dr. Wachman on the possible role of this methylation to the SP1 binding sites within the promoter region of the OPRM1 gene ***(Figure 21)***. In the conclusion, Dr, Wachman states:

<u>CONCLUSIONS:</u> "Increased methylation within the OPRM1 promoter is associated with worse NAS outcomes, consistent with gene silencing."



(Figure 22)

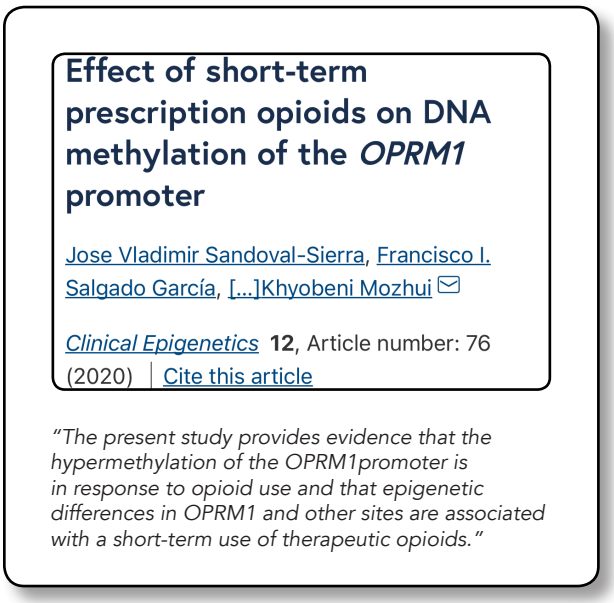
It is worth the time to read carefully the words within this conclusion by Wachman (2014). What these words are implying is the basic scientific concept of Cause and Effect. In essence, the methylation is the cause of the symptoms known widely as opioid withdrawal. We feel that the implications of these findings have not been fully realized to date by either the medical community at large, governmental leaders, or the general population.

Dr. Wachman must have felt that these findings were of real scientific importance. A second study was undertaken and completed in 2018. This second study, Wachman (2018) was quite similar to the first ***(Figure 22)***. The main difference in the second study is that Dr. Wachman chose to correlate not only the infants level of methylation within the promoter region of the OPRM1 gene to the severity of the withdrawal symptoms but also to correlate the mothers level of methylation within the promoter region of the OPRM1 gene and the severity of the withdrawal symptoms experienced by the infant. And here are the findings:

"This study shows associations between maternal and infant methylation levels in the OPRM1 promoter region and differences in NAS severity. Higher levels of methylation were observed at several CpG sites in infants who required pharmacologic treatment and correlated with infant LOS. These results obtained in an independent cohort confirm our prior findings in an independent cohort and, for the first time, show an association with maternal methylation levels and NAS severity."

The second Wachman (2018) study was successful in both confirming the prior correlation between infant methylation and severity of withdrawal and, for the first time, showing an association between maternal methylation and the severity of the withdrawal symptoms in the infant. We now have strong scientific evidence for the association between the opioids and DNA methylation (Nielsen (2008), Chorbov (2011)). We now have strong scientific evidence for the correlation between the opioids and DNA methylation (Wachman (2014) Wachman (2018)). The next step from the scientific perspective is evidence that it is the opioids themselves that are causing the DNA methylation. In other words, we need the scientific proof that when an individual takes the opioid, the methylation occurs as a result of the opioid exposure. This is known scientifically as causation. And for scientific evidence of causation, we turn to a study from 2020.

CAUSATION BETWEEN THE OPIOIDS & DNA METHYLATION WITHIN THE PROMOTER REGION OF THE OPRM1 GENE



(Figure 23)

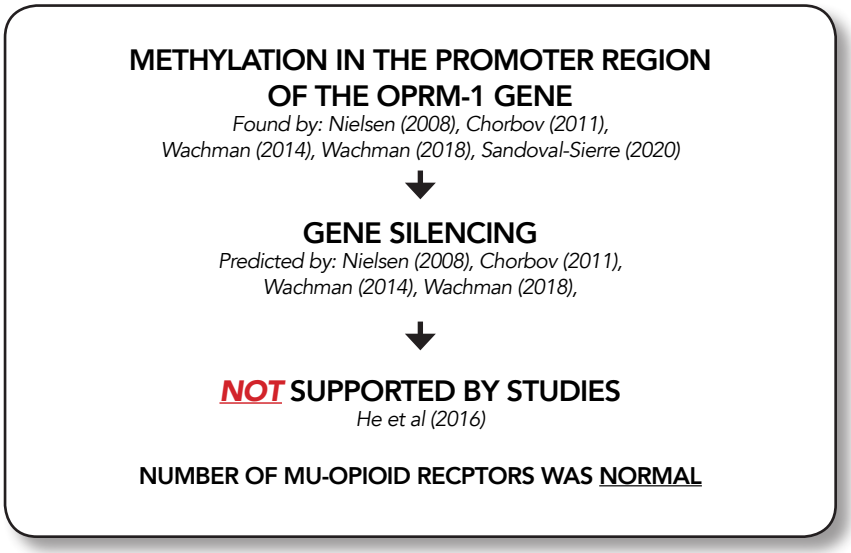
We have now seen scientific evidence for both an association and a correlation between the opioids and DNA methylation within the promoter region of the OPRM1 gene. The next question is whether or not it is the opioids themselves that are causing the DNA methylation. This would be known as causation. Do we have any scientific evidence for causation? This would require the evaluation of an opioid-naive individual who then takes the opioid and with DNA testing for methylation before, during, and after treatment with the opioids. And fortunately, we have exactly this study and done recently by Dr. Jose Vladimir Sandoval-Sierra et al and in 2020 ***(Figure 23)***. Sandoval-Sierra (2020) studied opioid-naive individuals undergoing dental surgery. Three DNA samples were obtained: before, during, and after standard treatment with the opioids. And the findings are highly significant. The opioids appear to be highly toxic to the DNA of humans and with measurable levels of methylation occurring with just a few doses. Apparently, none are immune to this methylation.

CONCLUSIONS: "The present study provides evidence that the hypermethylation of the OPRM1 promoter is in response to opioid use, and that epigenetic differences in OPRM1 and other sites are associated with short-term use of therapeutic opioids."

Again, we ask the reader to simply stop and think of the implications of these words. Look again at the quote "the hypermethylation of the OPRM1 promoter is in response to opioid use". We are now at a point wherein we have excellent scientific evidence for the triad of association, correlation, and causation between the opioids and DNA methylation within the promoter region of the OPRM1 gene.

SILENCE FROM THE DATA - THE CONCEPT OF PARTIAL GENE SILENCING

Sometimes in science, the observation is not about what is seen, rather the observation is about what is not seen ***(Figure 24)***. We have seen excellent data that the opioids result in a type of toxicity to the DNA known as methylation. And we know from science that methylation results in gene silencing. Three of the above four researchers predicted a lowering of the mu-opioid receptor population as a result of the methylation. Let's look now at the quotes from the authors referenced above as they speak of a reduction in the mu-opioid receptor:



(Figure 24)

(Figure 25)

"Methylation of these CpG sites may lead to reduced OPRM1 expression in the lymphocytes of these former heroin addicts." Nielsen (2008)

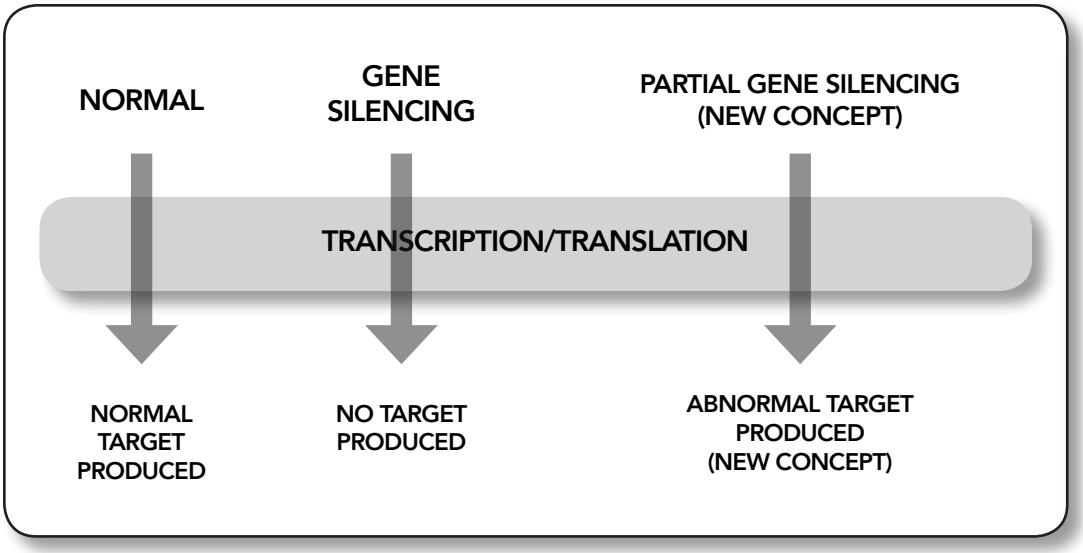
"Increased DNA methylation in the OPRM1 gene is associated with opioid dependence. Hypermethylated CpG sites located in OPRM1 promoter may potentially block the binding of Sp1 and other transcription activators, thus leading to OPRM1 silencing." Chorbov (2011)

"In this case, infants with hypermethylation at these specific CpG sites may have down-regulated OPRM1 gene expression leading to reduced levels of the mu-opioid receptor." Wachman (2014)

"Thus, we hypothesize that infants with hypermethylation at these sites may have downregulated OPRM1 gene expression and reduced levels of μ-opioid receptor." Wachman (2018)

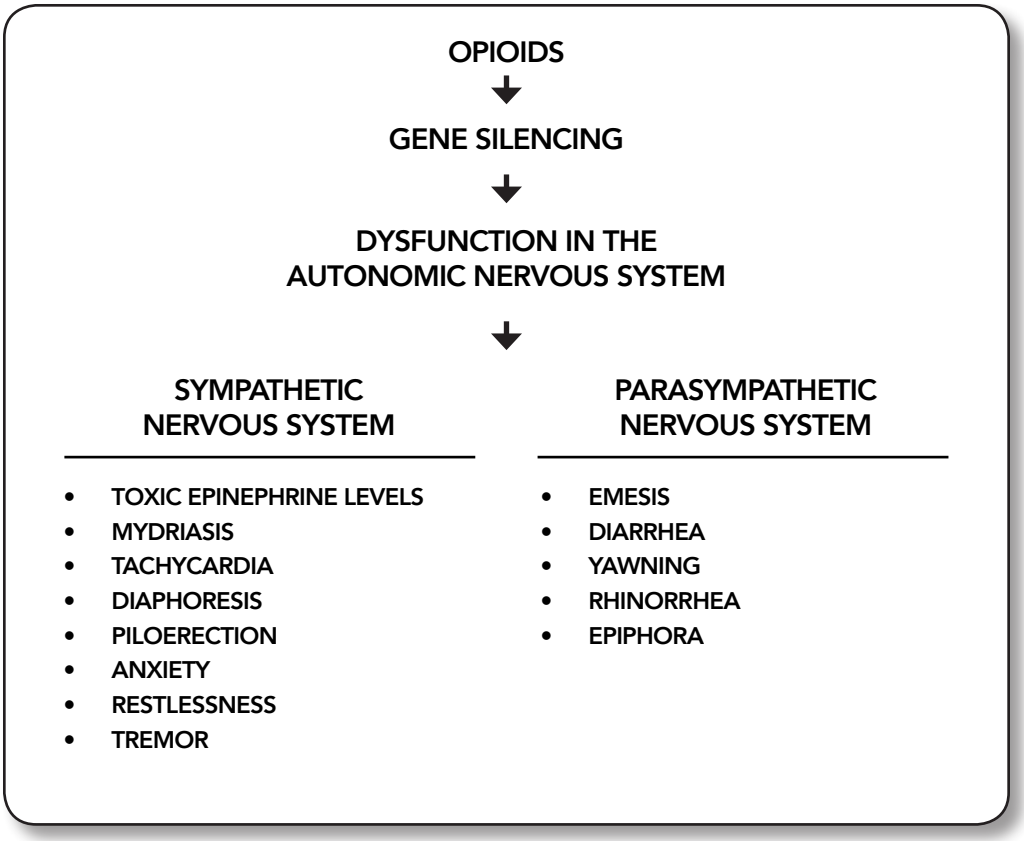
The outcome predicted by these excellent researchers is that the body of an individual suffering from the methylation in the promoter region of the OPRM1 gene would have fewer numbers of the mu-opioid receptors. Such a reduction in the number of mu-opioid receptors would be known as a down-regulation of the receptor. And this down-regulation of the mu-opioid receptor has simply not been found scientifically. We turn now to an article by He et al from 2016: ***(Figure 25)***

"Furthermore, numerous studies have demonstrated no substantial downregulation in the number of MORs (mu-opioid receptors) even in profoundly tolerant animals (for example, De Vries et al. 1993, Simantov et al. 1984; reviewed in Williams et al. 2001). Hence, it is unlikely that tolerance to morphine is mediated solely by desensitization and downregulation of the receptor." He et al (2016)



(Figure 26)

The opioids resulted in methylation in the promoter region of the OPRM1 gene. That is accepted science. This methylation results in a dysfunction within the Autonomic Nervous System. We feel that the scientific evidence is overwhelming for that statement. But, whatever the mechanism of the damage, this mechanism does not include mu-opioid receptor down regulation. We are proposing as a part of our hypothesis, a new concept to explain these findings. We believe that the population of the mu-opioid receptor remains at or near normal. We are hypothesizing, however, that these mu-opioid receptors have been rendered as abnormal by the methylation. We believe that something important simply was not generated. In other words, the receptor was encoded and generated, but a part is simply missing. It's like a car rolling off the assembly line but missing a wheel. We are calling this process **PARTIAL GENE SILENCING** ***(Figure 26)***. In partial gene silencing, the protein is created per the information encoded within the DNA. But, since some of the DNA was silenced by methylation, the protein will be missing the corresponding peptides. We feel that there is strong scientific evidence that the mu-opioid receptor in an opioid dependent individual is not able to function normally. And now we are saying that the mu-opioid receptor in an opioid dependent individual does not even look the same. There is a distinct possibility that the peptide sequence itself may even be altered. Partial gene silencing may result in a target molecule to be altered structurally, functionally, and structurally. For all we currently know, all three have occurred in the mu-opioid receptor in an opioid dependent individual.



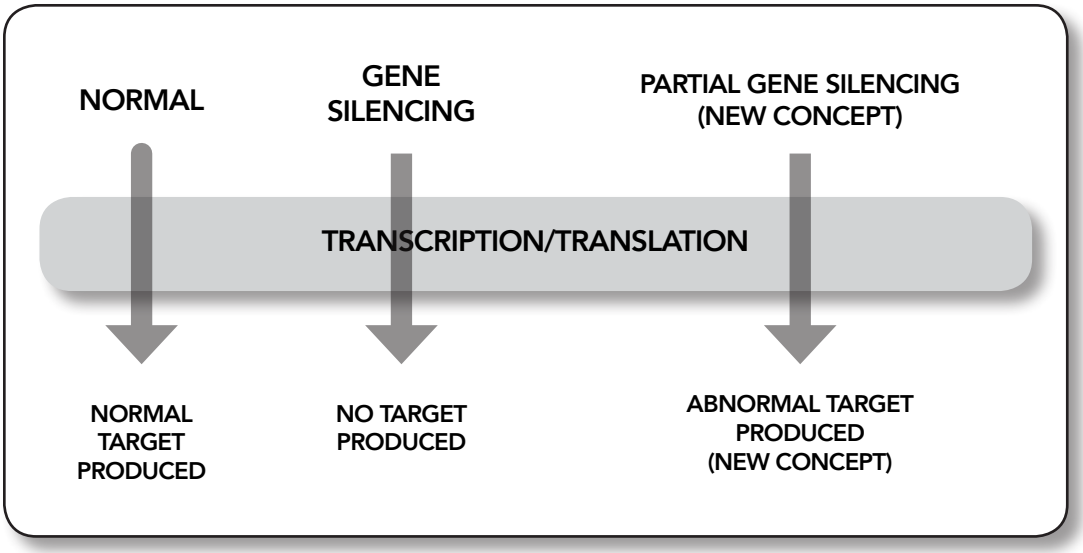
(Figure 27)

SUMMATION OF THE DATA

Szyf (2011) had warned us that some drugs are able to be toxic to our bodies through a type of poisoning known as DNA methylation. In the opinion of these authors, this is an exact description of the opioids effect upon the body.

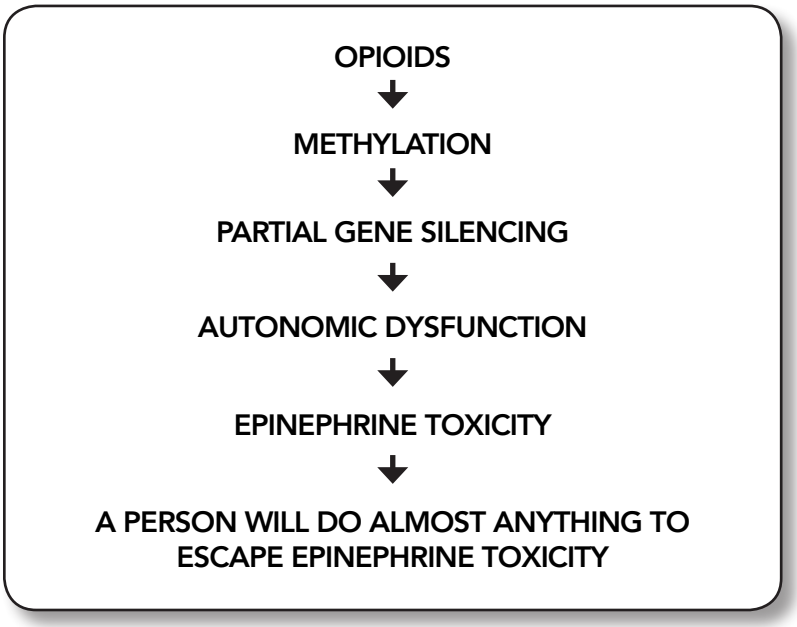
We have presented scientific evidence that the opioid dependency result in a dysfunction within the Autonomic Nervous System ***(Figure 27)***. This dysfunction is evident with abnormalities noted in both branches of the Autonomic Nervous System. In the Sympathetic Nervous System, we are seeing evidence of toxic epinephrine levels, mydriasis, tachycardia, diaphoresis, piloerection, anxiety, restlessness, and tremor. In the Parasympathetic Nervous System, we are seeing emesis, diarrhea, yawning, rhinorrhea, and epiphora. These symptoms appear when the opioid receptors are unbound to any opioid. These symptoms resolve when the opioid receptors are bound to an opioid.

We have also presented scientific evidence that exposure to the opioids results in a methylation within the promoter region of the OPRM1 gene. The level of this methylation is correlated to the severity of the withdrawal symptoms experienced by the individual. Furthermore, this methylation, this toxicity, this poisoning to the DNA is measurable even after just a few dosage of the opioids.
The challenge now is to coordinate these two seemingly disparate sets of scientific evidence into one plausible scientific hypothesis. And from this hypothesis, a prediction should be clear and able to be tested by the scientific method. Let's begin with a series of statements that are known or inferred:



(Figure 28)

1. Once an individual becomes opioid dependent, then when opioid abstinence is attempted, the symptoms of autonomic dysfunction begin ***(Figure 28)***. This is a fundamental observation and must be explained by any reasonable candidate for the hypothesis. How is it that the symptoms of autonomic dysfunction are present in the absence of opioids and resolve with the presence of opioids? Some of the authors noted in the methylation studies above made a strong point about the methylation occurring not only within the promoter region of the OPRM1 gene, but more specifically, within the SP1 binding sites. Several authors suggested this could result in a process known as gene silencing. The result of this gene silencing was presumably a reduction in the population of mu-opioid receptors. But it was further presumed that any mu-opioid receptors would be of normal anatomy and function. This would not explain why the administration of a full or partial opioid agonist would resolve the symptoms of autonomic dysfunction. However, if the gene silencing was only a partial gene silencing, then a population of abnormal mu-opioid receptors could be the result. The mu-opioid receptor was created. But something important is missing. The loss of this something important is, apparently, offset by the actions of either a full or partial opioid agonist. Full agonist opioids are fraught with risk. Partial agonist opioids have a much safer safety profile due mainly to the well documented ceiling effect. Partial agonist opioids also appear to be superior at suppressing the symptoms of autonomic dysfunction as seen by a lack of tolerance as opposed to the full agonist opioids.



(Figure 29)

2. It is apparent from the scientific evidence that at least a part of the autonomic dysfunction seen in opioid withdrawal is represented by a toxicity of epinephrine ***(Figure 29)***. Epinephrine is potent within the body. Epinephrine toxicity is a true Neuroendocrine Emergency. We know from other examples of epinephrine toxicity that epinephrine toxicity itself is described by the individual as a state difficult for the human body to endure. Many describe a state of apprehension known as impending doom. While difficult for individuals to describe or quantitate, epinephrine toxicity surely results in the individual wishing to stop this epinephrine toxicity. Much of what we know comes from the literature on the catecholamine secreting tumor known as the pheochromocytoma.

THE MU-OPIOID RECEPTOR HAS A LARGER ROLE IN MAINTAINING BALANCE AND HOMEOSTASIS IN THE AUTONOMIC NERVOUS SYSTEM THAN HAS BEEN PREVIOUSLY RECOGNIZED

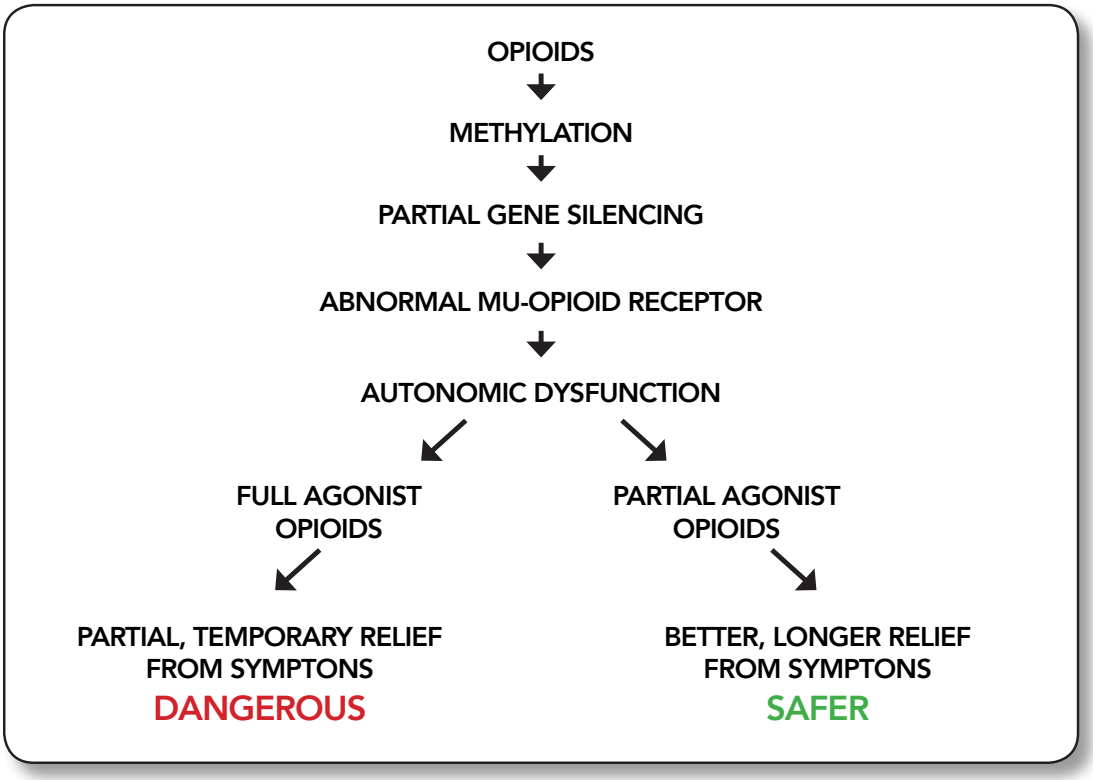
(Figure 30)

Pheochromocytoma tumors are known to periodically excrete toxic levels of epinephrine. Surgical resection remains the treatment of choice. There is a great deal of overlap between the symptoms of an epinephrine secreting pheochromocytoma and the symptoms seen in opioid withdrawal - mydriasis, tachycardia, diaphoresis, tremor, anxiety, restlessness. It is highly likely that the individual in a state of autonomic dysfunction and with epinephrine toxicity would be desirous of an opioid in order to bring about at least a partial relief from the negative symptoms. While the predominance of current thinking is that the individual is involved with a pathological consumption of opioids due to a "craving to get high", it seems more likely that the individual is craving the opioid simply to escape the Dante's Inferno of an autonomic dysfunction and with epinephrine toxicity.

3. There is an increasing body of scientific evidence that the opioids are associated with a clinical entity known as Opioid Induced Adrenal Insufficiency. The exact mechanism is unknown. But knowing that opioid withdrawal is associated with a 400% increase in Sympathetic Nerve Activity to the adrenal gland, multiple and prolonged episodes of autonomic dysfunction should be avoided.

4. One of the biggest leaps in faith occurs when trying to connect an abnormal mu-opioid receptor with a dysfunction within the Autonomic Nervous System ***(Figure 30)***. We do know that the primary function of the Autonomic Nervous System is to maintain balance and homeostasis within the internal organs. And we do know that the Autonomic Nervous System is millions of years old. The Autonomic Nervous System is a fundamental of the more complex life forms on this planet. It is of no surprise that a dysfunction within the Autonomic Nervous System is so poorly tolerated by the individual. And we also know that the mu-opioid receptor is also itself an older more primitive receptor. Perhaps what we are learning is that the mu-opioid receptor is more integral to the Autonomic Nervous System than was formally appreciated.

(Figure 31)



And if an abnormal mu-opioid receptor is the result of the methylation within the promoter region of the OPRM1 gene, then it should follow that the abnormal mu-opioid receptor would also lose its ability to maintain homeostasis and balance within the Autonomic Nervous System ***(Figure 31)***. This loss of control of the Autonomic Nervous System is partially restored, evidently, by the administration of a full agonist

opioid. This loss of control of the Autonomic Nervous System is more fully restored, evidently, by the administration of a partial agonist opioid.

5. We were able to account for all the symptoms listed in the Clinical Opiate Withdrawal Scale (COWS) as either sympathetic or parasympathetic in origin except for the abnormal perception of bone and joint pain. Now knowing that an abnormal mu-opioid receptor is at the center of our hypothesis, it should be of no surprise that an abnormal sensation of pain would be present during a period of opioid withdrawal.

We will now present our hypothesis

THE SMITH HYPOTHESIS

Methylation of certain CpG islands within the promoter region of the OPRM1 gene, as seen in response to the exposure to the opioids, results in gene silencing. This gene silencing is not producing a drop in the population of the mu-opioid receptor. Rather, this gene silencing results in the formation of an abnormal mu-opioid receptor. This production of an abnormal mu-opioid receptor is due to a process we are calling partial gene silencing - the receptor was produced but it was an abnormal receptor. This abnormal mu-opioid receptor is no longer able to maintain balance and homeostasis within the Autonomic Nervous System when Opioid Abstinence is attempted. This dysfunction within the Autonomic Nervous System results in a true Neuroendocrine Emergency known as Autonomic Dysfunction. This Autonomic Dysfunction is reflected in the abnormal activity in both branches of the Autonomic Nervous System, the Sympathetic Nervous System and the Parasympathetic Nervous System. Autonomic dysfunction, and the epinephrine toxicity that results, is a condition of extreme duress and cannot long be endured by the human body. The full agonist opioids offer a partial and temporary relief. But this partial and temporary relief comes with the associated risk of the full agonist opioids. The partial agonist, Buprenorphine, is able to maintain a more complete and longer lasting relief from the autonomic dysfunction but, due to the Ceiling Effect of Buprenorphine, at a higher level of safety. Untreated, there is concern that autonomic dysfunction could be a risk factor for the development of Opioid Induced Adrenal Insufficiency.

OUR PREDICTIONS

The Scientific Method involves the creation of a hypothesis. This hypothesis should lead to certain predictions. These predictions can then be tested and evaluated via a clinical trial. Utilizing the IRB system insures that these clinical trials are done a manner respectful all human rights. The following are our predictions drawn from the above Smith Hypothesis:

WE PROPOSE TO TEST FOR ELEVATED PLASMA EPINEPHRINE DURING OPIOID ABSTINENCE WITHDRAWAL IN HUMANS. THIS HAS BEEN CONFIRMED IN MICE, BUT NOT IN HUMANS YET. THIS DOES NOT REQUIRE ANY DEVIATION FROM STANDARD THERAPY

(Figure 32)

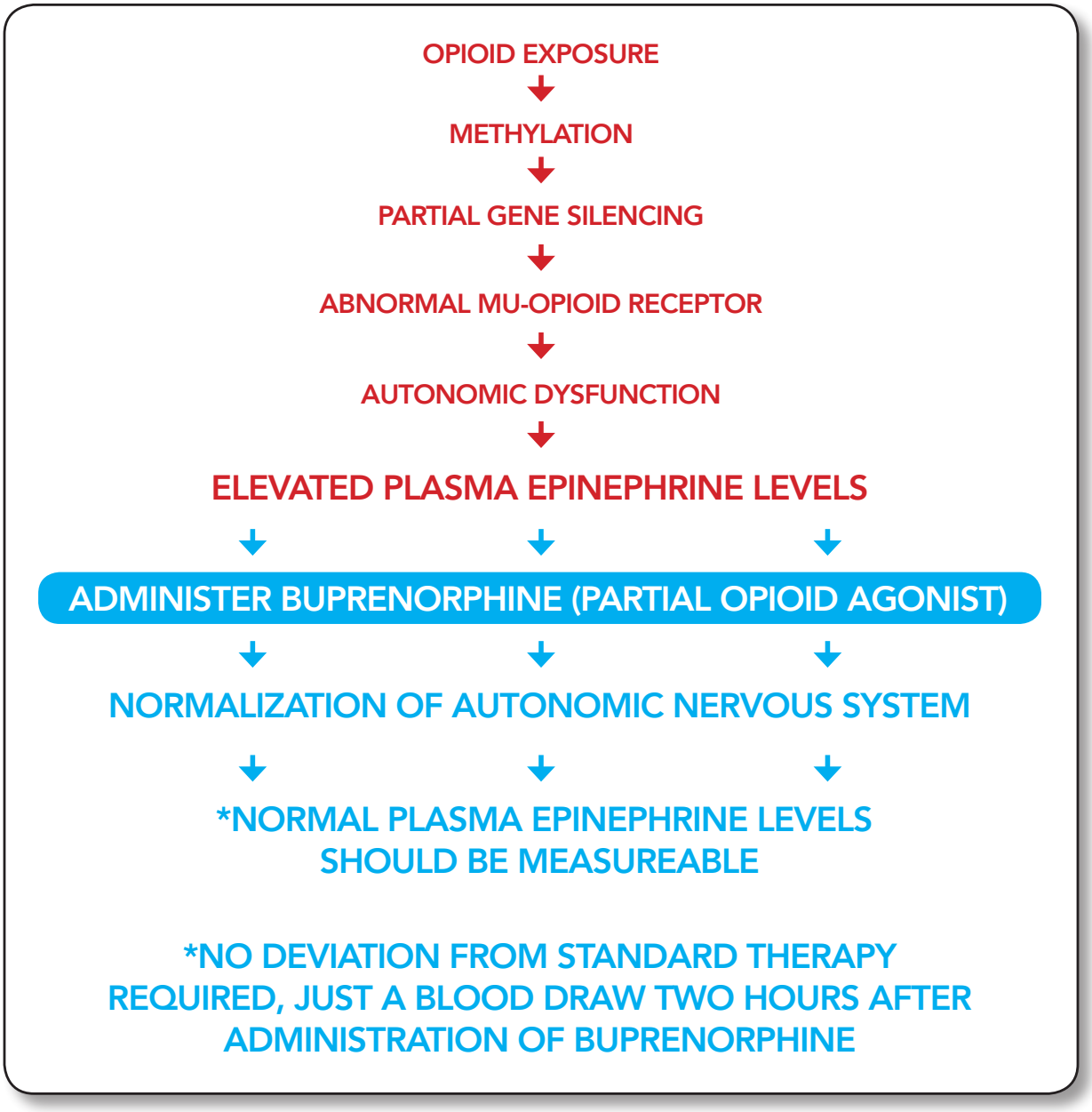
1. Elevated levels of epinephrine should be discoverable in the state of autonomic dysfunction that is referred to as opioid withdrawal ***(Figure 32)***. These elevated levels of epinephrine should be present regardless of the etiology of the opioid withdrawal, (opioid abstinence versus opioid antagonist induced). These elevated levels of epinephrine are due to the dysfunction within the Autonomic Nervous System. This dysfunction within the Autonomic Nervous System is due to the presence of abnormal mu-opioid receptors. These abnormal mu-opioid receptors are the result of the methylation occurring on the CpG islands within the promoter region of the OPRM1 gene due to the exposure to the opioids. This methylation results in gene silencing. This gene silencing is not resulting in a decrease in the number of mu-opioid receptors. Rather this gene silencing results in the formation of an abnormal mu-opioid

AUTONOMIC DYSFUNCTION SCALE

1. I am yawning more than normal	1 2 3 4 5
2. My eyes are watering more than normal	1 2 3 4 5
3. My nose is running more than normal	1 2 3 4 5
4. I am having stomach cramping	1 2 3 4 5
5. I am vomiting	1 2 3 4 5
6. I have diarrhea	1 2 3 4 5
7. I am sweating more than normal	1 2 3 4 5
8. The hair on my body is standing on end	1 2 3 4 5
9. My heart is beating hard and fast	1 2 3 4 5
10. I feel anxious	1 2 3 4 5
11. I feel hot then cold	1 2 3 4 5
12. I have a tremor (shaking)	1 2 3 4 5
13. I feel like something bad is about to happen	1 2 3 4 5
14. I can't stand feeling this way	1 2 3 4 5

(Figure 33)

receptor. Unless in a state of stimulation from either a full or partial opioid agonist, the abnormal mu-opioid receptor is unable to maintain balance and homeostasis within the Autonomic Nervous System. Autonomic dysfunction is the result of this loss of balance and homeostasis within the Autonomic Nervous System ***(Figure 33)*** The symptoms of autonomic dysfunction experienced by the individual should be measurable with a proper screening tool.



(Figure 34)

2. These levels of elevated epinephrine should rapidly diminish following the administration of either a full agonist opioid or a partial agonist opioid ***(Figure 34)***. This normalization of the epinephrine level is due to the normalization of the Autonomic Nervous System. This normalization of the Autonomic Nervous System will continue as long as the mu-opioid receptor is bound by either the full or partial opioid agonist. As the full or partial opioid agonist is metabolized by the body, the state of autonomic dysfunction will return. The epinephrine level will begin to rise again. Other symptoms of autonomic dysfunction will be experienced by the individual. These symptoms of autonomic dysfunction are difficult for the human body to long endure. The individual is highly motivated to bring about an end to the autonomic dysfunction. This desire to bring about an end to the autonomic dysfunction has been labeled as opioid craving.

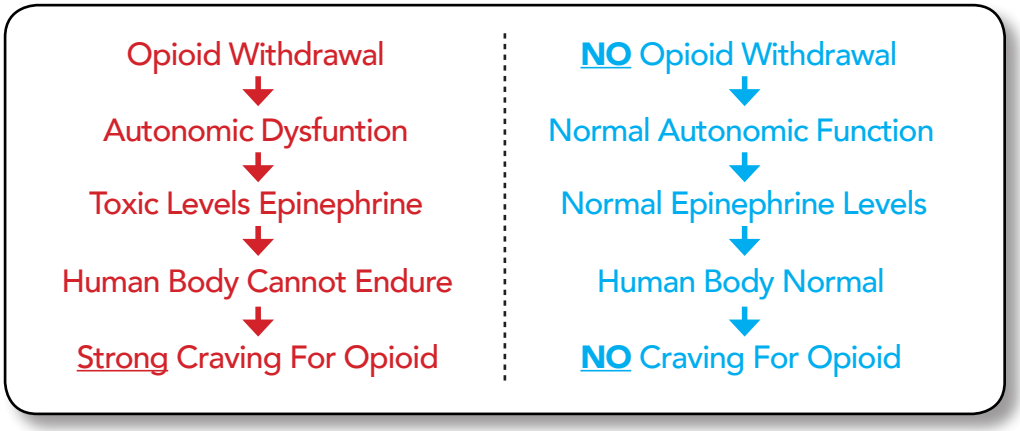
OPIOID CRAVING SCALE

1. If I had an opioid right now, I would take it.	1 2 3 4 5
2. I would not be able to stop myself from taking an opioid right now.	1 2 3 4 5
3. I would feel more in control of things if I could take an opioid right now.	1 2 3 4 5
4. Taking an opioid right now would make me feel better.	1 2 3 4 5
5. If I could take an opioid right now I would feel less restless	1 2 3 4 5
6. I am craving an opioid right now.	1 2 3 4 5
7. Using an opioid right now would make me feel better	1 2 3 4 5

(Figure 35)

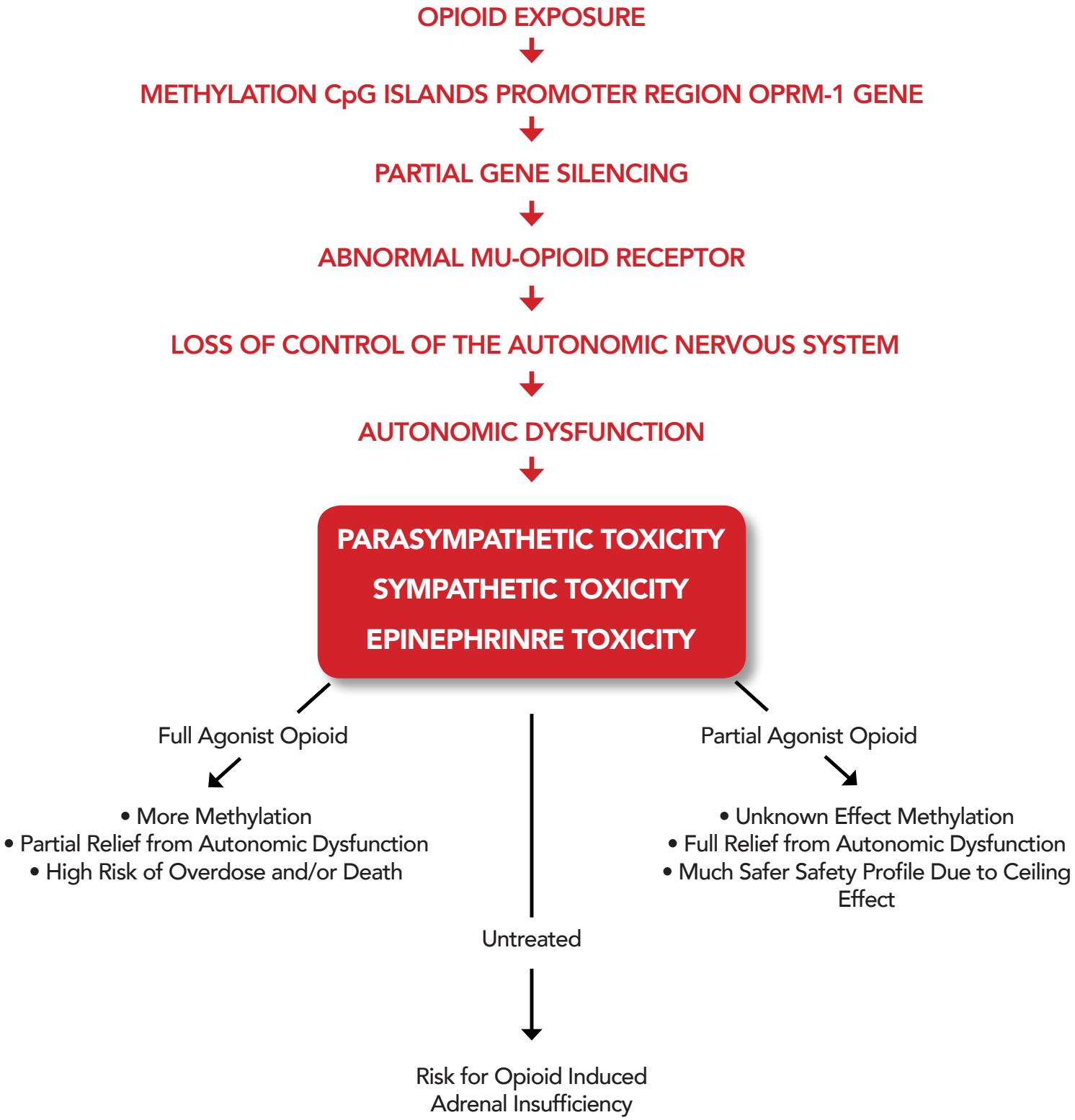
3. Opioid craving during opioid withdrawal is not a desire by the individual to pursue an, "opioid high" ***(Figure 35)***. Rather this opioid craving is merely reflective of the desire to escape the negative symptoms of the autonomic dysfunction and with epinephrine toxicity. This state of opioid craving, a desire to bring about an end to the autonomic dysfunction, should be a measurable event with a proper screening tool.

4. When the state of autonomic dysfunction is resolved and epinephrine levels have returned to normal, the desire to take another opioid is diminished ***(Figure 36)***. This return to a normalized state and the drop in opioid craving should be a measurable event with a proper screening tool.



(Figure 36)

GRAPHIC REPRESENTATION OF THE SMITH HYPOTHESIS & ITS PREDICTIONS



(Figure 37A)

PREDICTIONS FROM THE SMITH HYPOTHESIS

PREDICTION #1

- ✓ Epinephrine levels will be elevated in opioid withdrawal
- ✓ Epinephrine levels will normalize with the administration of Buprenorphine to a person in opioid abstinence withdrawal.

PREDICTION #2

- ✓ Opioid craving will be high during Autonomic Dysfunction
- ✓ Opioid craving will be low when the Autonomic Dysfunction resolves

PREDICTION #3

The mu-opioid receptor will be found to be altered in the opioid dependant. This alteration can either be functional, structural, or appearance